THE ROLE OF CALCIUM AND OXYGEN-DERIVED FREE RADICALS IN REPERFUSION INJURY TO THE ISCHAEMIC KIDNEY

by

Lisa Ann Cotterill, Bsc. (Hons)

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in the

University of London

Royal Free Hospital Medical School,

Hampstead, London

NW3 2QG

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ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

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The objective of this thesis was to investigate the possible roles of calcium and oxygen derived free radicals in tissue damage resulting from ischaemia and reperfusion using the rabbit kidney as a model. Kidneys were flushed and stored at $0^{o}C$ for 24-72 hours (cold ischaemia) in either hypertonic citrate solution (a good storage medium) or isotonic saline (a poor storage medium) and the extent of free radical induced lipid peroxidation was subsequently measured in vitro in tissue homogenates. The effects of a number of agents which affect calcium homeostasis or inhibit calcium-dependent processes were investigated. The results strongly suggested that altered calcium homeostasis during the ischaemic period, involving both extracellular influx and intracellular redistribution played an important role in the development of oxidative membrane damage upon reoxygenation.

Free fatty acids (FFAs), the products of phospholipase action were analysed by glc and a rise in unsaturated FFAs, particularly arachidonic acid, was found to occur in renal tissue during the storage period. Further studies implicated the involvement of a calcium-dependent phospholipase A2 in the mediation of increased lipid

peroxidation following ischaemia.

Experiments were designed to test the hypothesis that hypoxia/reoxygenation may alter the turnover of the phosphatidylinositol (PI) secondary messenger system. Reoxygenation of rabbit kidney cortical slices exposed to hypoxia in vitro resulted in a rapid increase in PI hydrolysis to secondary messenger products. This effect appeared to be calcium-dependent and paralleled the increase in lipid peroxidation observed upon reoxygenation of hypoxic slices.

The results of this investigation strongly suggest that altered calcium homeostasis and the production of oxygen-derived free radicals act synergistically and contribute significantly to the pathogenesis of renal damage resulting from ischaemia and reperfusion. Modulators of calcium-dependent processes may therefore be of therapeutic value in preventing organ damage during renal transplantation.

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CHAPTER 1

INTRODUCTION

Renal Transplantation: An Historical Background

Since the original attempts at solid tissue grafting in the late 18th century (Hunter, 1778) the science of transplantation has made enormous progress. The earliest record of a renal allograft in man was performed by the Russian surgeon Voronoy in 1933 and involved the unsuccessful transplantation into the groin of a patient suffering from mercuric poisoning (Kahan, 1983). This was followed by the connection of a cadaveric kidney to the brachial artery and cephalic vein of a young woman with acute renal failure by Landsteiner, Hufnagel and Hume in 1945, but this organ failed to function. In the early 1950s, several series of human kidney transplants were performed (Murray et al, 1955; Merrill et al, 1956), but no immunosuppressive drugs were available to prevent rejection, and all but one of the patients died early. However, out of these studies a successful allotransplantation technique was established by Murray and co-workers using grafts between identical twins.

Although great technical advances were being made, progress was severely hindered by the immunological process mediating allograft rejection. Attempts to control the immune response, initially with irradiation of the whole body and later with chemical immunosuppression using

azathioprine and prednisolone, still yielded only 50% survival of the grafts for one year by 1970, and the mortality rate due to infection was 15% (Kahan, 1983). The discovery of the immunosuppressive properties of cyclosporine (Borel, 1976) offered new impetus to transplantation, since the agent possessed selectivity for T lymphocytes not obtained with non-specific agents such as azathioprine or steroids. The one year graft survival rates are now over 80% in kidney, heart and liver transplants in patients treated with cyclosporine.

Poor preservation techniques are still a major cause of limited primary function of grafts. Most organs transplanted in the UK are taken from cadavers which have been certified brain-stem dead. Even though about four thousand suitable donors die every year, only a small number of these organs are actually used for transplantation, and so the waiting lists continue to grow and the shortfall in supply results in many patients who are waiting for a transplant dying as a result of renal, cardiac or hepatic failure. Many patients with renal failure can be kept alive by haemodialysis, but it is estimated that the cost of kidney disease in the U.K. is 744 million pounds a year, with 19 million working days lost (Green, 1988). Improved methods of organ storage and preservation are therefore urgently required.

Current Methods of Renal Preservation

In the late 1960s two important studies demonstrated that kidneys could be safely preserved for 30 hours by simple infusion followed by cold storage (Collins, Bravo-Shugarman and Terasaki, 1969) and for as long as 72 hours by continuous hypothermic perfusion (Belzer, Ashby and Dumphy, 1967). Kidneys can currently be stored successfully for up to 48 hours whilst matching is performed and the organ transported to a suitable recipient, although occasionally storage extends into the third day. However, shorter periods are preferable for optimum post-transplant renal function.

Kidneys are initially flushed free of blood with a cold electrolyte solution via the renal artery and then the kidney is immersed in cold liquid. Kidneys can thereafter be preserved either by continued storage with ice immersion at $0^{o}C$ to slow metabolism as completely as possible, or by continuous cold perfusion at $5\text{-}10^{o}C$ to provide for diminished metabolism and removal of waste products. The use of isotonic solutions for perfusion is thought by some workers (Green and Pegg, 1979a) to result in intracellular oedema, with increased leakage of intracellular ions and enzymes into the extracellular spaces. Others, however, have reported that isotonic solutions may be effective in experimental preservation of some organs (Howden et al, 1983). This question is still unresolved although many workers have shown that prevention of hydropic degeneration

by use of hypertonic solutions is important in organ preservation (Green and Pegg, 1979a; Sheil and d'Apice, 1987). Successful flushing solutions have been isosmolar or hyperosmolar electrolyte solutions (Collins et al, 1969; Ross et al, 1976: Jablonski et al, 1980; Squifflet et al, 1984). These flush solutions are effective by controlling cell volume and composition through modification of the transmembrane movement of water and ions, and by their buffering capacity.

Hypertonic citrate solution has been used successfully in clinical renal preservation for several years. Developed by Ross, Marshall and Escott (1976), it is hypertonic with respect to plasma and contains 186mM mannitol which permeates the cell membrane slowly, thus preventing cell swelling. Normally, cells are bathed in an extracellular solution high in sodium and low in potassium compared to intracellular levels. These gradients are maintained by the sodium/potassium ATPase, which uses much of the energy (ATP) derived from oxidative phosphorylation. The sodium pump keeps sodium largely outside the cell which counteracts the colloidal osmotic pressure derived from the intracellular proteins and other impermeable anions. The calculated osmotic pressure derived from the intracellular proteins and impermeable anions is about 110-140 mOsm/kg (MacKnight and Leaf, 1977). Anaerobic-hypothermic preservation suppresses the activity of the sodium pump and decreases the membrane potential of the plasma membrane

(Martin et al, 1972). Consequently, sodium and chloride ions enter the cell down a concentration gradient, and the cell swells because it accumulates water. This tendency to swell can be counteracted by adding 110-140mmol/l (110-140mOsm/kg osmotic force) of substances providing they are non-ionised, such as mannitol that are relatively impermeable to the cell.

Continuous hypothermic perfusion using colloidal solutions such as cryoprecipitated plasma (Belzer et al, 1967) or simpler colloidal solutions (Pegg et al, 1977) has obvious disadvantages of complexity and expense, but undoubtedly provides better immediate life-sustaining function after transplantation. It is potentially a more successful method of renal preservation if storage periods are to be extended to 4 or more days.

Immediate function after renal transplantation is desirable but not obligatory, since the transplanted recipients can be kept alive on dialysis. The main objectives for improving organ preservation are: to increase organ availability, decrease organ wastage, increase organ sharing and reduce cost. One possible avenue for the improvement of storage techniques would be the formulation of a solution containing agents which theoretically might prevent the damage to tissues during hypothermic storage of the organ. This has been attempted in the new University of Wisconsin cold storage solution (Belzer and Southard, 1988). In addition to the inclusion

of agents which prevent cell swelling (raffinose and lactobionate) the solution also contains a variety of agents which are thought to protect against biochemical damage to organs following preservation. The solution appears to be equally effective for preserving the kidney, liver and pancreas and potentially could be used as a general storage solution.

The commonest cause of early non-function in transplanted organs is acute tubular necrosis (ATN), and its frequency is influenced by the degree of ischaemic damage occurring to the donor kidney and the duration of the storage period. In order to fully understand the nature of renal damage during transplantation, it is first necessary to examine the structure and function of the organ.

The Kidney - Anatomy and Physiology

The kidneys are paired organs lying retroperitoneally on the posterior abdominal wall. They control the composition and volume of body fluids within narrowly-defined limits by excreting unwanted substances, either produced endogenously or ingested, and regulating the excretion of water and essential solutes such as sodium, chloride, phosphate and bicarbonate ions, amino acids and glucose (Gabriel, 1977). The kidneys also have a number of other important roles, such as endocrine functions related particularly to blood volume and composition, and metabolic

functions. Efficient functioning of these organs is therefore essential to the well-being of the organism. The unique functions of the kidney result from the interrelationships between various highly specialised structural components.

Bisection of the kidney reveals the hilus, where the renal artery, vein and pelvis join the organ (Figure 1). The kidney is composed of cortex and medulla, which are sharply demarcated where the pyramids are situated. The pyramid is that portion of the medulla which projects towards the pelvis, the distal part being called the papilla (Figure 1). Faint striations are visible in the cortex running in the direction of the hilus. These are medullary rays which contain collecting tubules and the ascending limbs of the nephron (Figure 2).

The nephron is the functioning unit of the organ (Figure 2), and there are approximately one million per kidney in the human (Morgan et al, 1987). The upper end lies in the cortex where it is invaginated by a mesh of capillaries called the glomerulus. The glomerular tuft is composed of a coil of specialized capillaries fed by arterioles and lying within Bowman's capsule (Figure 3). Ultrafiltration occurs across the capillary tuft and the fluid then passes into the proximal convoluted tubule, whose lining of epithelial cells is continuous with the glomerulus. The proximal tubule is the largest segment of the nephron. It is a coiled structure lined with a single

FIGURE 1: Structure of the Cut Surface of the Kidney

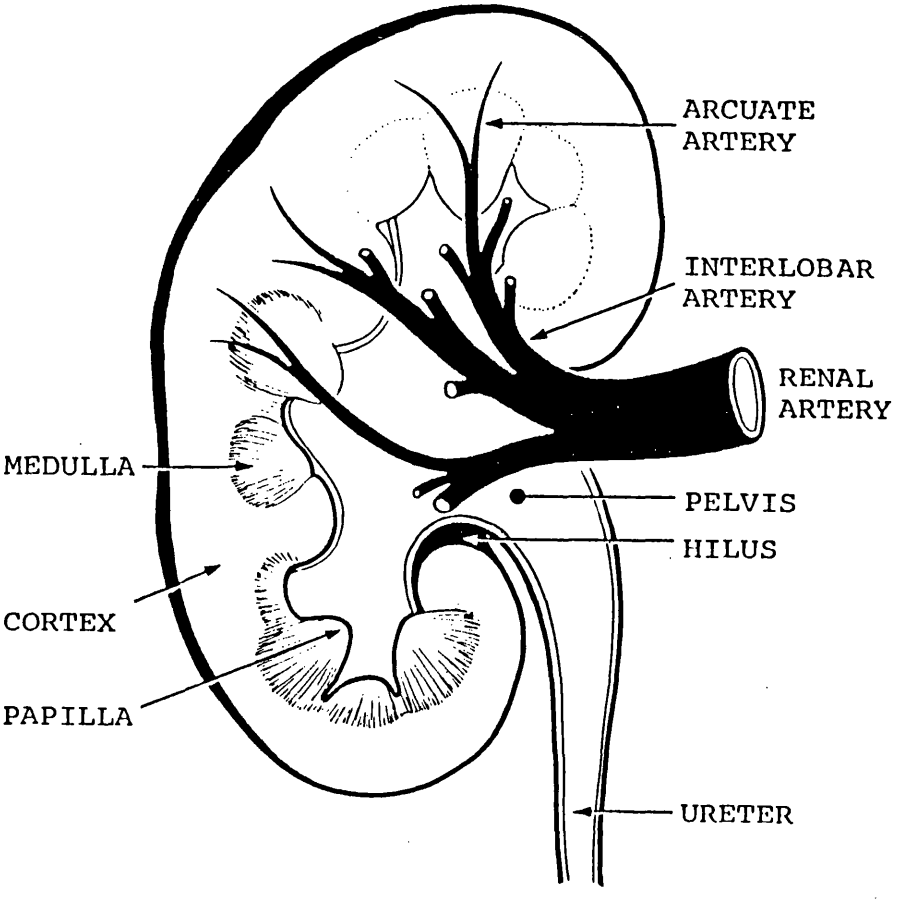
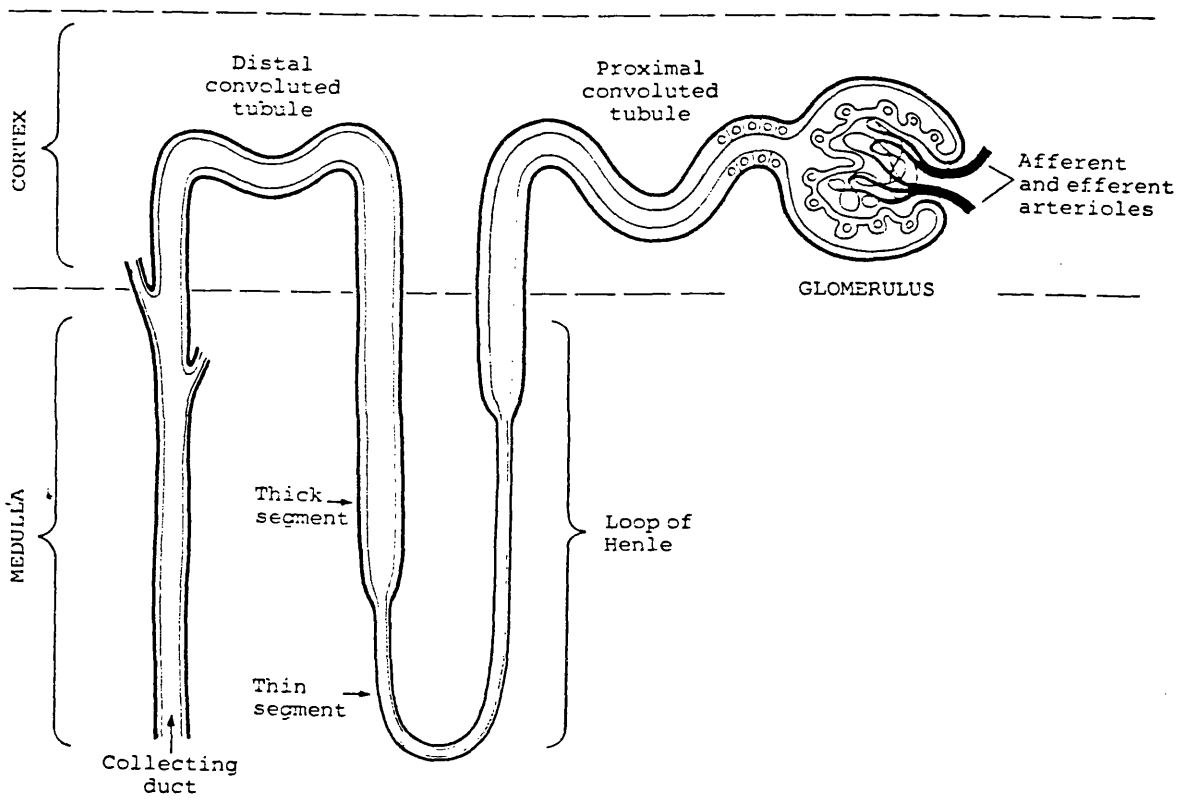


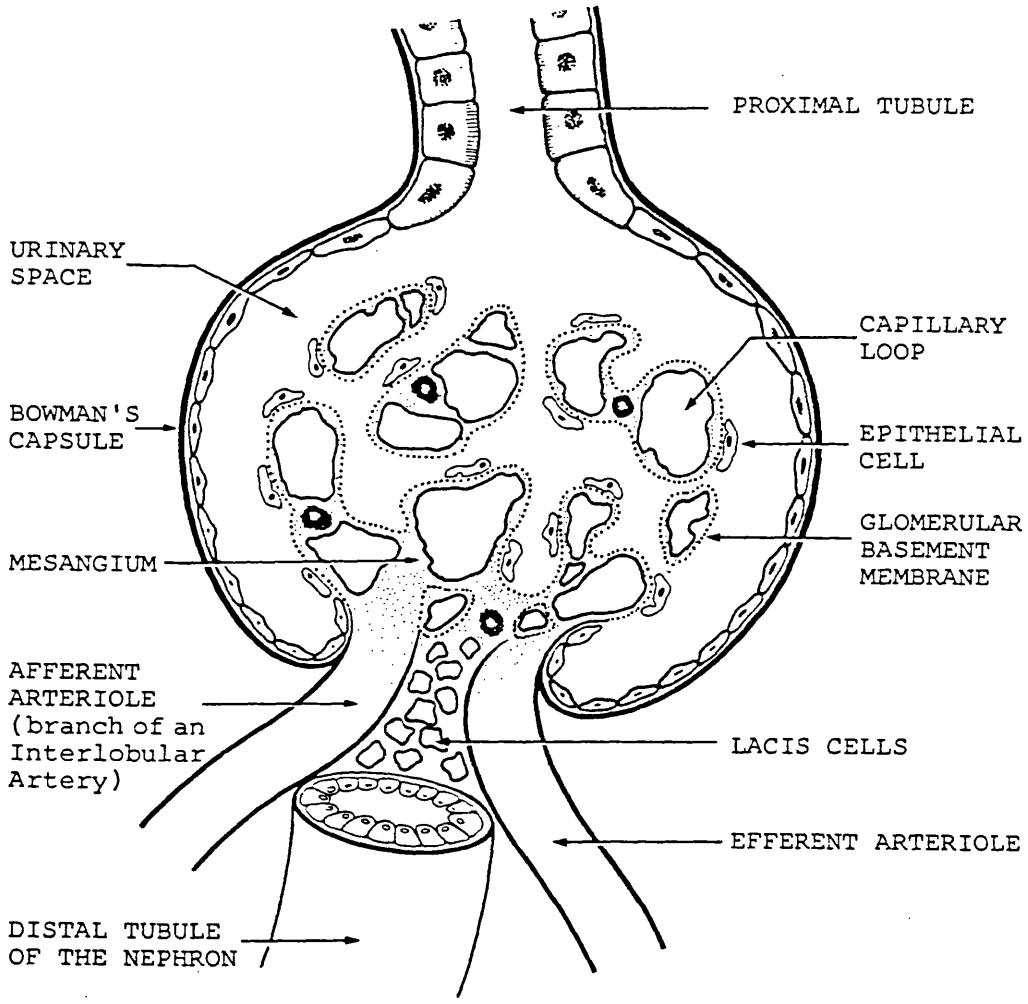
FIGURE 2: Diagrammatic Representation of a Nephron, Showing its Location in the Cortex and Medulla



layer of cells, and has a prominent brush border composed of long microvilli projecting into the lumen to increase the surface area. Between 70-90% of the sodium, chloride, potassium and water are reabsorbed here so that the composition of the filtrate remains similar to plasma, and in addition there is selective reabsorption of other important metabolites. Towards the distal part of the proximal tubule, the secretion of weak acids and weak bases becomes more prominent.

As the nephron passes into the medulla, it forms a U-shaped bend called the loop of Henle, from which the nephron runs back towards the cortex as the distal convoluted tubule. The loop of Henle appears to function as a counter current multiplier. Fluid in the descending limb becomes progressively more concentrated during its passage from the cortico-medullary junction to the tip of the loop. In the ascending limb, sodium is reabsorbed more rapidly than water by an ion exchange mechanism. The fluid passing into the distal tubule is more dilute than that which entered the descending limb, and so a gradient is created between the limbs. Water then diffuses out of the descending limb, thus concentrating the contents until an equilibrium is reached between the tubule and the interstitial fluid. After leaving the distal tubule, the urine then enters the collecting ducts and is concentrated as it passes through the medulla before leaving the kidney via the ureter.

FIGURE 3: Diagram of the Glomerulus and Juxtaglomerular Apparatus



Kidney function is rigidly controlled by a variety of hormones, in particular those hormones which activate α_1-adrenoceptors to evoke a cellular response through the mobilization of calcium. In the kidney, activation of α_1-adrenoceptors evokes a multiple response: increased sodium reabsorption (Osborn et al, 1983; Hesse and Johns, 1984), prostanoid production (Cooper and Malik, 1985), gluconeogenesis (Kessar and Saggerson, 1980; McPherson and Summers, 1982), renal vasoconstriction (Schmitz et al, 1981; Smyth et al, 1984; Cooper and Malik, 1985) and inhibition of renin release (Matsumura et al, 1985).

There are therefore many delicate structures present in the nephron which are finely tuned to perform their individual functions. If any of these are damaged, which may occur for example, during ischaemia, it can be seen that the functional ability of the kidney would be considerably impaired.

Ultrastructural Renal Damage During Ischaemia and Reperfusion

During renal transplantation, kidneys are subjected to periods of ischaemia, which can be defined as the reduction or complete cessation of the blood supply, with concomitant starvation of oxygen and nutrients, to the tissue. There are two distinct types of ischaemia. Cold ischaemia (CI) occurs during storage of the organ (usually at a temperature of 0-4^{o}C) whilst awaiting transplantation,

whereas normothermic or warm ischaemia (WI) results when the blood supply is arrested at around 37°C. WI is of clinical importance in reconstructive renal surgery for removal of tumours or for correction of ureteric stricture, when the renal blood supply may be interrupted for considerable periods; indeed, if surgery lasting more than 60 minutes is anticipated, it is essential to cool the kidney at the outset to reduce the extent of damage. It is almost inevitable that some WI damage will occur during the surgical removal of organs prior to hypothermic preservation, but this is always minimized as far as practicable. Both CI and WI progress to the same end point of non-function, differing primarily in the rate at which the injury occurs, since lesions develop much more slowly in cooled ischaemic tissues.

Ischaemia results in an impairment of the energy-dependent functions of the cell. In metabolically active cells, energy sources are quickly depleted and, as a result, cell membrane transport becomes impaired and cell structural integrity may be breached (Smolens and Stein, 1981). Following renal transplantation, during which there is usually a short period of WI followed by a prolonged period of CI, a frequent complication is acute renal failure (ARF).

ARF was first recognized during the Second World War (Bywaters and Beall, 1941) when post-traumatic renal failure was observed in victims of crush injuries and bomb

blasts. ARF is a syndrome in which there is a rapid, but frequently reversible reduction in renal function, as opposed to chronic renal failure which develops over a longer period and is essentially irreversible. Renal ischaemia has been shown to precipitate ARF in a variety of animal models (Stein et al, 1978). Macroscopically, as a result of ARF, kidneys appear enlarged with a pale cortex and a dark and congested medulla. Studies have shown that there is a correlation between the degree of renal ischaemia and the amount of morphological injury and functional impairment that result. For example, total clamping of the renal artery for 25 minutes in rats causes functional impairment which is reversed within hours, whereas application of the clamps for 60 minutes results in renal dysfunction which persists for several weeks (Venkatachalam et al, 1980).

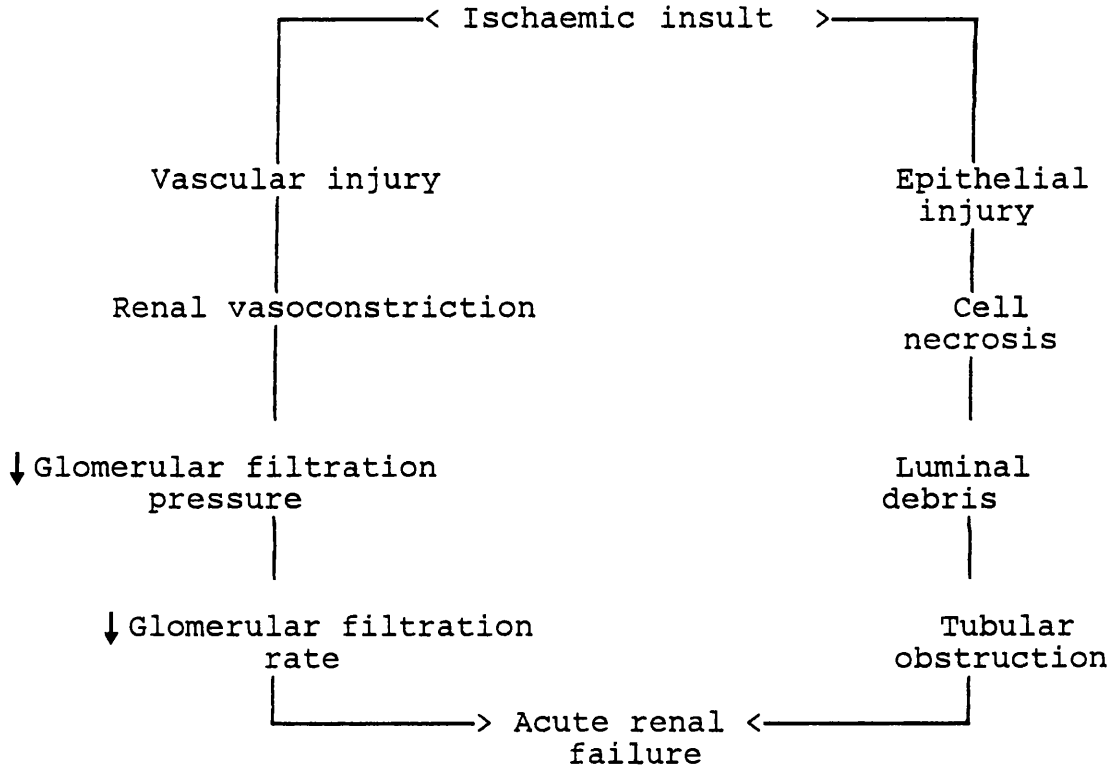
In the kidney, metabolic work is primarily related to the reabsorption of solutes by the renal tubule (Lassen et al, 1961). The cells most susceptible to ischaemic damage in these regions are the tubular cells. If a rat kidney is clamped for 40-60 minutes, a lesion is produced resembling ARF in humans, both clinically and histopathologically (Cronin et al, 1978). Two distinct lesions have been identified: intratubular cast formation, which leads to a rise in intratubular pressure and a fall in the glomerular filtration rate (GFR); and tubular wall necrosis, resulting in loss of the brush border membrane and leakage of tubular

fluid back into the peritubular space and capillaries (Venkatachalam et al, 1978). More recently, Galat et al (1988) demonstrated that 10 minutes WI resulted in a fall in GFR, oliguria and severe tubular dysfunction in the rat kidney. As a result of this, the excretory capacity of the kidney is drastically impaired (Stein et al, 1978). Increased concentrations of urinary sodium have also been observed following 30 minutes WI (Gregg et al, 1986) indicating a breakdown in the reabsorption mechanism.

There is also some evidence that vascular injury plays a role in ARF. Studies of the pathogenesis of the injury incriminate a vascular component in the initial phase of ischaemic ARF which is maintained due to tubular obstruction. Figure 4 shows the potential interaction between the vascular injury and tubular injury of ischaemic ARF. Glomerular injury has also been shown to occur following kidney preservation (Lambert et al, 1986), but most damage is thought to be located in the tubular cells. In the kidney, post-ischaemic injury can result in necrosis of proximal renal tubules and accumulation or "trapping" of erythrocytes in the outer medulla (Jacobsson et al, 1988). In most human studies, renal blood flow during ARF is reduced by 70% and the GFR falls to almost zero (Linton, 1987).

Calcium entry blockers have been shown to protect against ARF in several models (Schrier et al, 1987). Antagonists prevent or attenuate vasoconstrictor

FIGURE 4: Vascular and Epithelial Components of Ischaemic Renal Cell Injury Which Lead to Acute Renal Failure



(norepinephrine, vassopressin and angiotensin II) mediated contraction of glomerular mesangial cells and thus allow preservation of glomerular capillary permeability.

It can therefore be seen that the effects of any length of ischaemia on the kidney are deleterious, and that the more extended the ischaemic period, the greater the ultrastructural damage that occurs.

Biochemical Damage During Ischaemia and Reperfusion

With the onset of ischaemia, the cell enters a potentially reversible phase of catabolism with depletion of high energy phosphate stores. As the ischaemic period continues, metabolic alterations ultimately lead to the accumulation of toxic products, ionic shifts, membrane destabilization and irreversible injury resulting in cell death. This sequence of pathological events has been widely investigated and reported. Calman et al (1973) observed the depletion of cellular stores of high energy nucleotides, and found that the total adenine nucleotide content correlated with subsequent kidney viability. With its rich, high-flow blood supply, the cortex operates primarily under conditions of aerobic metabolism. The medulla, however, shows a strong adaptation to anaerobic glycolysis (Bonventre, 1984), presumably because of the lower flow rate through its vasculature and the higher energy demands in this region. Consequently, with the onset of ischaemia, there is less of a fall in tissue ATP

levels in the medulla as compared to the cortex, and a correspondingly lower accumulation of tissue lactate levels. Although this phenomenon might be expected to protect the medulla from ischaemic stress, metabolic studies in the rat heart (Neely and Feuvray, 1981) have shown that glycolytic products such as NADH, H^+, and lactate may themselves be initiators of ischaemic cell damage.

Various metabolites have also been shown to accumulate during ischaemia, especially H^+ ions (Chan et al, 1983), and intrarenal pH was shown to fall rapidly and progressively during ischaemia of rat kidneys, to levels as low as pH 6.3 (Sehr et al, 1979). Warm ischaemia and hypothermic reperfusion of canine kidneys were both found to induce an increase in free lysosomal enzymes in renal cortex (Pavlock et al, 1984), resulting in tissue damage by autolysis. Phospholipids are major components of cellular membranes, modulating both structure and function. Damage to membranes has been demonstrated in ischaemic canine kidneys, with subsequent loss of the constituent phospholipids of cortical tissue and mitochondria (Southard et al, 1984). Free fatty acid accumulation has been demonstrated in a variety of ischaemic tissues (Rehncrona et al, 1982; Shui, Nemmer and Nemoto, 1983; Nemoto et al, 1987), probably as a result of calcium-dependent phospholipase activation and the blockade of fatty acid esterification due to shortage of ATP.

Enzymes are also inactivated during ischaemia. ATPase

and 5'-nucleotidase, enzyme markers for mitochondria and the plasma membrane respectively, declined rapidly in the cortex of rat kidneys following WI, and the activities of the microsomal enzymes glucose-6-phosphatase and NADPH-cytochrome c reductase were also reduced, but less rapidly (Jung and Beyer, 1985). The early decrease in ATPase and 5'-nucleotidase may have been due to membrane alterations during ischaemia, but other effects such as acidosis, the action of proteases or the breakdown or loss of catalytic function are also possible mechanisms affecting enzyme inactivation. The level of marker enzymes circulating in the plasma has been utilized to assess the extent of ischaemic cell damage in renal allografts at the onset of reperfusion (Maessen et al, 1989). Vascular injury resulting in oedema, loss of circulating proteins and the leakage of blood cells into the extravascular surroundings have also been observed as a direct result of ischaemia (Green, 1976).

Cytosolic calcium levels are kept low compared with the extracellular medium and this is controlled by a variety of mechanisms, many of which require ATP (Humes, 1986). Hence, during ischaemia depleted ATP levels may contribute to a redistribution of intracellular calcium resulting in increased cytoplasmic calcium concentrations. Investigations in a number of systems have linked irreversible cell injury with increases in the concentration of calcium in the cytoplasm (Farber, 1981).

This suggests the possibility that elevated intracellular calcium levels may be the common denominator which triggers cell death and it is therefore possible that calcium ions play a role in the degenerative processes resulting from ischaemia and reperfusion (this will be discussed later).

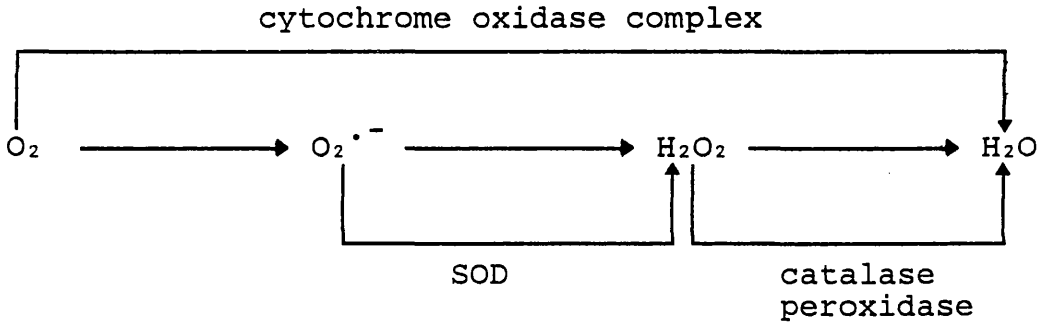
Oxygen-derived free radicals have also been implicated in ischaemia and reperfusion damage to tissues although the mechanisms of damage are yet to be fully elucidated.

Production and Reactivity of Oxygen-Derived Free Radicals

There is now considerable evidence that the production of oxygen-derived free radicals may be a primary cause of the tissue damage which accompanies ischaemia and reperfusion. A free radical can be defined as any species capable of independent existence which contains one or more unpaired electrons (Halliwell and Gutteridge, 1985). This renders the species paramagnetic, and often makes these species highly reactive.

The oxygen molecule itself is a radical, having two unpaired electrons. More reactive forms of oxygen, known as singlet oxygen, can be generated by an input of energy. If a single electron is added to the ground-state O_2 molecule, the product is the superoxide anion ($O_2^{\cdot -}$). Adding a further electron gives the peroxide ion (O_2^{2-}) which is not a radical. The O-O bond in hydrogen peroxide (H_2O_2), which exists in biological systems, is relatively weak; hence, H_2O_2 readily decomposes and may, in the

presence of catalytic transition metals, produce the hydroxyl radical ($OH^{\cdot}$). During normal metabolism, mitochondrial cytochrome c oxidase transfers four electrons, in one electron steps to molecular oxygen with the production of water and the conversion of NADH to NAD^{+} (Antonini et al, 1970). However, some single electrons may leak from the transport chain to produce $O_2^{\cdot -}$. To protect against free radical damage to the tissue, endogenous enzymes and antioxidants are present which will scavenge these free radicals eventually to form water:



Superoxide dismutase (SOD) "dismutes" two superoxide anions to produce hydrogen peroxide (Fridovich, 1975):

$$O_2^{\cdot -} + O_2^{\cdot -} + 2H^{+} \longrightarrow H_2O_2 + O_2$$

Hydrogen peroxide is then prevented from decomposing to generate hydroxyl radicals by the action of catalases and peroxidases, particularly glutathione peroxidase, which convert it into water:

Catalases:			$2H_2O_2$	——————>	$2H_2O$	+	O_2
Peroxidases:	AH_2	+	H_2O_2	——————>	$2H_2O$	+	A
glutathione peroxidase	2GSH	+	H_2O_2	——————>	$2H_2O$	+	GSSG

However, on reperfusion of ischaemic tissues, the sudden burst of molecular oxygen may liberate a substantial amount of $O_2^{\bullet -}$. It is possible that the endogenous defence mechanisms are overwhelmed as a result, leading to the uncontrolled formation of free radicals.

The most damaging oxygen-derived free radicals are the hydroxyl radical ($OH^{\bullet}$) and iron-complexed radicals (Gutteridge, 1984; Minotti and Aust, 1987). The production of $OH^{\bullet}$ from H_2O_2 is catalyzed by transition metals such as copper and, in particular, iron (Halliwell and Gutteridge, 1984), via the metal-catalysed Haber-Weiss reaction (Haber and Weiss, 1934) (Figure 5). $O_2^{\bullet -}$ is initially protonated to yield H_2O_2 and O_2 (step 1). Ferric ions then react with a further molecule of $O_2^{\bullet -}$ to produce ferrous ions (step 2), which then react with H_2O_2 to yield $OH^{\bullet}$ and regenerate ferric ions (step 3), which can then participate again in step 2. It can therefore be seen that a relatively small quantity of catalytic iron could produce a large flux of $OH^{\bullet}$. This can then react at extremely high rate constants with almost every type of molecule found in living cells, including sugars (Halliwell and Gutteridge, 1981), amino acids, DNA bases (Loeb et al, 1988), phospholipids

FIGURE 5: Production of the Hydroxyl Radical via the Iron-Catalysed Haber-Weiss Reaction

$$2O_2^{\bullet -} + 2H^+ \longrightarrow H_2O_2 + O_2 \qquad (1)$$

$$Fe^{3+} + O_2^{\bullet -} \longrightarrow Fe^{2+} + O_2 \qquad (2)$$

$$Fe^{2+} + H_2O_2 \longrightarrow OH^{\bullet} + OH^- + Fe^{3+} \qquad (3)$$

Key: $O_2^{\bullet -}$: superoxide anion; $OH^{\bullet}$: hydroxyl radical; H_2O_2 : hydrogen peroxide; Fe^{2+}; ferrous ions; Fe^{3+} : ferric ions.

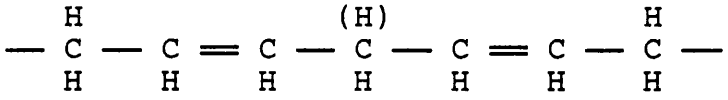
(Wills, 1969; Gutteridge, 1984) and constituents of erythrocyte membranes (Rice-Evans and Baysal, 1987).

Endogenous antioxidants provide excellent protection against oxygen-derived free radical attack; these include vitamin E (α-tocopherol), ascorbate, uric acid, and glutathione. Vitamin E protects the membrane against lipid peroxidation, a chain reaction initiated by free radicals (Porter, and Whelan, 1983). Vitamin E quenches singlet O_2 and also reacts with $O_2^{\cdot -}$ although this reaction is slow and $O_2^{\cdot -}$ does not itself initiate lipid peroxidation. Probably of the greatest importance in most membranes is the fact that vitamin E reacts with lipid peroxy radicals, which are produced during lipid peroxidation. Vitamin E radicals are formed which are insufficiently reactive to abstract H atoms from the membrane lipids and thus interrupts the chain reaction of lipid peroxidation. The vitamin E radicals produced are fairly stable because the unpaired electron on the oxygen atom can be delocalized into the aromatic ring structure and vitamin E may be regenerated by interaction of the vitamin E radical with ascorbate (Tappel, 1968).

Free Radical-Mediated Lipid Peroxidation

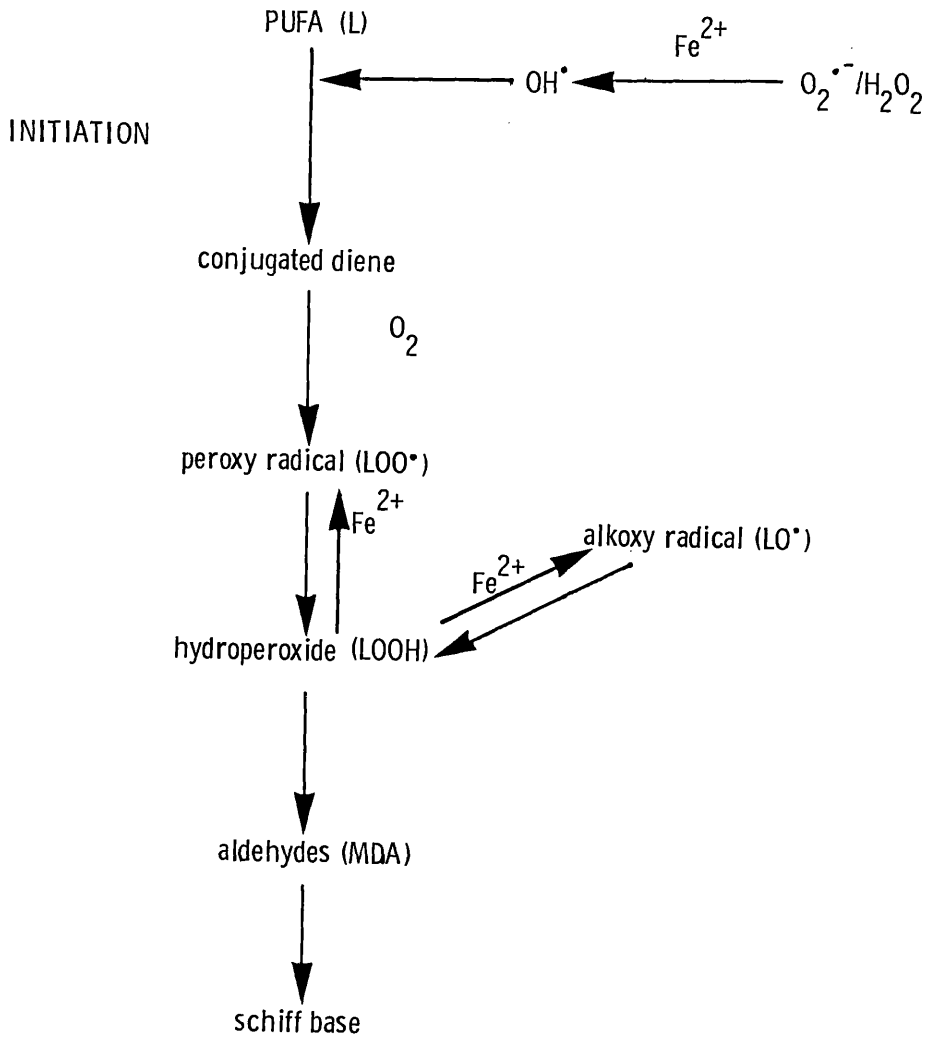
Biological membranes consist of a lamellar lipid bilayer, with proteins and antioxidants such as vitamin E associated with (Diplock and Lucy, 1973). Both the plasma membrane and subcellular organelles are major sites of

lipid peroxidation. Mitochondrial membranes contain relatively large amounts of polyunsaturated fatty acids (PUFAs) in their phospholipids, with up to 6 double bonds in each fatty acid structure. It is because of this unique structure of methylene-interrupted double bonds in PUFAs that biological membranes are highly susceptible to autoxidation:



OH• will abstract hydrogen atoms (H) from membrane PUFAs, leaving an unpaired electron on the carbon atom. This carbon radical is then stabilized by molecular rearrangement to produce a conjugated diene (L•) (Figure 6), which rapidly reacts with O_2 to give hydroperoxy (LOO•) or alkoxy radicals (LO•). Hydroperoxy radicals can then abstract hydrogen atoms from other lipid molecules, thus setting up the chain reaction known as lipid peroxidation (Figure 6). The hydroperoxy radical combines with the hydrogen atom that it abstracts to give a lipid hydroperoxide (LOOH). Lipid hydroperoxides are relatively stable, but transition metals such as iron or complexes of iron will catalyze their decomposition, thus propagating the peroxidative cascade.

FIGURE 6: Lipid Peroxidation



Lipid hydroperoxides will also fragment to form a large variety of aldehydes including malondialdehyde (MDA):

$$LOOH \longrightarrow OHC\text{-}CH_2\text{-}CHO$$

(MDA)

Aldehydes can then undergo a condensation reaction with two molecules of primary amine donors such as amino acids, lipids, proteins or DNA to form an N,N^1-disubstituted 1,3 imino propene structure, commonly known as a Schiff base:

$$R_1NH_2 + (OHC\text{-}CH_2\text{-}CHO) + H_2NR_2 \longrightarrow R_1N{=}CH{-}CH{=}CH{-}NHR_2 \text{ (Schiff base) } +2H_2O$$

Malondialdehyde, Schiff bases and diene conjugates can all be detected spectrophotometrically and used as markers of lipid peroxidation and hence free radical damage.

Peroxidation of membrane phospholipids is a highly destructive process which may render the membrane permeable and has been shown to increase membrane rigidity (Demopoulos et al, 1980). Furthermore, previous investigations have established that the activity of rat liver microsomal calcium-dependent phospholipase A2 is higher in rigid membranes and decreases when the membrane is fluidized (Momchilova, Petkova and Koumanov, 1986a; 1986b). Phospholipase A2 hydrolyses phospholipids

releasing free fatty acids and leaving behind residual lysophosphatides, both of which can be deleterious to cell function (see later).

Free Radical Damage During Ischaemia and Reperfusion

There is mounting evidence for the involvement of oxygen-derived free radicals in ischaemic/reperfusion damage to kidneys. It has been suggested that free radicals may degrade the glomerular membrane and alter glomerular and tubular cell function (Laurent and Ardaillou, 1986), thus implicating these species as a cause of ARF. Membrane-bound sodium/potassium ATPase activity is depressed by free radicals and during renal ischaemia (Kako et al, 1988) which would upset the cellular sodium concentration. Free radical-mediated damage to the endothelial cells in the outer medulla may be particularly important in reperfusion injury (Ratych and Bulkley, 1986). However, most evidence for free radical involvement in ischaemic and reperfusion damage comes indirectly from the examination of the effects of free radical scavengers, such as superoxide dismutase (SOD).

The effect of SOD on various parameters of kidney function have been examined following ischaemia. Paller et al (1984) demonstrated that inulin clearance and renal blood flow increased and tubular injury decreased in rat kidneys subjected to WI when SOD was administered prior to reperfusion. An improved survival rate of 60% compared to

19% and a lowering of serum creatinine was observed in a similar model when comparing SOD-treated to untreated ischaemic controls (Baker et al, 1985). Another animal model commonly used is the porcine kidney. In an experiment designed to mimic human cadaveric renal transplantation, both SOD and allopurinol improved creatinine clearance when administered at the start of reperfusion following 24 hours cold storage in Euro-Collins solution (Koyama et al, 1985) A combined infusion of SOD and catalase during reperfusion also improved renal function following 45 minutes WI in pig kidneys (Bosco and Schweizer, 1988).

As already mentioned, cellular stores of high energy phosphates become rapidly depleted during ischaemia. ATP is degraded to AMP, which is subsequently catabolized to adenosine, inosine and hypoxanthine. Under normal metabolic conditions, hypoxanthine is then irreversibly converted to uric acid by the enzyme xanthine dehydrogenase (Roy and McCord, 1983):

$$\text{hypoxanthine} + H_2O + NAD^+ \longrightarrow \text{uric acid} + NADH + H^+$$

However, during ischaemia, without the energy supply to maintain ion gradients, intracellular calcium levels may rise, activating calmodulin-regulated enzymes. In the ischaemic intestine a calcium-dependent protease (Roy and McCord, 1983) and in other tissues a sulfhydryl oxidase

(Clare et al, 1981) can promote the conversion of xanthine dehydrogenase to xanthine oxidase. This conversion has been demonstrated in ischaemic rat tissues (Engerson et al, 1987). Xanthine oxidase also catalyzes the breakdown of hypoxanthine but, unlike xanthine dehydrogenase, utilizes molecular oxygen which is in abundance on reperfusion of the tissue, and liberates the superoxide radical ($O_2^{\cdot -}$):

$$\text{hypoxanthine} + H_2O + 2O_2 \longrightarrow \text{uric acid} + 2O_2^{\cdot -} + 2H^+$$

The conversion of xanthine dehydrogenase to xanthine oxidase is organ-dependent, taking only 1 minute in the ischaemic rat intestine, but up to 30 minutes in the ischaemic rat kidney (Battelli et al, 1972). Renal xanthine oxidase content varies between species, being highest in the rat at 1,188 mU/g protein and lowest in man at 96mU/g protein (Krenitsky et al, 1974). As a result of the irreversible conversion of hypoxanthine to uric acid, the former is lost from the available substrate pool, and hence is no longer available for reconversion to adenine nucleotides during cell recovery. It has been demonstrated that allopurinol, an inhibitor of xanthine oxidase, improved recovery of renal function in experimental animals following warm ischaemia (Vasko et al, 1972; Owens et al, 1974).

It has recently been recognized that significant tissue injury can also occur during reperfusion. It is

fashionable to talk of reperfusion damage almost as if it occurs independently of the ischaemic period. However the severity of reperfusion injury is highly dependent on the pathological processes occurring as a result of ischaemia. Postischaemic renal failure may be mediated by a variety of oxygen-derived products including oxygen-free radicals and arachidonic acid metabolites such as thromboxane and prostacyclin (Lelcuk et al, 1985a; Kaufmen et al, 1988). Leukocytes, particulary neutrophils, are a rich source of toxic agents and have been implicated as mediators of postischaemic renal injury (Klausner et al, 1989). An additional mechanism of cell injury during ischaemia and reperfusion is via the abnormal metabolism of oxygen by the mitochondrial electron transport chain. During normal metabolism, mitochondrial cytochrome c oxidase transfers four electrons from NADH to molecular oxygen with the production of water and the conversion of NADH to $NAD^{\cdot}$. However, some single electrons may leak from the transport chain to produce $O_2^{\cdot -}$. During ischaemia some of the components of the electron transport chain accumulate in a reduced state which may result in an increased leakage of electrons onto molecular O_2 during reperfusion, and hence an increased production of $O_2^{-\cdot}$ may occur.

There is also a wealth of evidence for free radical damage during ischaemia and reperfusion in many other organs. Oxidative damage has been demonstrated in a feline model of partial intestinal ischaemia (Granger et al, 1981)

and in the ischaemic myocardium (Shlafer et al, 1982; Rao et al, 1983). It has also been shown that protons and free radicals are responsible for a specific defect in the sarcoplasmic reticulum of the ischaemic myocardium, uncoupling calcium transport from ATP hydrolysis (Hess et al, 1982), and that this situation can be improved by the administration of SOD (Stewart et al, 1983). Administration of six different anti-free radical agents reduced the incidence of reperfusion-induced arrhythmias (Bernier et al, 1986). SOD also protected against a fall in sarcolemmal sodium/potassium ATPase activity during ischaemia and reperfusion (Kim and Akera, 1987), and free radical damage plus histamine release has recently been proposed as a cause of reperfusion damage in the guinea pig heart (Masini et al, 1989). Ischaemic/reperfusion damage due to free radicals has also been reported in lungs (Stuart et al, 1985; Ward et al, 1986), liver (Marubayashi et al, 1982), pancreas (Sanfey et al, 1984), small intestine (Parks et al, 1982; Schoenberg et al, 1983) and skin (Manson et al, 1983). A role for oxygen free radicals has also been suggested in skeletal muscle damage (Jackson et al, 1985) and circulatory shock (Parks et al, 1983; McCord, 1985); the latter can be viewed as "whole body partial ischaemia" since all tissues may be insufficiently perfused. In the pathogenesis of rheumatoid disease, $OH^{\bullet}$ formation is thought to occur in the synovial fluid (Rowley and Halliwell, 1983; Blake et al, 1985).

It would therefore appear that oxygen-derived free radicals may play an active role in the pathogenesis of ischaemic/reperfusion damage in many different organs. Furthermore, an alteration in calcium homeostasis appears to be an important determinant of damage.

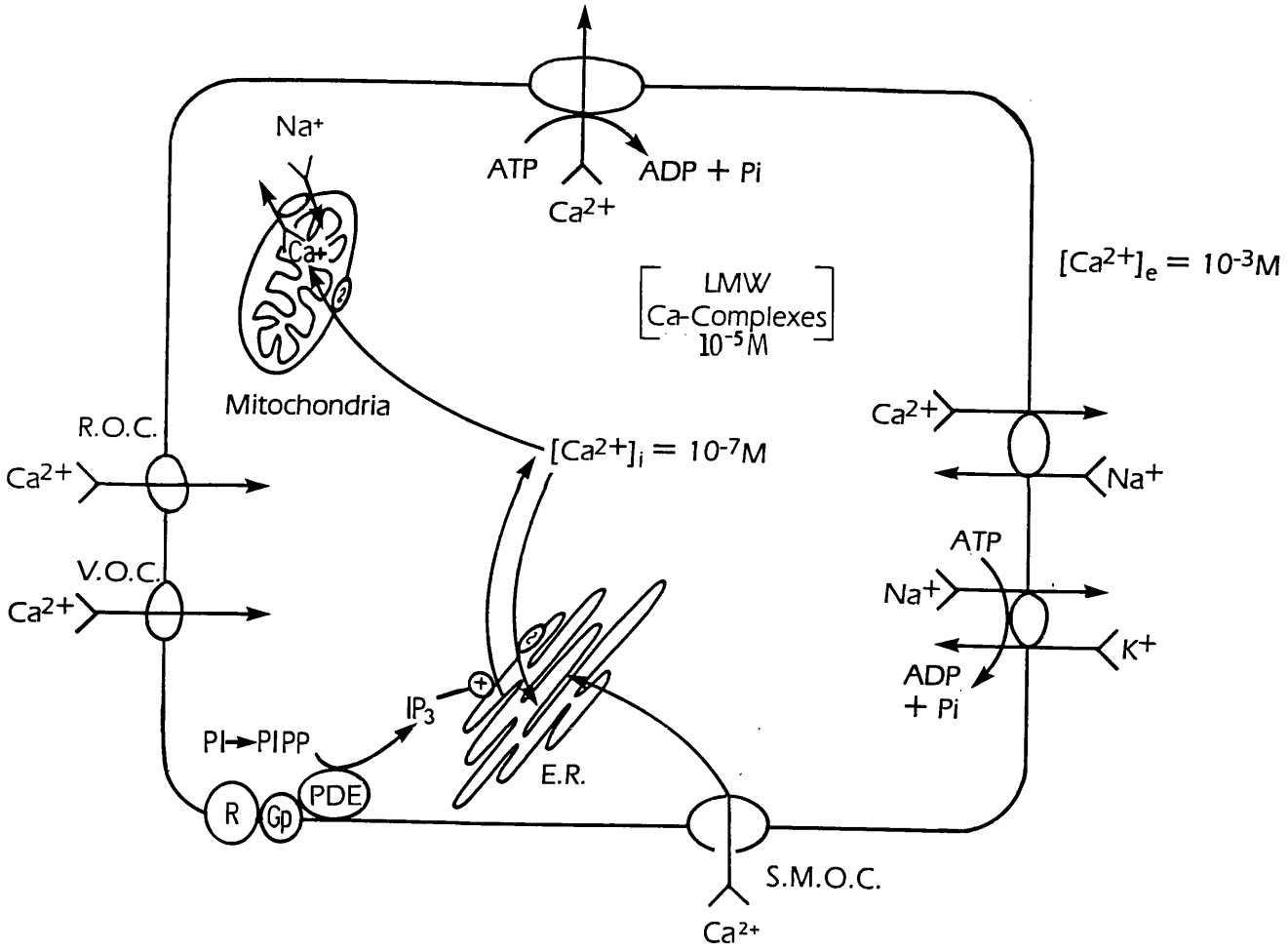
The Role of Calcium in the Cell

Calcium ions are now known to play a crucial role in the regulation of many biological functions including cell activation, stimulation of motile and contractile systems, excitation-contraction of muscle cells, hormonal secretion and control, and regulation of cell metabolism (Bygrave, 1978; Carafoli, 1987). Accurate control of the cytosolic calcium concentration is vital as persistent, large elevations in intracellular calcium have deleterious effects and can ultimately lead to cell death (Farber, 1981).

Normally, in intact cells, the gradient between relatively high (10^{-3}M) extracellular concentrations and a low (10^{-7}M) cytosolic concentration is maintained by various control mechanisms (Figure 7). Intracellular calcium is unevenly distributed in cells being found mostly in one of three major calcium pools: one bound to plasma membranes, another bound to or sequestered within intracellular organelles and a third pool either bound or free in the cytosol. Table 1 gives an indication of the distribution of calcium in a typical mammalian cell. The figures are

Cytosolic calcium levels are maintained at relatively low concentrations (10^{-7}) by active removal via a plasma membrane ATPase and also sequestration by the mitochondria and ER. Calcium may also leave the cell passively via a calcium/sodium exchanger which is closely related to a sodium/potassium exchange mechanism. Calcium entry is facilitated through receptor-operated channels (ROCs), voltage-operated channels (VOCs) and also secondary messenger-operated channels (SMOCs) which become operational, for example, with increased PI turnover.

FIGURE 7: The Regulation of Calcium Homeostasis in a Normal Mammalian Cell



only approximate and vary depending on cell type.

Soluble proteins involved in calcium regulation and signalling are called "calcium-modulated proteins". Examples are troponin C, parvalbumin and calmodulin (Carafoli, 1987). Calmodulin is probably the most important of the high affinity calcium binding proteins since it is present in considerable amounts in all eukaryotic cells. It is now known to interact with a large number of cellular enzymes, and to mediate their function; for example the calcium pumping ATPase on the plasma membrane (Klee and Vanaman, 1982).

TABLE 1: Estimated Calcium Distribution in a Typical Mammalian Cell

Intracellular compartment		Percentage of total cell ion content
Nucleus		50
Mitochondria		30
Microsomes		14
Cell membrane (mainly extracellular)		5
Cytoplasm	bound	0.5
	free	0.005

Opening of various channels in the plasma membrane allows calcium to diffuse passively into cells, down its steep electrochemical gradient. Voltage-operated calcium channels (VOCs) are gated by the electrical potential across the plasma membrane and can be blocked by benzothiazepines and dihydropyridines. Calcium entry through the plasma membrane may also be facilitated through receptor-operated channels (ROCs) in which receptor and channel functions coexist in one single or two closely adjacent molecules and therefore receptor activation and channel opening are intimately interconnected. There also exist secondary messenger-operated channels (SMOCs) which are believed to be opened by second messengers generated within the cells in response to receptor activation allowing calcium influx (Meldolesi and Pozzan, 1987). Influx of calcium is balanced by active calcium pumping as well as by calcium exchange with sodium. The calcium ATPase extrudes calcium from the cell electroneutrally in exchange with protons. The ATPase in most cell types has a high affinity but a low capacity for calcium, and each of these are increased by calmodulin (Schatzmann, 1966). The sodium/calcium exchange mechanism exchanges three sodiums per calcium. Since it is electrogenic, the directions in which the ions move depend on the transmembrane potential. Upon depolarization, calcium influx occurs, whereas with a polarized membrane potential (negative inside) calcium is reflected out of the cell (Reuter and Seitz, 1968).

Calcium can also be specifically segregated within membrane-bounded organelles. Mitochondria avidly transport available calcium (Rasmussen and Goodman, 1977) and are important in the maintenance of calcium homeostasis. Calcium also plays a major role in mitochondrial membrane function. The mechanism of mitochondrial calcium uptake is directly linked to oxidative phosphorylation with calcium uptake taking precedence (Chance, 1965). Moderate accumulation of calcium by mitochondria causes an alteration in the permeability characteristics of the inner mitochondrial membrane that permit it to engage in efficient oxidative phosphorylation (Parce et al, 1980). Transfer of protons across the inner mitochondrial membrane sets up an electrochemical gradient which allows the synthesis of ATP from ADP and inorganic phosphate and also drives mitochondrial calcium uptake (Humes, 1986). Calcium uptake occurs via an electrophoretic uniport pathway which can be inhibited by magnesium ions and the polysaccharide dye ruthenium red (Richter and Frei, 1988). Mitochondrial calcium overload is deleterious resulting in a spontaneous release. This calcium efflux occurs via both ruthenium red-sensitive and ruthenium red-insensitive pathways.

The endoplasmic reticulum (ER) is thought to be the most important organelle for the regulation of cytosolic calcium homeostasis. It has been shown to be the major intracellular calcium store (Somlyo et al, 1985), actively sequestering calcium via an ATP-dependent pump (Berridge,

1986). Furthermore, isolated mitochondria lower the external calcium to about 0.5μM, while addition of ER reduces it to 0.2μM (Richter and Frei, 1988). The ER also provides the important calcium store for the phosphatidylinositol-derived secondary messenger system (Berridge, 1984).

In the kidney, control of the filtration and transport functions are modulated by a variety of hormones, many of which evoke a secondary messenger response involving the hydrolysis of inositol lipids to form inositol-1,4,5-trisphosphate (IP_3) and diacyl glycerol (DAG) (Berridge, 1987). IP_3 binds to a receptor on an IP_3-sensitive microsomal store (probably the ER) which then releases calcium into the cytosol by a mechanism not requiring energy (Berridge, 1986). Calcium is constantly cycling across the ER membrane with the passive efflux component being balanced by the active uptake of calcium by the ATP-dependent pump. In the liver, calcium cycling appears to be very dependent on potassium functioning as a counter ion to neutralise the charge movement which occurs during the uptake or efflux of calcium (Muallem et al, 1985).

With such a universal messenger as calcium, it is clearly important for the cell to rigidly control calcium homeostasis by the mechanisms described above. However, under certain circumstances, for example ischaemia, the cell may lose this control and this may eventually lead to calcium overload and ultimately to cell death.

The Polyphosphatidylinositol Cycle

The initial discovery that hormones have effects on phosphoinositide metabolism was made by Hokin and Hokin (1954). Since then many researchers have shown that a large variety of agonists including neurotransmitters, peptide hormones and growth factors (Berridge, 1984) can stimulate an increase in the metabolism of inositol lipids. Agonists seem to be specific for a particular receptor class such as the muscarinic cholinergic receptor, the α_1-adrenergic receptor, the H_1-histaminergic receptor or the V_1-vasopressin receptor. In the kidney, activation of α_1-adrenoceptors evokes a multiple response: increased sodium reabsorption (Osborn et al, 1983; Hesse and Johns, 1984), prostanoid production (Cooper and Malik, 1985), gluconeogenesis (Kessar and Saggerson, 1980; McPherson and Summers, 1982), renal vasoconstriction (Schmitz et al, 1981; Smyth et al, 1984; Cooper and Malik, 1985) and inhibition of renin release (Matsumura et al, 1985).

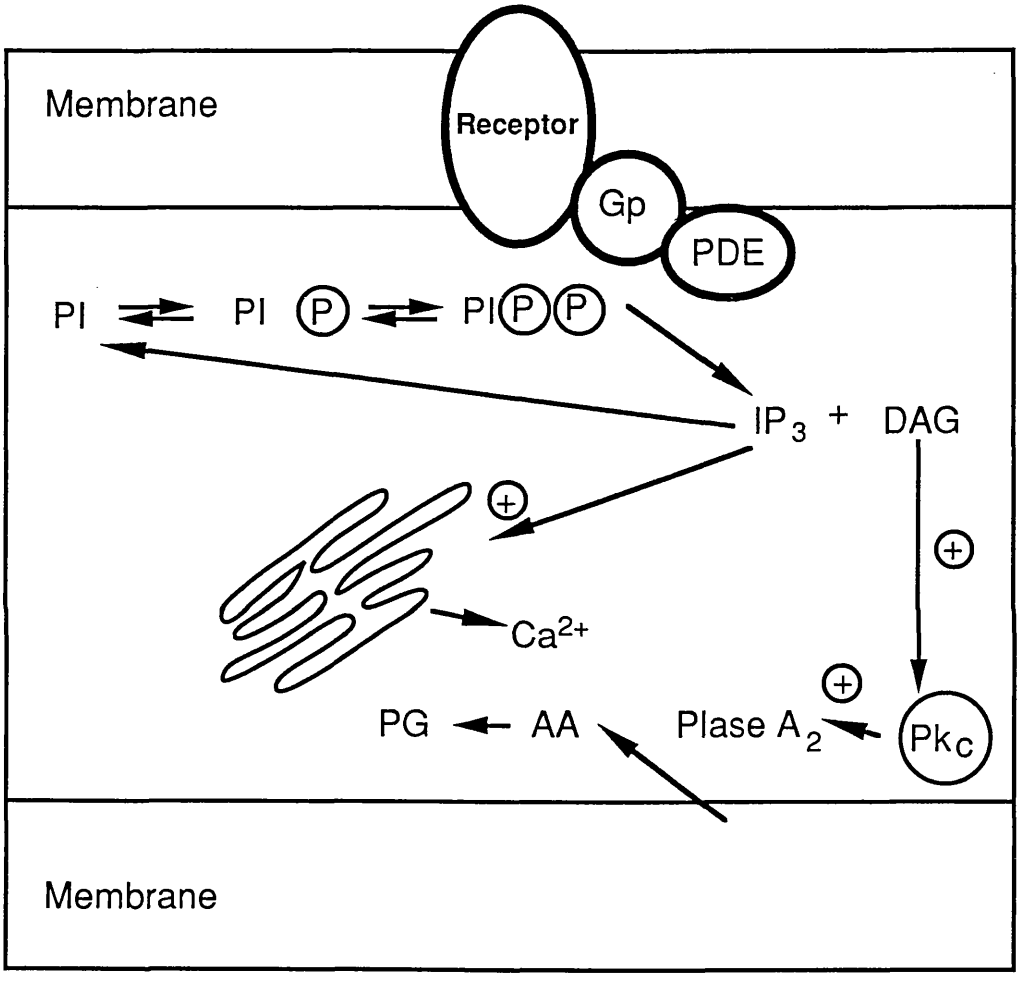
A primary function of the inositol lipids in cell signalling is to function as a precursor for the generation of second messengers such as inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). These are derived from the agonist-dependent hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) which releases IP_3 while DAG remains within the plane of the membrane where it activates protein kinase C (Figure 8). The binding of an agonist to the receptor induces a conformational change in the receptor,

which in turn perturbs the membrane sufficiently to make PIP_2 more accessible to a PIP_2-specific phosphodiesterase (PDE). The coupling of the receptor to the phosphodiesterase is thought to be controlled by a GTP-binding protein (G_p) (Gomperts, 1983).

The intracellular levels of DAG and IP_3 are determined by a balance between their rate of formation and removal by two pathways which channel them back to phosphatidylinositol. DAG is converted by DAG kinase to phosphatidic acid which reacts with CTP to form CDP-DAG. This in turn reacts with inositol to re-form phosphatidylinositol. IP_3 is converted to inositol 1,4-bisphosphate (IP_2) by inositol trisphosphatase and then to inositol 1-phosphate (IP_1) by inositol bisphosphatase. IP_1 is finally converted to free inositol by an inositol 1-phosphatase that is markedly inhibited by lithium, which therefore results in a build up in inositol phosphates (Berridge et al, 1982).

Calcium mobilization from intracellular calcium stores is brought about by IP_3 binding to a receptor on the ER and stimulating a release of calcium from this store which acts through calmodulin to phosphorylate certain proteins, so evoking a particular cellular response. The mechanism of IP_3-induced calcium release from the ER still requires further elucidation, but it seems to depend on an increase in the efflux pathway rather than inhibition of the calcium

FIGURE 8: The Polyphosphatidylinositol Cycle



pumps. This efflux is independent of ATP (Berridge, 1986) but has been shown to be enhanced by GTP (Dawson, 1985). Calcium mobilization is comprised of an initial release of calcium from an intracellular calcium pool, followed by a sustained entry of calcium from the extracellular space via a SMOC (Putney, 1986a), resulting in net calcium influx. It was suggested that calcium entry might be activated by the emptying of the intracellular pool (Putney, 1986b). Takemura et al (1989) have recently tested this hypothesis using thapsigargin which inhibits the ER ATPase and depletes the intracellular pool by a mechanism independent of receptor activation and the generation of inositol phosphates. The results indicated that depletion of the intracellular calcium pool alone was capable of initiating a net extracellular calcium influx, although the mechanism of calcium entry is as yet not characterized.

Under normal physiological conditions, protein kinase C is activated by DAG in the presence of membrane phospholipids and calcium. Activated protein kinase C is responsible for the phosphorylation of specific proteins (Berridge, 1984) that then contribute to various physiological processes. Protein kinase C can also be activated by phorbal esters which mimic the stimulatory effect of DAG. DAG can either be phosphorylated to phosphatidic acid by DAG kinase as described previously or it can be hydrolysed by a DAG lipase:

DAG ——————————> MAG + Arachidonic acid

DAG lipase (monoacylglycerol)

During hormonal stimulation there would appear to be a preferential degradation of inositol lipids which carry arachidonic acid on the 2-position. Hence DAG lipase would invariably produce this fatty acid as an end product.

The relationship between stimulated phosphoinositide metabolism and cell functions remains incompletely understood. In the kidney, a variety of cellular functions or biochemical changes associated with stimulated phosphoinositide metabolism in the kidney have now been identified (discussed previously) making it an interesting organ for study.

Calcium Ions and Ischaemic/Reperfusion Damage

In various animal models of ischaemia followed by reperfusion, the consistent biochemical feature is the gain of cellular calcium when the ischaemic cells are reperfused (Nayler, 1988). Furthermore, investigations in a number of cellular systems have linked irreversible cell injury with an increase in cytosolic calcium concentration (Farber, 1981). During ischaemia, ATP levels are rapidly depleted leading to the slowing or inhibition of all cellular energy-dependent processes. Hence the energy-requiring

transport systems located in the ER, plasma membrane and mitochondria would be unable to control cytosolic free calcium levels which would therefore increase. It has been shown that when kidneys are subjected to periods of warm ischaemia, mitochondrial dysfunction coupled with ATP depletion results in failure of the calcium uptake processes of this organelle (Arnold et al, 1986). Renal ischaemia also decreases the ability of ER to sequester calcium (Schieppati et al, 1985). It may also be postulated that as a result of anaerobic inhibition of the ER ATPase, extracellular calcium influx would be activated via the mechanism described by Takemura (1989) providing this mechanism is not energy-dependent. Thus a redistribution of intracellular calcium takes place and previous reports have demonstrated altered calcium homeostasis in tissues subjected to warm ischaemic insults (Bourdillon and Poole-Wilson, 1981) and increased cytosolic calcium levels in cultured kidney cells exposed to anoxia (Snowdowne et al, 1985).

Cytosolic calcium accumulation is highly deleterious to the cell and is responsible for the initiation of a cascade of events which may ultimately result in cell death (Farber, 1981). Mitochondria act as a high capacity calcium sink during conditions where cytosolic calcium is increased. As described previously, mitochondria have been shown to selectively transport calcium at the expense of oxidative phosphorylation (Chance, 1965). This would

further decrease cellular energy available as ATP and so compromise other energy dependent processes. The calcium/sodium exchange mechanism, although indirectly dependent on ATP is also likely to be compromised since sodium/potassium ATPase activity is the predominant determinant of cellular sodium concentration.

Calcium entry blockers have been shown to be effective in reducing damage caused by ischaemia and reperfusion, implicating the involvement of calcium channels on the plasma membrane in facilitating an extracellular calcium influx under these circumstances. Furthermore, mitochondrial β-oxidation of fatty acids is blocked during ischaemia resulting in accumulation of acyl-CoA in mitochondria and of acyl carnitines in the cytosol (Idell-Wenger et al, 1978; Liedtke et al, 1978). Palmitoyl carnitine in particular has been shown to activate calcium channels and therefore may contribute to the deleterious calcium overload which can occur during reperfusion following ischaemia (Spedding and Mir, 1987).

The mechanisms of calcium-induced tissue injury are complex and multifactorial. One probable mechanism is increased calcium-dependent activation of phospholipases, proteases and nucleases resulting in degradation to membranes, proteins and nucleic acids respectively. Activation of phospholipases results in an increase in free fatty acids (FFAs) and lysophosphatides both of which possess detergent-like properties and are deleterious to

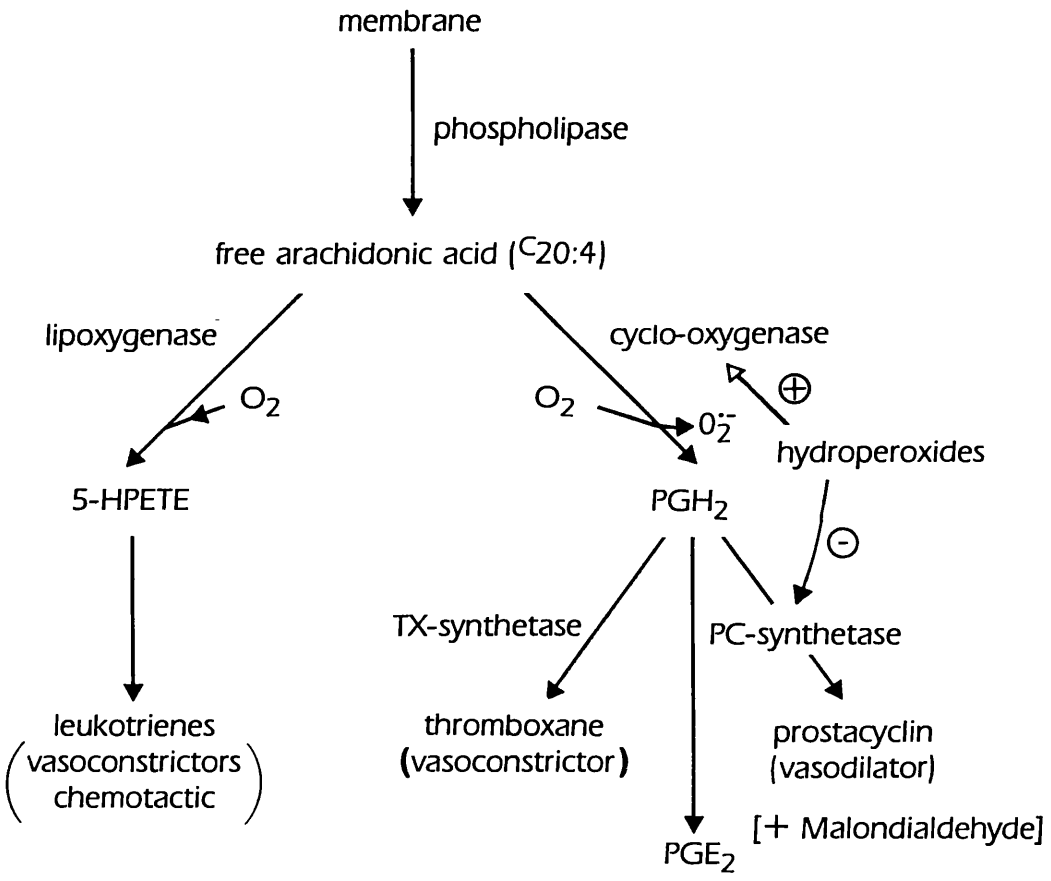
cell function. FFAs are known to accumulate during periods of ischaemia in several tissues (Rehncrona et al, 1982; Shui et al, 1983; Nemoto et al, 1987) and have been implicated in causing mitochondrial injury following anoxia, for example, in the myocardium (Piper and Das, 1986). It has also been shown that phospholipase A2 activity and markers of lipid peroxidation increase in rat small intestinal mucosa during ischaemia (Otamiri et al, 1987). The residual lysophosphatides remaining in the membrane following phospholipase action also increase during ischaemia and are themselves damaging species (Shaikh and Das, 1981). In particular altered configuration of membranes induced by phospholipid hydrolysis may render them more susceptible to attack by free radicals during reperfusion (Ungemach, 1985). Calcium also activates the calcium-dependent protease responsible for the conversion of xanthine dehydrogenase to xanthine oxidase, as described previously. Upon reperfusion, xanthine oxidase catalyzes the breakdown of hypoxanthine utilizing molecular oxygen to liberate the superoxide radical ($O_2^{\cdot -}$) so contributing to reperfusion damage (Roy and McCord, 1983).

Phospholipase activity is the rate limiting step in prostaglandin synthesis and lipoxygenation (Craven and DeRubertis, 1983) and thus these pathways may be stimulated following ischaemia. Free arachidonic acid is the substrate for cyclooxygenase, which utilizes oxygen-

derived free radicals and produces $O_2^{\cdot -}$ (Kontos et al, 1985). The cyclooxygenase-catalysed breakdown of arachidonic acid results initially in the formation of the endoperoxide prostaglandin G_2 (PGG_2). Further enzymic metabolism of PGG_2 produces PGH_2 and subsequently PGE_2, prostacyclin or thromboxanes (Figure 9). This range of products, under normal physiological conditions, maintains normal blood flow through the capillary bed. Prostaglandin E_2 and prostacyclin are both vasodilators, and thromboxane is a vasoconstrictor and induces platelet aggregation (Lefer, 1985). An imbalance in the formation of these products would therefore be detrimental to tissue perfusion and hence indirectly to cellular function. Indeed, PGE_2 has been shown to increase in hypoxic renal mesangial cells (Jelkmann, et al, 1985) and thromboxane A_2 increased following limb ischaemia upon reperfusion (Lelcuk et al, 1985b). In postischaemic renal failure, thromboxane A_2 was elevated, whereas a high prostacyclin to thromboxane A_2 ratio offered protection (Lelcuk et al, 1985a). Furthermore prostacyclin has been shown to be beneficial in renal (Langkopf et al, 1986), hepatic (Okabe et al, 1986) and cerebral ischaemia (Pluta, 1986).

Lipoxygenase also catalyzes the breakdown of arachidonic acid, which results in the production of leukotrienes some of which are chemotactic and cause vasoconstriction (Lefer, 1985; Piper et al, 1985) (Figure 9). The amount of membrane-bound arachidonic acid

FIGURE 9: Cyclooxygenase- and Lipoxygenase-Catalyzed Oxidation of Arachidonic Acid



converted to cyclooxygenase and lipoxygenase products is only a small fraction of the total (Schlondorff and Ardaillou, 1986). An alteration in prostaglandin homeostasis and the formation of lipoxygenase products are likely to result in vascular changes which may contribute to the "no-flow" phenomenon often observed upon reperfusion.

There is growing evidence that oxygen-derived free radicals are responsible, at least in part, for reperfusion injury. Lipid peroxidation affects calcium homeostasis in a number of ways. Peroxidation can render the cell membrane more permeable to calcium ions which would readily leak in from the extracellular milieu. Oxidative damage to the mitochondrion and ER partially destroys their ability to sequester calcium, so leading to an increase in cytoplasmic free calcium (Masini et al, 1985; Bellomo et al, 1985). It has recently been postulated that 4-hydroxynonenal (HNE), an aldehyde produced during lipid peroxidation stimulates adenylate cyclase, guanylate cyclase and PIP_2 breakdown (Dianzani et al, 1989) possibly through modification of the G-protein. Hence, lipid peroxidation might augment PIP_2 breakdown through the regulatory G-protein, increasing cellular calcium and leading to a cellular response. Furthermore, recent work has suggested that the hepatic α_1-receptor is vulnerable to free radical stress which may lead to an impairment of hormonally regulated calcium homeostasis in hepatocytes

(Pruijn and Bast, 1988).

Membrane-bound sodium/potassium ATPase activity is depressed by free radicals during renal ischaemia (Kako et al, 1988) which would upset the cellular sodium concentration. This would also indirectly affect the calcium/sodium exchange mechanism and ultimately lead to an alteration in calcium homeostasis. Hydroperoxides have been shown to induce calcium release from liver mitochondria via oxidation of pyridine nucleotides (Richter and Frei, 1988). This may be particularly relevant during reperfusion where a sudden burst of oxygen occurs. Impairment of the ability of mitochondria to retain calcium may contribute to increased cytosolic calcium levels following reperfusion. Furthermore, Malis and Bonventre (1986), have suggested that calcium and free radicals work synergistically to bring about mitochondrial dysfunction, an effect partially mitigated by an inhibitor of phospholipase A2, suggesting that this enzyme may be an important mediator of calcium potentiated free radical damage. The relationships between lipid peroxidation, calcium ions and cell death are complex and as yet not fully understood. It is hoped that this thesis will significantly contribute to understanding in this field.

The Use of Calcium Antagonists in the Prevention of Ischaemic/Reperfusion Damage

Prevention of ischaemic damage before transplantation

continues to pose a major challenge and there is currently considerable interest in the use of calcium antagonists. Calcium antagonists are a nonhomogeneous group of drugs with widely differing structures, potencies and tissue specificity (table 2). Their common property is the ability to interfere with slow calcium channels. One such slow calcium channel blocker is verapamil and it has been shown that this agent affords some protection to kidneys subjected to periods of cold (Shapiro et al, 1985) or warm (Burke et al, 1983; Goldfarb et al, 1983; Shapiro et al, 1985; Gingrich et al, 1986) ischaemia followed by reperfusion. Other calcium antagonists have been shown to be effective in protecting against ischaemia and reperfusion damage. Nisoldipine decreased ATP loss following ischaemia and reperfusion in myocardium (De Jong, 1985) and in cerebral ischaemia nicardipine, flunarizine and lisoflazine also conferred protection (Alps et al, 1988).

Introduction of calcium antagonists at reperfusion can reduce various aspects of reperfusion injury. In the heart this includes: a reduction in the loss of noradrenalin stores (Nayler and Sturrock, 1985); decreased entry of calcium into cells in the reperfusion period (Nayler et al, 1988); diminished enzyme release (Fitzpatrick and Karmazyn, 1984) and less mechanical 'stunning' after reperfusion (Przyklenk and Kloner, 1988). The beneficial effects of calcium antagonists may be due to changes in renal

TABLE 2: Classification of Calcium Modulators

Agents acting on the plasma membrane

Calcium entry blockers
Group I: Selective calcium entry blockers

(a) Agents selective for slow calcium channels
Phenylalkylamines: verapamil, emopamil.

Dihydropyridines: nifedipine, nicardipine, nitrendipine
Benzothiazepines: diltiazem, fostedil

(b) Agents with no perceived actions on the slow calcium inward current
Diphenylpiperazines: cinnarizine, flunarizine

Group II: Non-selective calcium entry blockers

(a) Agents acting at similar concentration on calcium channels and fast sodium channel

bencyclane, bepridil, caroverine, etafenone.

(b) Agents interacting with calcium channels while having another primary site of action

phenytoin, benextramine, fluperamide, amrinone

Sodium-calcium exchange inhibitors
Amiloride and derivatives

Agents acting within the cell

Acting on sacroplasmic reticulum
Dantrolene, TMB-8

Acting on mitochondria
Ruthenium red

Calmodulin antagonists
Phenothiazines: trifluoperazine, chlorpromazine
Naphtaoene derivatives: W7
Dopamine antagonists: pimozide, haloperidol
Calmidazolium (R 14571)

haemodynamics in the reperfusion period as well as direct effects on calcium fluxes. Previous studies have indicated that verapamil may augment renal blood flow and increase glomerular filtration rate (Bell and Lindner, 1984: Steele and Challoner-Hue, 1985), counteract the intrarenal vasoconstriction produced by norepinephrine (Malis et al, 1983) and reverse the renal cortical actions of angiotensin II (Ichikawa et al, 1979). Recently Opie (1989) suggested that the gain of calcium in reperfusion injury is only to a small extent dependent on voltage-dependent calcium channels, and that other mechanisms such as calcium entry via sodium/calcium exchange must also be considered. Although there are no specific inhibitors of this system, amiloride and its derivatives can inhibit the sodium/hydrogen exchanger thought to be involved in the accumulation of intracellular sodium during the ischaemic period (Tani and Neely, 1987; Neubauer et al, 1987) and therefore indirectly affect the sodium/calcium exchange mechanism.

There are many agents which affect calcium movements as shown in Table 2. There are a multitude of intracellular sites at which calcium mobilization or the effects of calcium might be influenced. Inhibition of the receptor for IP_3 on the ER would inhibit the receptor-mediated discharge of internal calcium. A different approach would be to inhibit the effect of elevated intracellular calcium on target proteins such as calmodulin

and troponin. There are a very large number of compounds, including the phenothiazines, which can be shown to inhibit calmodulin-dependent function in vitro and in cells. However, these compounds have a multiplicity of other actions and it is seldom clear that their cellular effects reflect an action on calmodulin rather than on membrane processes or for example on protein kinase C (Sanchez, Hallam and Rink, 1983). Furthermore, calmodulin is present at exceedingly high concentrations, tens of micromolar in most cells, so that very large amounts of drug would be required to achieve substantial blockade.

Calcium antagonists and other modulators of calcium movements could well have an important role to play in the protection of organs subjected to ischaemia and reperfusion. Further work is needed to gain a better understanding of their mode of action and tissue specificity which would allow for an improvement in specific drug targeting.

Objectives of the Study

There is growing evidence for the involvement of oxygen-derived free radicals in tissue damage following ischaemia and reperfusion. However the possible role for calcium in mediating oxidative damage to stored organs has received relatively little attention. The objectives of this study were:

(1) to obtain evidence for altered intracellular calcium homeostasis in rabbit kidneys subjected to cold ischaemia

(2) to investigate the possible relationships between calcium and oxygen-derived free radical-mediated damage to rabbit kidneys as a result of ischaemia and reperfusion

(3) to investigate the mechanisms by which altered calcium homeostasis may potentiate free radical damage to membranes in the ischaemic rabbit kidney

(4) to provide a better understanding of the temporal sequence of biochemical events occurring during ischaemia and reperfusion

(5) to afford protection against ischaemia/reperfusion damage using a variety of therapeutic agents.

CHAPTER 2:

MATERIALS AND METHODS

Materials

Verapamil hydrochloride was purchased from Abbot Laboratories Ltd, Kent. Ruthenium red, A23187, dibucaine hydrochloride, and fatty acid methyl ester standards were purchased from Sigma Chemical Company Ltd., Dorset. Chloroform and methanol were special spectral grade and obtained from BDH Chemicals Ltd, Essex. Dimethyl-acetamide (DMA), methyl iodide, pyridine and collagenase were purchased from Boehringer Mannheim, Sussex. Leibovitz's L-15 medium was obtained from Flow Laboratories, Middlesex. Myo-(2-^{3}H) inositol was obtained from Amersham International, Bucks. All other chemicals were of analytical grade and obtained from either Sigma Chemical Company Ltd. or BDH Chemicals Ltd.

Surgical Technique

Rabbit Kidneys

New Zealand White (NZW) rabbits (average weight 3kg) were anaesthetized by intramuscular injection of 0.2ml/kg fentanyl-fluanisone (Hypnorm$^{(R)}$), followed by slow intravenous injection of diazepam (Valium 20) at 1.0mg/kg. Frusemide (3mg/kg) and heparin (300i.u./kg) were also administered by intravenous injection. Oxygen flowing at 2L/min was supplied by face mask throughout the operations. The abdomen was opened by a mid-line incision and the

kidneys were dissected free and removed with their vascular pedicle. The renal artery of each kidney was cannulated and the organs flushed with either 30ml of sterile isotonic sodium chloride (0.9%) solution (saline) or 30ml of sterile hypertonic citrate (HCA) (Ross, Marshall and Escott, 1976) solution already cooled to 4°C. The kidneys were placed in sterile beakers containing 60ml of the identical flush solution and stored in ice at 0°C for up to 72 hours. In some experiments verapamil (100μM), $CaCl_2$ (1mM), ruthenium red (5μM), dibucaine (250μM), A23187 (10μM) or allopurinol (5mM) were added previously to the flush and storage solutions.

Rat Liver

Sprague-Dawley rats were killed by cervical dislocation. The abdomen was immediately opened by a mid-line incision and the liver exposed. The hepatic artery was cannulated and the organ flushed with 500ml of calcium-and magnesium-free Hanks basic salt solution (NaCl (137mM), KCl (5.37mM), Na_2HPO_4 (0.13mM), $NaHCO_3$ (4.17mM), KH_2PO_4 (0.44mM), glucose (5.55mM) and N-2-hydroxyethyl piperazine-N-2-ethane sulphonic acid (HEPES) (1.0mM) pH 7.4) followed by 500ml Liebowitz's L-15 culture medium (containing 10mM HEPES to provide a buffer). Both solutions were previously warmed to 37°C. The softened liver was carefully dissected free from the animal, leaving the cannula inserted, in readiness for hepatocyte preparation (see Tissue Preparation) (Innes,

Fuller and Hobbs, 1988).

Tissue Preparation

Homogenization

Kidneys were removed and immediately dissected into cortex and medulla. Thin slices were taken and blotted on filter paper before being weighed. They were then added to phosphate-saline buffer (PBS) (40mM KH_2PO_4: K_2HPO_4: pH 7.4) to give an homogenate concentration of 50mg/ml wet weight. The samples were homogenized for 1.0 minute using a Potter-Elvehjem homogenizer and then incubated at 37°C in open vessels with mechanical shaking for 60 minutes. Samples of homogenate were also stored at -70°C for later determination of protein content.

Crushing under Liquid Nitrogen

Kidney specimens were processed either immediately after the storage period or following flushing of the organ after removal from the rabbit. Kidneys were dissected into cortex and medulla and thin slices were immediately clamped in the jaws of metal tongs previously cooled in liquid nitrogen (-196°C). In this way the tongs rapidly froze the tissue preventing metabolic changes. Samples were ground to a powder with a pestle and mortar in an insulated tray containing liquid nitrogen to keep the samples frozen. All samples were stored in liquid nitrogen until required.

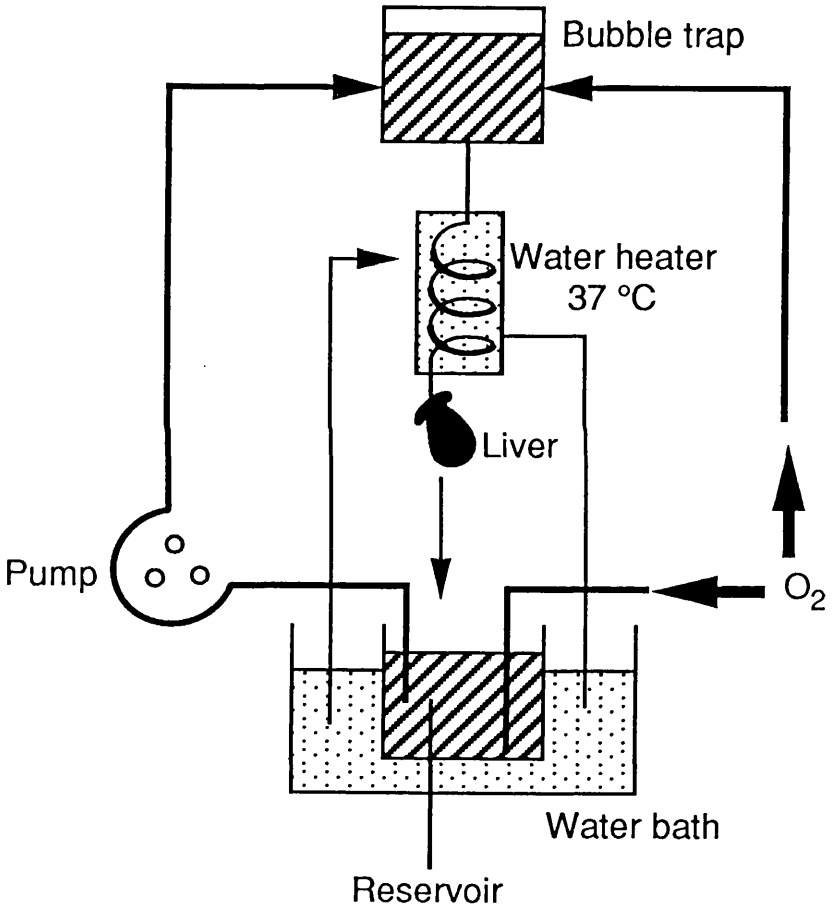
Kidney Slice Preparation

Kidney cortical slices were prepared from fresh kidneys flushed with an ice-cold modified Krebs-Ringer bicarbonate solution (KRB) (NaCl (118.46mM), KCl (4.75mM), $CaCl_2$ (2.54mM), KH_2PO_4 (1.19mM), $MgSO_4.7H_2O$ (2.44mM), $NaHCO_3$ (40.38mM) and pyruvate (10mM) previously gassed with 95% O_2: 5% CO_2 (carbogen) for 20 minutes. Tissue was sliced using a McIlwain tissue chopper to create slices 1000µm x 400µm x 400µm in size. Slices were kept on ice in KRB as a 25% suspension (v/v).

Preparation of Rat Hepatocytes

A single cell suspension of hepatocytes was prepared by the method described by Seglen (1976). Freshly excised rat livers were tied to a perfusion apparatus (Figure 10). The liver was continuously perfused (15ml/min) with L-15 medium containing 0.1% (w/v) collagenase at 37°C for 30 minutes. Following perfusion, the organ was placed on ice, and gently dissected free from fatty and fibrous tissue and agitated to disperse the hepatocytes. The hepatocytes were incubated with L-15 containing 0.1% collagenase in a trypsin flask at 37°C for 20 minutes to ensure maximal digestion. A simple tea strainer was used to remove undigested material and large debris, and the isolated cells were placed in pre-cooled tubes and washed with L-15 medium by low force centrifugation (500 rpm for 2 minutes) 4 times to remove the enzyme. Further filtration through a 100µm pore nylon mesh

FIGURE 10: Liver Perfusion Apparatus used for the Preparation of Hepatocytes



removed clumps of cells and resulted in a cell population of isolated single cells, which were re-suspended in fresh medium. The ability of hepatocytes to exclude trypan blue dye was used as a criterion for viability. Hepatocyte viability was then determined and suspensions were diluted in L-15 medium containing 10% foetal calf serum (GIBCO, Paisley, UK.), Gentamycin 80U/ml (Roussel, Dublin, Republic of Ireland) and Fungizone 0.5µg/ml (Flow Laboratories) to give 5×10^5 viable cells/ml. Aliquots (5ml) containing 2.5×10^6 viable hepatocytes were plated out into $25cm^2$ plastic tissue culture flasks (Primaria, Falcon, Becton Dickinson Labware, Lincoln Park, NJ). All cultures were incubated at 37^oC in a 5% CO_2/ air mixture for 3 hours to allow hepatocyte attachment (Innes, Fuller and Hobbs, 1988), whereupon they were washed to remove non-viable cells and fresh L-15 (5ml) was added.

Measurement of Lipid Peroxidation

Assay for Schiff Base (SB) Formation

Kidney homogenates were incubated at 37^oC in open vessels with mechanical shaking for 60 minutes. Duplicate aliquots (1.0ml) were removed after 0 and 60 minutes of incubation and immediately added to chloroform:methanol (4ml, 2:1 v/v). The glass tubes used for lipid extraction had previously been washed with chloroform:methanol (2:1 v/v) to remove possible contamination. Lipid extracts were

obtained by vortex mixing for 2 minutes followed by centrifugation at 3000 rpm for 10 minutes. The lower chloroform layer was then monitored for a fluorescence maximum between 400-450nm when excited at 360nm (Bidlack and Tappel, 1973) in a Perkin Elmer LS-3B fluorescence spectrometer standardized against a fluorescent polymer block. The change in fluorescence intensity following the incubation period was taken as an indication of susceptibility of the tissue to lipid peroxidation.

Assay for TBA-Reactive Material (TBARs)

Kidney homogenates were incubated at 37^{o}C in open vessels with mechanical shaking for 60 minutes. Duplicate aliquots (1.0ml) were removed at 0 and 60 minutes of incubation and immediately added to 0.1M HCl (1ml), 0.67% (w/v) thiobarbituric acid (0.5ml), 10% (w/v) phosphotungstic acid (0.15ml) and 7% (w/v) sodium dodecyl sulphate (0.1ml) and after mixing, were incubated at 95^{o}C for 60 minutes. Following incubation, samples were allowed to cool before the addition of butan-1-ol (2.5ml). Samples were vortexed for 2 minutes followed by centrifugation at 3000 rpm for 10 minutes. The upper butanol phase was then monitored for a fluorescence maximum at 553nm when excited at 515nm in a Perkin Elmer LS-3B fluorescence spectrometer (Suematsu and Abe, 1982). The change in fluorescence intensity following the incubation period was taken as an indication of susceptibility of the tissue to lipid peroxidation. This

assay was calibrated using malondialdehyde tetraethyl acetal (Sigma Chemical Company Ltd).

Lipid Peroxidation in Rabbit Kidney Cortical Slices

Lipid peroxidation was stimulated either with CCl_4 or Fe^{2+}/ascorbate. Rabbit kidney cortical slices were suspended in ice-cold KRB (25% v/v). Aliquots of slices (12ml) were transferred to flat-bottomed flasks containing either 5µl carbon tetrachloride (CCl_4, approximate concentration: 5.7mM) or 50µM $FeSO_4$, 4mM ADP and 1mM ascorbic acid (Orrenius, Dallner and Ernster, 1964). All samples were incubated on a rotary shaker for 60 minutes. Duplicate aliquots (1ml) were taken at 0, 20, 40 and 60 minutes of the incubation period and assayed for TBARs (as described above). 1mM ferrous sulphate was added to the thiobarbituric acid solution in order to ensure that the TBA-test was not affected by differences in the iron content of the samples (Wills, 1964).

Lipid Peroxidation in Rabbit Kidney Cortical Slices Exposed to Hypoxia/Reoxygenation

Rabbit kidney cortical slices were suspended in ice-cold KRB (10% v/v). Aliquots of slices (12ml) were transferred to flat-bottomed flasks and gassed either with 95% O_2:5% CO_2 (carbogen) for 2 minutes (control) or N_2 for 5 minutes to create hypoxic conditions. All flasks were tightly stoppered. Samples were incubated for 120 minutes

at 37°C on a rotary shaker. Following incubation, control slices and half the hypoxic slices were placed in separate flasks and gassed with carbogen. The rest of the hypoxic slices remained in an atmosphere of N_2. All samples were incubated for a further 30 minutes. Duplicate aliquots (750µl) were removed (aliquots of hypoxic slices were removed under N_2 and the flasks were again tightly stoppered) at 0, 30, 60, 90 and 120 minutes of the incubation period and following the final incubation period. All samples were immediately assayed for TBARs.

Protein Determination

All results were corrected for variability in the protein content of the homogenates (Lowry et al 1951). Stored homogenates were re-homogenized and diluted 20-fold in PBS buffer. Duplicate aliquots (200µl) were then added to 3ml of a reaction mixture containing potassium sodium-tartrate (0.02%), $CuSO_4$ (0.01%) and Na_2CO_3 (2%) in 0.1M NaOH. After incubation at room temperature for at least 15 minutes, Folin and Ciocalteu's phenol reagent (2.0N) (300µl) was added, and the absorbance read at 650nm on a Uvikon 810P spectrophotometer exactly 30 minutes later. The protein concentration was calculated by comparison with a range of standard solutions of bovine serum albumin (0-200 µg protein) subjected to the same procedure.

Free Calcium Determination

The calcium chelating ability of the HCA solution was determined by adding a range of known amounts of $CaCl_2$ and measuring the resultant 'free' calcium concentration using an ion-selective electrode (Orion model 811) calibrated with calcium-EGTA buffers in the range of 10^{-8} to 10^{-4} Molar.

Free Fatty Acid (FFA) Analysis

Extraction

Extraction and methylation techniques were modified from those described by Allen (1984). Kidneys were dissected into cortex and medulla, immediately frozen in liquid nitrogen and ground to a powder with a pestle and mortar (see Tissue Preparation). Methanol (6ml), hexane (12.5ml) and the internal standard pentadecanoic acid (1µg), were added without delay to 80mg of the powdered samples. The mixture was shaken vigorously for 1 minute and centrifuged (1000rpm for 10 minutes). The lower phase was removed and the remaining hexane phase was washed twice with 0.1M phosphoric acid (8ml). The hexane layer was removed to another tube and extracted with 0.5M potassium hydroxide (KOH) solution (40µl). The KOH layer containing the FFAs was removed for methylation.

Methyl Iodide Methylation

N,N-Dimethyl-acetamide (DMA) (100µl) and methyl iodide (60µl) were added to the extracted fatty acids present in

the KOH. The mixture was incubated for 10 minutes in a shaking water bath at 65°C. Pyridine (120µl) was then added and a further incubation was carried out at 65°C for 10 minutes. After cooling, 0.1M phosphoric acid (1ml) was added and the FFA methyl esters were extracted into ethylene chloride (35µl). Samples were washed 3 times with distilled water equilibrated with ethylene chloride. The upper phase was removed and the sample volume reduced in a rotary evaporator (Uniscience, London). Samples were stored under N_2 at -70°C until required. After resuspension in hexane (20µl), 2µl of the FFA methyl esters was injected into the gas liquid chromatograph (glc).

Gas-Liquid Chromatography

Glc was performed with a Pye Unicam series 104 chromatograph connected to a Trivector chromatography computing integrator (Vinten Analytical Systems Ltd., Bedfordshire). A glass column (1.5m x 6mm) packed with 10% diethylene glycol stearate (DEGS) on chromosorb WAW 80-100 mesh (Phase Separations Ltd, Clwyd) was used. The temperatures of the injector and detector were 250°C and the column was maintained at 190°C. Fatty acid methyl esters were identified on the basis of their retention times compared to known standards of fatty acid methyl esters. Amounts of FFAs were calculated by comparison of their peak areas with that of the internal standard.

Determination of Phosphatidylinositol Turnover in Tissues

Measurement of PIP_2 Breakdown

(i) Hplc Analysis of Radiolabelled Inositol Phosphates:

Hplc analysis of inositol phosphates was performed according to Irvine et al (1985) on an LKB system consisting of a controller (model 2152) connected to an hplc pump (model 2150). Samples (50µl) were loaded onto a 0.46 cm x 25cm Partisil SAX 10 high pressure anion exchange chromatography column (HPLC Technology Ltd, Cheshire). All samples were spiked with adenine nucleotide markers (AMP, ADP and ATP) which were detected by their absorption at 254nm on a UV detector (LKB 2510 uvicord SD) in order to check the chromatographic separation. A gradient system was employed with a flow rate of 1.25ml/minute. Initially, water was allowed to flow through for 6 minutes, then, over 24 minutes a linear gradient was applied rising from water to 100% 1.0M ammonium formate buffered to pH 3.7 with orthophosphoric acid (i.e. phosphate content approx 0.5M). The formate/phosphate buffer was passed through the column for a further 5 minutes, and then over the following 2 minutes the eluent was returned linearly to water (Irvine et al, 1985). Finally, a further 10 minutes of water elution was employed to re-equilibrate the column for the next injection. The eluent was collected in 1.25ml fractions by a LKB 2212 Helirac fraction collector and the radioactivity of each fraction was measured by adding LKB Optiphase 'Hisafe 3' liquid scintillant (4.5ml) to each fraction and

the radioactivity counted in a scintillation counter (LKB 1216 Rackbeta 11 Liquid Scintillation Counter).

(ii) Analysis of Radiolabelled Inositol Phosphates by a Simple 2-step Chromatographic Procedure:

Chromatograpy was carried out according to Berridge et al (1983). A 2ml volume of a 50% (w/v) slurry of Dowex-1 resin (BDH Chemicals Ltd; 100-200 mesh, X8 in the formate form stored in 0.1M formic acid) was placed into plugged glass Pasteur pipettes (4mm diameter). The columns were washed with distilled water (10ml) and 1ml samples applied. The columns were then washed with 60mM ammonium formate/5mM sodium borate (12ml) which eluted inositol and glycerophosphoinositol into glass tubes. Duplicate aliquots (1ml) of this eluent was transferred to scintillation vials containing Optiphase 'Hisafe 3' liquid scintillant (4.5ml) and the radioactivity counted in a scintillation counter. The columns were then washed with 1M ammonium formate/0.1M formic acid (12ml) to elute the radiolabelled inositol phosphates (inositol-trisphosphate, inositol-bisphosphate and inositol-monophosphate). Duplicate aliquots (1ml) of this fraction were transferred to scintillation vials containing liquid scintillant (4.5ml) and the radioactivity counted in a scintillation counter. The ratio of the radioactivity in fraction 2 : the radioactivity in fraction 1 was calculated (after taking into account differences in counting efficiency) and taken as a measure of PIP_2

breakdown. This method was checked by analysing products in both fractions by hplc to ensure fraction 1 contained inositol and glycerophosphoinositol and fraction 2 contained inositol phosphates.

Accumulation of Radiolabelled Inositol Phosphates into Tissues

Myo-(2-^{3}H)-inositol incorporation into cells and inositol phosphate accumulation was assessed in three models.

(i) Rabbit Kidney Cortical Slices

A 25% (v/v) suspension of rabbit kidney cortical slices in ice-cold KRB was placed in a 100ml flask which was gassed (95% O_2: 5% CO_2 for 2 minutes) and immediately tightly stoppered. As inositol uptake by the kidney is an energy-dependent process, the slices were then incubated on a rotary shaker at 37^{o}C for 30 minutes to increase ATP levels (Brown et al, 1984). Following incubation, 57µl of myo-(2-^{3}H)-inositol (0.32µM; 19.9 Ci/mmol) was added and the slices were re-gassed, stoppered and incubated on a rotary shaker for a further 60 minutes at 37^{o}C. The slices were then washed four times with KRB to remove unincorporated ^{3}H-inositol and re-suspended in fresh KRB (25% v/v). Duplicate aliquots (1ml) were added to 20% (w/v) trichloroacetic acid (TCA) (2ml). Samples were centrifuged (1000rpm for 5min) and the supernatant washed 5 times with water saturated

diethyl ether to remove the TCA (Neylon and Summers, 1987). Sample volumes were then reduced in a rotary evaporator and 50µl injected onto the hplc column (See Hplc Analysis of Radiolabelled Inositol Phosphates).

(ii) Rat Hepatocytes in Culture:

Cultured hepatocytes were incubated with 32µl myo-(2-^{3}H)-inositol (0.32µM; 19.9 Ci/mmol) per flask (2.5 x 10^{6} viable cells/5ml L-15 medium) for 60 minutes at 37^{o}C. The flasks were washed 3 times with L-15 medium following the incubation period, and the cells were removed and lysed with 20% (w/v) TCA (3ml). Samples were centrifuged (1000rpm for 5 minutes) and the supernatant was washed 5 times with water saturated diethyl ether. Sample volumes were then reduced in a rotary evaporator. 50µl samples were injected onto the hplc column.

(iii) Rabbit Kidney <u>In Vivo</u>:

NZW rabbits were anaesthetized (as in Surgical Technique) and injected (i.p) with myo-(2-^{3}H)-inositol (50µl, 19.9Ci/mmol) into the abdomen. Animal were allowed to recover and 24 hours later were anaesthetized and the kidneys removed. The kidneys were dissected into cortex and medulla and homogenized in 20% (w/v) TCA (5ml). Samples were centrifuged (1000rpm for 5 minutes) and the supernatant was washed 5 times with water saturated diethyl ether. Sample volumes were then reduced in a rotary evaporator and

50µl injected into the hplc column.

Incorporation of Myo-(2-^{3}H)-Inositol into Lipids in Rabbit Kidney Slices

A 25% (v/v) suspension of rabbit kidney cortical slices in ice-cold KRB was placed in a 100ml flask, gassed (95% O_2:CO_2 for 2 minutes) and immediately tightly stoppered. The slices were then incubated on a rotary shaker at 37^{o}C for 30 minutes to increase ATP levels (Brown et al, 1984). Following incubation, 57µl of myo-(2-^{3}H)-inositol (0.32µM; 19.9 Ci/mmol) was added and the slices were re-gassed, stoppered and incubated on a rotary shaker for a further 135 minutes at 37^{o}C. Duplicate aliquots (1ml) were removed at intervals of 15 minutes, washed four times with KRB and re-suspended in fresh KRB (25% v/v). Following washing, chloroform:methanol (2:1 v/v, 4ml) was added and the samples were vortexed for 2 minutes followed by centrifugation at 3000rpm for 10 minutes. 1ml of the upper aqueous layer, containing inositol, and 1ml of the lower chloroform layer (containing radiolabelled extracted lipids) were transferred to scintillation vials containing liquid scintillant (Optiphase 'Hisafe 3'; 4.5ml) and the radioactivity counted in a scintillation counter.

PIP_2 Breakdown in Rabbit Kidney Slices Exposed to CCl_4

In order to investigate the effect of CCl_4 on PIP_2 breakdown, rabbit kidney cortical slices were loaded with

myo-(2-^{3}H)-inositol as previously described. In addition, the calcium requirement of this process was studied by suspending slices in either ice-cold KRB buffer, or calcium-free KRB buffer containing 10mM EGTA. Aliquots of slices (12ml, 25%v/v with added LiCl (10mM) to accumulate inositol phosphate products) with or without CCl_4 (5µl, approximate concentration: 5.7mM) were incubated on a rotary shaker for 60 minutes at 37^{o}C. Duplicate aliquots (1ml) were taken at 0, 20, 40 and 60 minutes of the incubation period and transferred to glass tubes containing chloroform:methanol (2:1 v/v). The tubes were vortexed for 2 minutes followed by centrifugation at 3000 rpm for 10 minutes. 1ml of the aqueous layer was applied to Dowex columns and analysed for inositol phosphates (see Measurement of PIP_2 Breakdown part(ii))

PIP_2 Breakdown in Rabbit Kidney Slices During Hypoxia/Reoxygenation

In order to investigate the effect of hypoxia/reoxygenation on PIP_2 breakdown, rabbit kidney cortical slices were loaded with myo-(2-^{3}H)-inositol as previously described. In addition, the calcium requirement of this process was studied by suspending slices in ice-cold KRB buffer, or calcium-free KRB buffer containing 10mM EGTA. Aliquots of slices (12ml, 10% v/v) were transferred to flat-bottomed flasks and gassed either with carbogen for 2 minutes (control) or N_2 for 5 minutes to create hypoxic

conditions. All flasks were tightly stoppered. Samples were incubated for 120 minutes at 37°C on a rotary shaker. Following incubation, control slices and half the hypoxic slices were gassed with carbogen. The rest of the hypoxic slices remained in an atmosphere of N_2. All samples were incubated for a further 30 minutes. Duplicate aliquots (750µl) were removed at 0, 30, 60, 90, and 120 minutes of the initial incubation period and again following the final incubation period. Aliquots of hypoxic slices were removed under N_2 and the flasks were again tightly stoppered. Samples were immediately added to chloroform:methanol (2:1 v/v), vortexed for 2 minutes and centrifuged for 10 minutes. 1ml of the aqueous layer was applied to Dowex columns and analysed for inositol phosphates (see Measurement of PIP_2 Breakdown part(ii)).

ATP Determination

ATP was measured using ATP bioluminescence CLS kits (Boehringer).

The assay utilises the enzyme luciferase from *Photinus pyralis* (American firefly) which catalyses the following reaction:

$$\text{ATP} + \text{D-luciferin} + O_2 \xrightarrow[\text{luciferase}]{Mg^{++}} \text{oxyluciferin} + PP_i + \text{AMP} + CO_2 + \text{light}$$

The quantum yield of this reaction, defined as the ratio of

the number of emitted photons and the number of converted ATP molecules, is almost 100%. The maximum of the emitted light is 562 nm with a broad peak between 500 and 600 nm. The intensity of the emitted light is directly proportional to the ATP concentration.

A standard curve was set up with ATP concentrations ranging from 0 to 5 x 10^{-7} M. Duplicate aliquots (1ml) of rabbit kidney cortical slices were added to 12% (w/v) trichloroacetic acid (TCA) (2ml). Samples were centrifuged (1000rpm for 5min) and the supernatant, containing the ATP, was washed 5 times with water saturated diethyl ether to remove TCA and neutralize the sample (Neylon and Summers, 1987). All samples were kept on ice until required. Duplicate aliquots (300µl) were added to 500µl of the reagent solution which contained HEPES buffer, pH 7.75 (40mM), luciferase (1.6 µg/ml), D-luciferin (700µM), magnesium chloride (20mM), EDTA (4mM), dithiothreitol (0.36mM) and AMP (0.3mM). Readings were taken on a Perkin-Elmer LS-50 luminescence spectrometer in phosphorescence mode with the emission slit at 20nm and the total emission mirror in place to achieve maximum sensitivity.

Statistical Analysis

Statistical analysis was performed either by analysis of variance, comparison within groups by Student's t-test or, where appropriate, by t-test of paired samples (Armitage and Berry, 1987).

RESULTS SECTION

CHAPTER 3: THE EFFECT OF CALCIUM MODULATORS ON LIPID PEROXIDATION IN RABBIT KIDNEYS SUBJECTED TO COLD ISCHAEMIA

Investigations in a number of cellular systems have linked irreversible cell injury with an increase in cytosolic calcium concentrations (Farber, 1981). Normally, in intact cells, the gradient between relatively high (10^{-3}M) extracellular concentrations and a low (10^{-7}M) cytosolic concentration is maintained by membrane bound ATP-dependent pumps which remove calcium from the cytosol and sequester excess calcium in mitochondria and endoplasmic reticulum. During ischaemia ATP levels are rapidly depleted (Calman et al, 1973) and this could lead to changes in calcium sequestration. Previous reports have demonstrated altered calcium homeostasis in tissues subjected to warm ischaemic insults (Bourdillon and Poole-Wilson, 1981) and increased cytosolic calcium levels in isolated cells exposed to hypoxia (Snowdowne et al, 1985).

The experiments in this chapter were designed to examine the possible role of calcium in mediating oxidative damage to membranes following cold storage of rabbit kidneys and test the hypothesis that deliberate manipulation of cell calcium homeostasis under these conditions might affect biochemical markers of lipid peroxidation. Rabbit kidneys

were flushed and stored at 0°C in either isotonic saline (0.9% NaCl) (a poor storage solution) or hypertonic citrate (a good storage solution). Various agents were added which affect calcium distribution and the extent of lipid peroxidation was measured following in vitro incubation of homogenates of the stored kidneys.

Verapamil (100μM), a calcium-channel blocker, was added to the flush and storage solutions with the intention of inhibiting influx of extracellular calcium into the cells through voltage operated channels during cold ischaemia; $CaCl_2$ (1mM) was added to the solutions to investigate the importance of extracellular calcium levels; the ionophore A23187 (10μM) was used to render both plasma and intracellular organelle membranes permeable to calcium; the effect of the polysaccharide dye ruthenium red (5μM), which inhibits mitochondrial calcium uptake was investigated; and inhibition of the calcium-dependent phospholipase A2 by dibucaine (250μM) was also investigated. A study was also carried out using allopurinol which inhibits xanthine oxidase activity. Xanthine oxidase may be produced from xanthine dehydrogenase by a calcium-dependent proteolysis (Roy and McCord, 1983). Allopurinol was added with or without A23187. Verapamil and dibucaine were also added directly to homogenates of kidneys stored in saline or HCA solution, to test the possibility that any changes in fluorescent markers of lipid peroxidation observed after incubation could be due to direct antioxidant properties of

traces of these drugs remaining in the kidneys following the storage period.

Fresh and treated stored kidneys were paired against non-treated kidneys from the same rabbit to remove variation between animals in all the experiments in this chapter. Hence, values were expressed as difference from stored, non-treated values. Control (stored, non-treated) values (mean ± 95% confidence intervals) for kidneys stored in HCA were: TBARs Cortex, 1.04 ± 0.28 nmol malonaldehyde/hr/mg protein; and medulla, 1.35 ± 0.33 nmol malonaldehyde/hr/mg protein. SB Cortex 2.06 ± 0.78 fluorescence U/hr/mg protein; and medulla, 2.80 ± 0.73 fluorescence U/hr/mg protein.
Control (stored, non-treated) values (mean ± 95% confidence intervals) for kidneys stored in saline were:
TBARs Cortex, 1.25 ± 0.81 nmol malonaldehyde/hr/mg protein; and medulla, 1.75 ± 1.01 nmol malonaldehyde/hr/mg protein. SB Cortex 4.6 ± 1.21 fluorescence U/hr/mg protein; and medulla, 5.70 ± 1.45 fluorescence U/hr/mg protein.
A Student's paired t-test was carried out in each case to determine significance. Six determinations were carried out in all experiments.

The Effect of Verapamil and $CaCl_2$ on Lipid Peroxidation in Rabbit Kidneys Stored for 72 Hours in HCA at $0^{o}C$

Calcium may enter cells from the external milieu through a variety of channels: via voltage-operated fast and slow channels (Fleckenstein, 1977); through receptor

operated channels (Bolton, 1979); and in exchange for sodium (Baker, et al, 1969) or protons (Hinnen et al, 1979). Verapamil blocks the entrance of calcium into cells via voltage-dependent slow calcium channels and may therefore be of therapeutic value in the prevention of ischaemic tissue injury caused by influx of extracellular calcium.

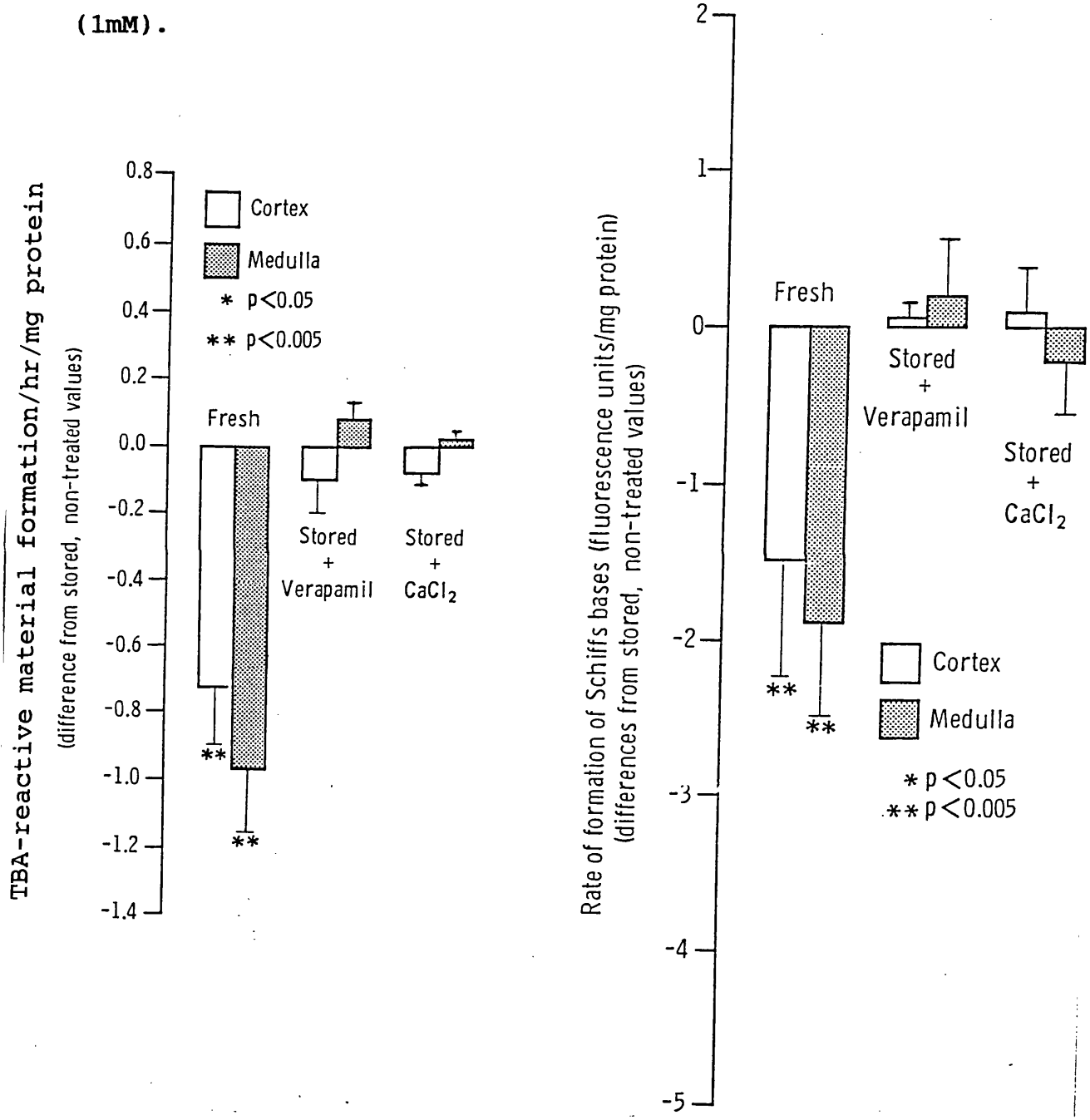
Rabbit kidneys were flushed with ice-cold HCA, placed in sterile beakers containing 60ml of HCA and stored at $0^{o}C$ for 72 hours. Fresh control kidneys were obtained under identical conditions except that the organs were flushed but not stored. Cold storage of rabbit kidneys for 72 hours in HCA resulted in significantly ($P<0.005$) elevated levels of SB and TBAR markers of lipid peroxidation in homogenates of both cortex and medulla compared to fresh kidneys (Figure 11). The prevention of extracellular calcium entry into kidney cells through voltage-operated channels by the addition of verapamil to the HCA flush and storage solution had no effect on the rise in markers of lipid peroxidation, the levels remaining close to stored non-treated values in both areas of the kidney (Figure 11). The addition of verapamil directly to homogenates of kidney stored in HCA for 72 hours had no effect on lipid peroxidation (Table 3). Thus this agent at a concentration of 100μM was not exerting any direct antioxidant effect in the homogenate alone.

Increasing the extracellular calcium concentration by addition of $CaCl_2$ (1mM) to the HCA flush and storage solution had no significant effect on the extent of lipid

FIGURE 11: The Rate of Formation of TBA-Reactive Material and Schiff Bases in Homogenates of Cortex and Medulla from Kidneys Flushed and Stored for 72 Hours at 0°C in Hypertonic Citrate Solution Containing Verapamil (100µM) and $CaCl_2$ (1mM).

Experiment	Section of kidney	Formation of lipid peroxidation products mean + SD (difference from stored, nontreated values)	
		SB Change in fluorescence intensity/hr /mg protein	TBARs /hr /mg protein
Fresh	cortex	-1.480 ± 0.932	-0.721 ± 0.213
	medulla	-1.884 ± 0.983	-0.967 ± 0.233
Stored + verapamil	cortex	0.042 ± 0.084	-0.141 ± 0.078
	medulla	0.233 ± 0.443	0.091 ± 0.052
Stored + $CaCl_2$	cortex	0.089 ± 0.342	-0.084 ± 0.044
	medulla	-0.444 ± 0.453	0.022 ± 0.02

FIGURE 11: The Rate of Formation of TBA-Reactive Material and Schiff Bases in Homogenates of Cortex and Medulla from Kidneys Flushed and Stored for 72 Hours at 0°C in Hypertonic Citrate Solution Containing Verapamil (100μM) and $CaCl_2$ (1mM).



Values are expressed as the mean difference from stored, nontreated kidneys ± 95% confidence interval.

TABLE 3: The Effect of Verapamil (100μM) on Lipid Peroxidation in Homogenates of Kidneys after Storage

Experiment	Section of kidney	Formation of lipid peroxidation products mean ± 95% confidence interval			
n=6		SB Change in fluorescence intensity/hr /mg protein		TBARs /hr /mg protein	
		control	treated	control	treated
100μM verapamil added to the homogenate after storage in HCA	cortex	2.81 ± 0.85	3.04 ± 0.93	0.81 ± 0.18	0.86 ± 0.23
	medulla	2.23 ± 0.51	1.92 ± 0.41	1.00 ± 0.22	0.98 ± 0.22
100μM verapamil added to the homogenate after storage in saline	cortex	2.22 ± 0.58	1.82 ± 0.54	0.83 ± 0.15	0.75 ± 0.77
	medulla	2.28 ± 0.72	2.65 ± 1.13	0.72 ± 0.16	0.82 ± 0.10

peroxidation in either the cortex or medulla of the stored organs (Figure 11). These results therefore suggested that extracellular calcium did not play a role in mediating oxidative damage to kidneys when stored in HCA.

The Effect of Verapamil and $CaCl_2$ on Lipid Peroxidation in Rabbit Kidneys Stored for 24 Hours in Saline at 0^oC

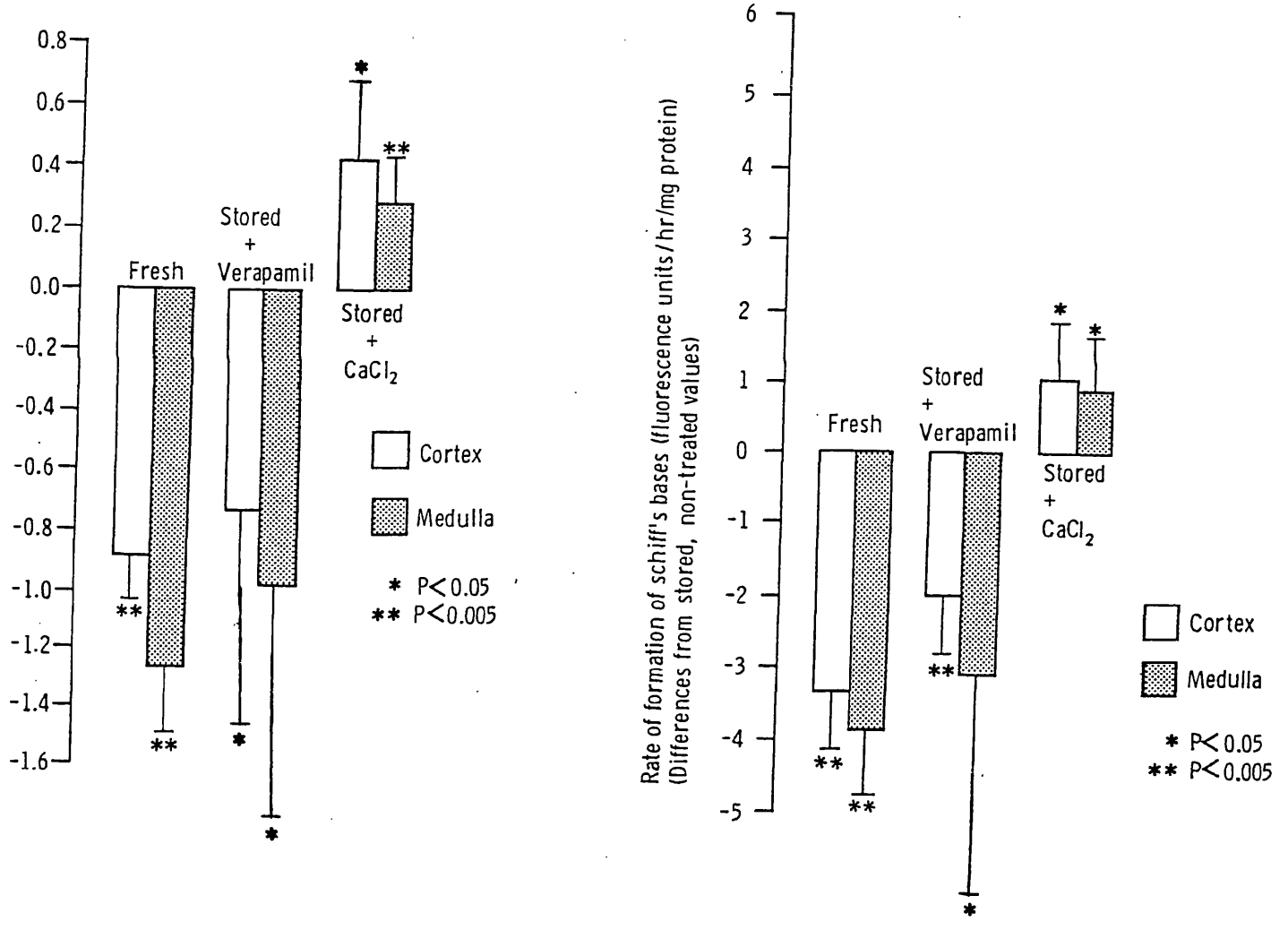
Rabbit kidneys were flushed with ice-cold saline and placed in sterile beakers containing 60 ml of saline and stored at 0^oC for 24 hours. Fresh, control kidneys were obtained under identical conditions except that the organs were flushed but not stored. Cold storage of rabbit kidneys in this poor storage medium for 24 hours resulted in significantly ($P<0.005$) elevated levels of SB and TBARs markers of lipid peroxidation in homogenates of both cortex and medulla compared to fresh kidneys (Figure 12). In complete contrast to the results obtained when kidneys were stored in HCA, the addition of verapamil to the saline storage solution very effectively inhibited the rise in markers of lipid peroxidation in kidneys stored in this medium (Figure 12) and the levels of both SB and TBARs were very similar to those found in fresh kidneys. The addition of verapamil directly to homogenates of kidneys stored in saline had no effect on lipid peroxidation (Table 3). Thus it seems likely that this agent was exerting its effect on the intact cells of the organ during the storage period.

Addition of $CaCl_2$ (1mM) to the saline flush and storage

FIGURE 12: The Rate of Formation of TBA-reactive Material and Schiff Bases in Homogenates of Cortex and Medulla from Kidneys Flushed and Stored for 24 Hours at 0°C in Isotonic Saline Solution Containing Verapamil (100µM) and $CaCl_2$ (1mM).

Experiment	Section of kidney	Formation of lipid peroxidation products mean + SD (difference from stored, nontreated values)	
		SB Change in fluorescence intensity/hr /mg protein	TBARs /hr /mg protein
Stored + verapamil	cortex	-2.000 ± 1.196	-0.758 ± 0.884
	medulla	-3.141 ± 3.743	-0.974 ± 0.944
Stored + $CaCl_2$	cortex	1.062 ± 1.034	0.409 ± 0.334
	medulla	0.883 ± 0.871	0.280 ± 0.161

FIGURE 12: The Rate of Formation of TBA-reactive Material and Schiff Bases in Homogenates of Cortex and Medulla from Kidneys Flushed and Stored for 24 Hours at 0°C in Isotonic Saline Solution Containing Verapamil (100µM) and $CaCl_2$ (1mM).



Values are expressed as the mean difference from stored, nontreated kidneys ± 95% confidence interval.

solution resulted in levels of lipid peroxidation in both cortex and medulla which were significantly greater than those in kidneys stored for 24 hours in saline alone (Figure 12). Thus it appears that extracellular calcium was mediating, to a significant extent, oxidative damage to kidneys when stored in saline solution.

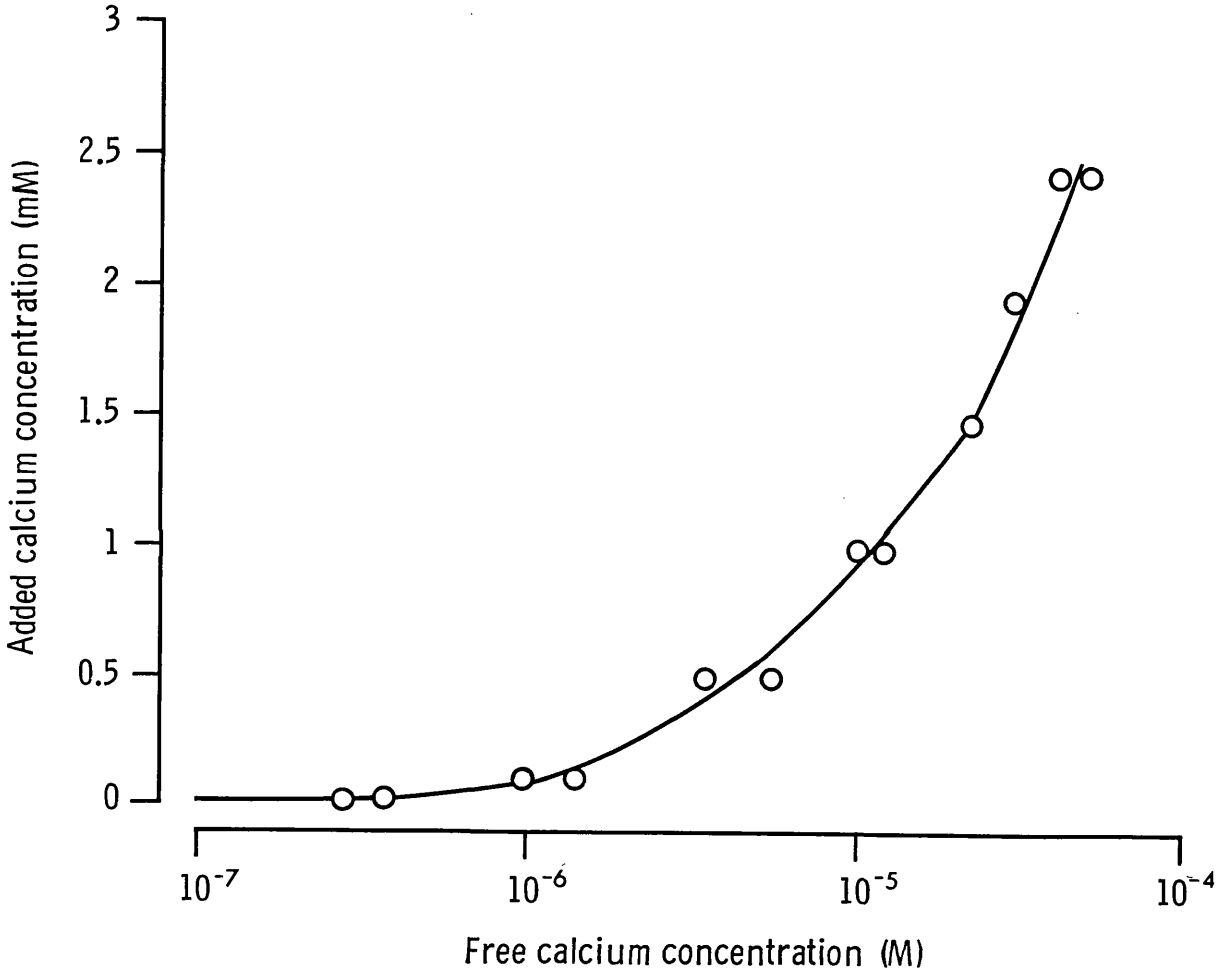
Determination of the Calcium Buffering Capacity of HCA

One important difference between these two storage solutions may be the ability of the large excess of citrate (55mM) in HCA to chelate calcium during the storage period thus preventing, or at least slowing, the entry of extracellular calcium into the renal cells. It was therefore decided to measure free calcium ion concentrations by means of an ion-specific electrode in HCA to which various amounts of calcium were added. As can be seen in Figure 13, the calcium buffering capacity of HCA proved highly effective; for example, the free calcium concentration was only 1μM in the presence of 100μM total calcium and only 10μM when 1mM was added.

The Effect of A23187 on Lipid Peroxidation in Rabbit Kidneys Stored for Either 72 Hours in HCA or 24 Hours in Saline at 0°C

Experiments were carried out to investigate the effect of the calcium ionophore A23187 which renders both plasma membranes and intracellular organelles permeable to calcium,

FIGURE 13: Relationship Between the Added Calcium Concentration and the Free Calcium Concentration in Hypertonic Citrate Solution.

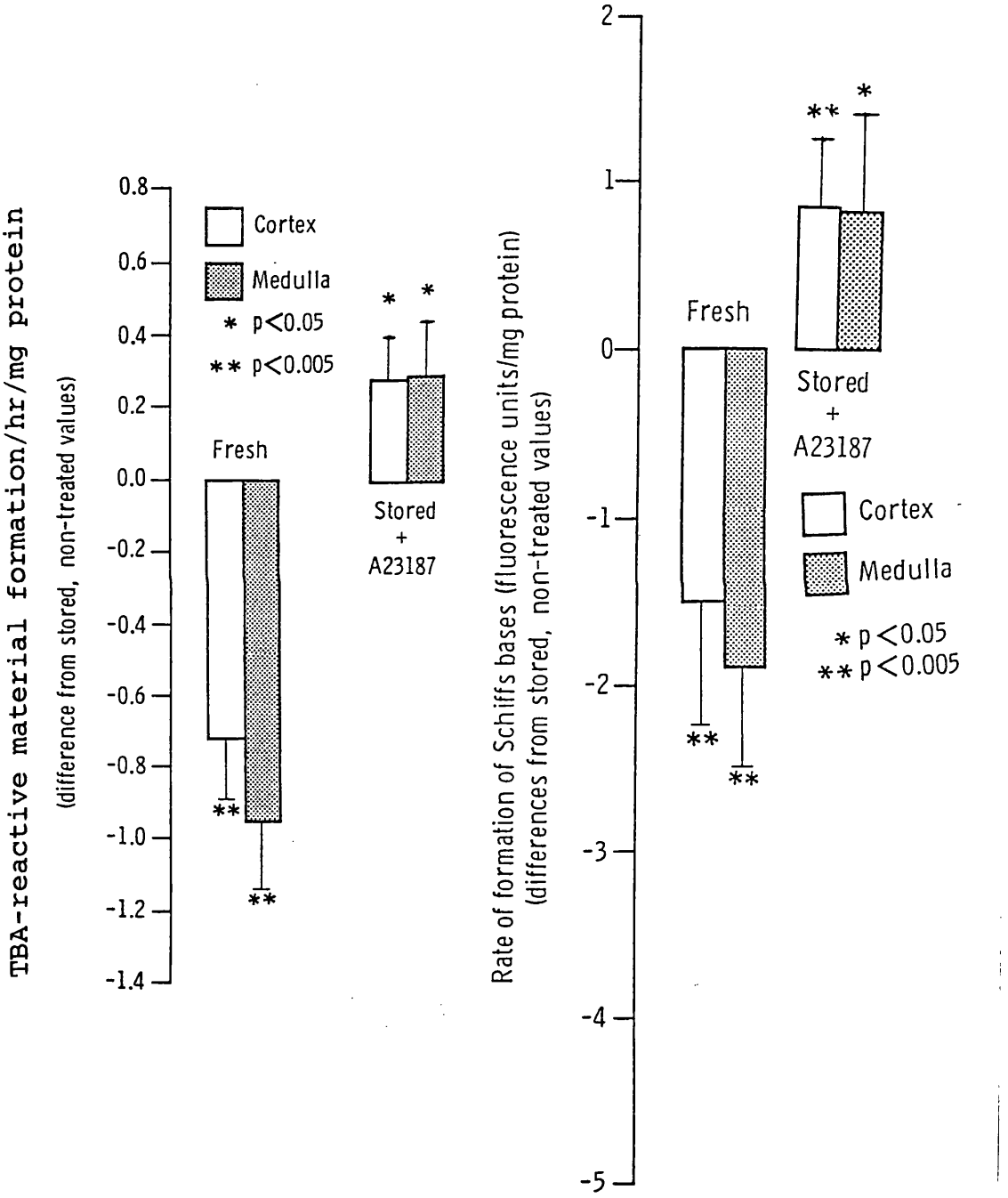


Values represent mean where n=3.

FIGURE 14: The Rate of Formation of TBA-Reactive Material and Schiff Bases in Homogenates of Cortex and Medulla from Kidneys Flushed and Stored for 72 Hours at 0°C in Hypertonic Citrate Solution Containing A23187 (10μM).

Section of kidney	Formation of lipid peroxidation products mean + SD (difference from stored, nontreated values)	
	SB Change in fluorescence intensity/hr /mg protein	TBARs /hr /mg protein
cortex	0.844 ± 0.467	0.277 ± 0.13
medulla	0.828 ± 0.685	0.281 ± 0.17

FIGURE 14: The Rate of Formation of TBA-Reactive Material and Schiff Bases in Homogenates of Cortex and Medulla from Kidneys Flushed and Stored for 72 Hours at 0°C in Hypertonic Citrate Solution Containing A23187 (10μM).

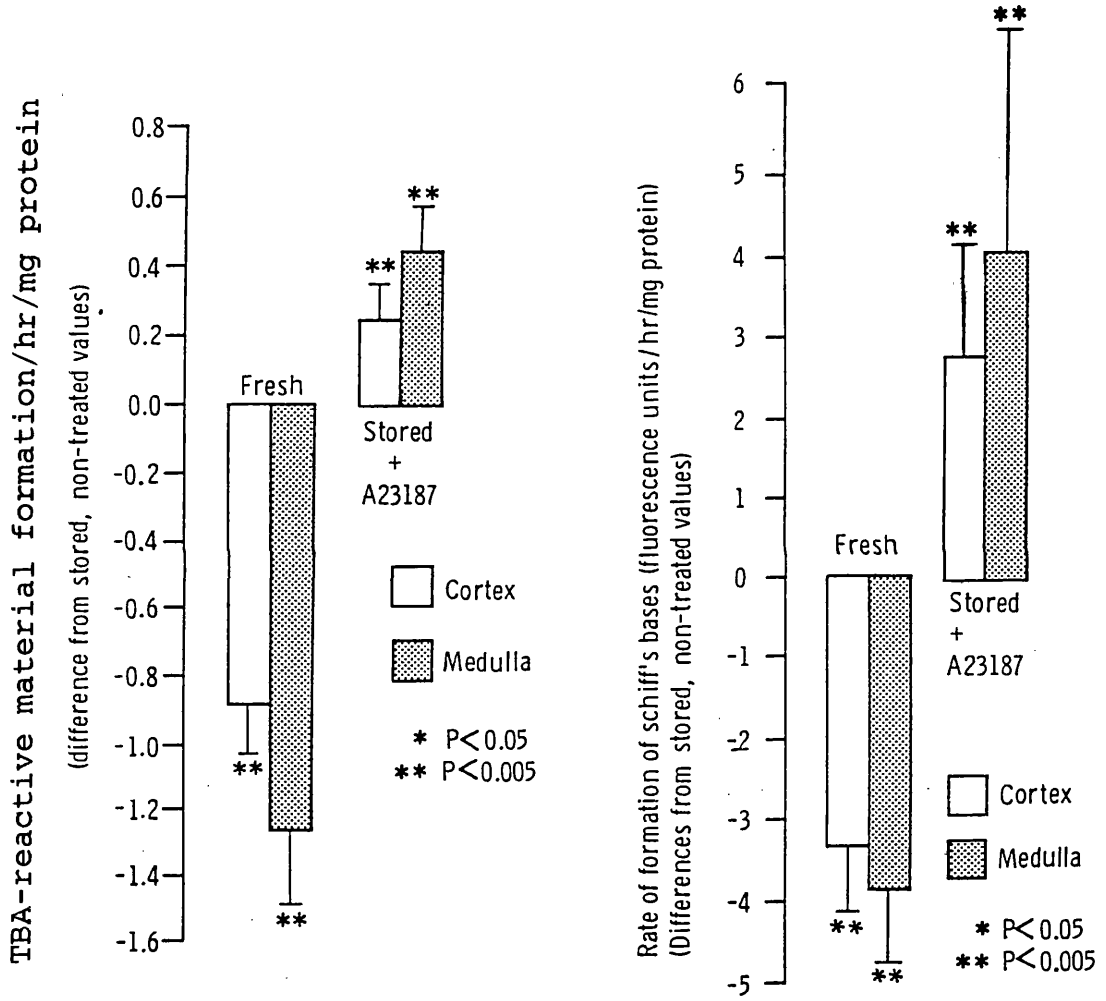


Values are expressed as the mean difference from stored, nontreated kidneys ± 95% confidence interval.

FIGURE 15: The Rate of Formation of TBA-reactive Material and Schiff Bases in Homogenates of Cortex and Medulla from Kidneys Flushed and Stored for 24 Hours at 0°C in Isotonic Saline Solution Containing A23187 (10μM).

Section of kidney	Formation of lipid peroxidation products mean + SD (difference from stored, nontreated values)	
	SB Change in fluorescence intensity/hr /mg protein	TBARs /hr /mg protein
cortex	2.821 ± 1.675	0.248 ± 0.128
medulla	4.109 ± 3.336	0.451 ± 0.188

FIGURE 15: The Rate of Formation of TBA-reactive Material and Schiff Bases in Homogenates of Cortex and Medulla from Kidneys Flushed and Stored for 24 Hours at 0°C in Isotonic Saline Solution Containing A23187 (10μM).



Values are expressed as the mean difference from stored, nontreated kidneys ± 95% confidence interval.

producing an overall increase in cytosolic calcium (Reed and Lardy, 1972). Addition of this agent to the flush and storage solutions resulted in increased production of both markers of lipid peroxidation during incubation of homogenates of both cortex and medulla to levels significantly greater ($P<0.05$) than those observed in kidneys stored for either 72 hours in HCA alone (Figure 14) or 24 hours in saline alone (Figure 15). This suggested that increasing the levels of cytosolic calcium by A23187 in kidneys subjected to cold storage resulted in increased lipid peroxidation upon subsequent incubation of homogenates.

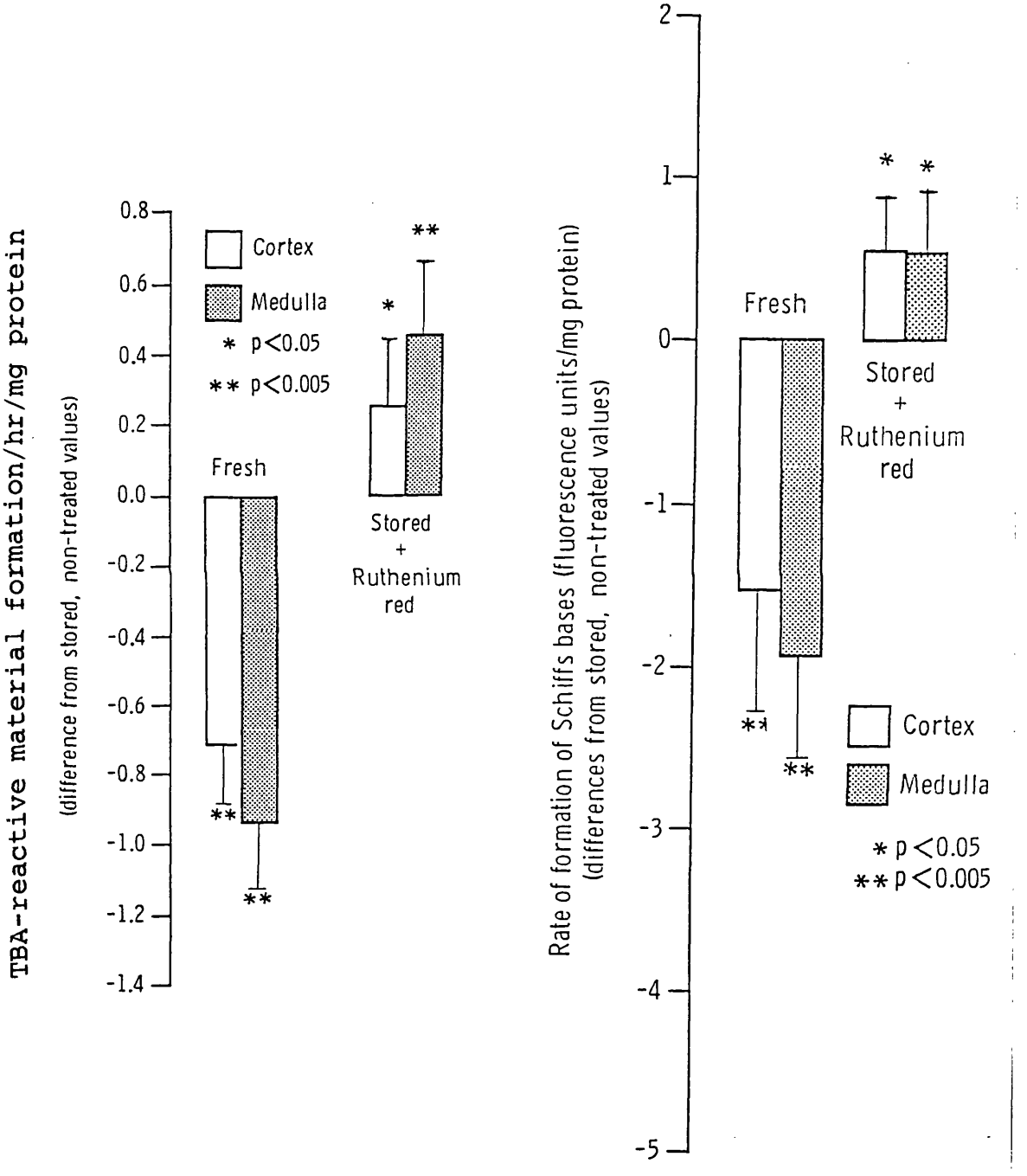
The Effect of Ruthenium Red on Lipid Peroxidation in Rabbit Kidneys Stored for Either 72 Hours in HCA or 24 Hours in Saline at $0^{o}C$

One important organelle which sequesters calcium and keeps cytosolic calcium levels low is the mitochondrion. Dysfunction of this uptake mechanism, possibly as a result of ischaemic damage, would therefore lead to an upset in calcium homeostasis. This possibility was investigated using ruthenium red, a polysaccharide dye which inhibits mitochondrial calcium uptake and efflux (Humes, 1986). Addition of this agent to the flush and storage solutions resulted in significantly ($P<0.05$) increased markers of lipid peroxidation in the medulla and cortex of organs stored in either HCA for 72 hours (Figure 16) or saline

FIGURE 16: The Rate of Formation of TBA-Reactive Material and Schiff Bases in Homogenates of Cortex and Medulla from Kidneys Flushed and Stored for 72 Hours at 0°C in Hypertonic Citrate Solution Containing Ruthenium Red (5μM).

Section of kidney	Formation of lipid peroxidation products mean + SD (difference from stored, nontreated values)	
	SB Change in fluorescence intensity/hr /mg protein	TBARs /hr /mg protein
cortex	0.536 ± 0.388	0.242 ± 0.233
medulla	0.530 ± 0.437	0.475 ± 0.255

FIGURE 16: The Rate of Formation of TBA-Reactive Material and Schiff Bases in Homogenates of Cortex and Medulla from Kidneys Flushed and Stored for 72 Hours at 0°C in Hypertonic Citrate Solution Containing Ruthenium Red (5μM).

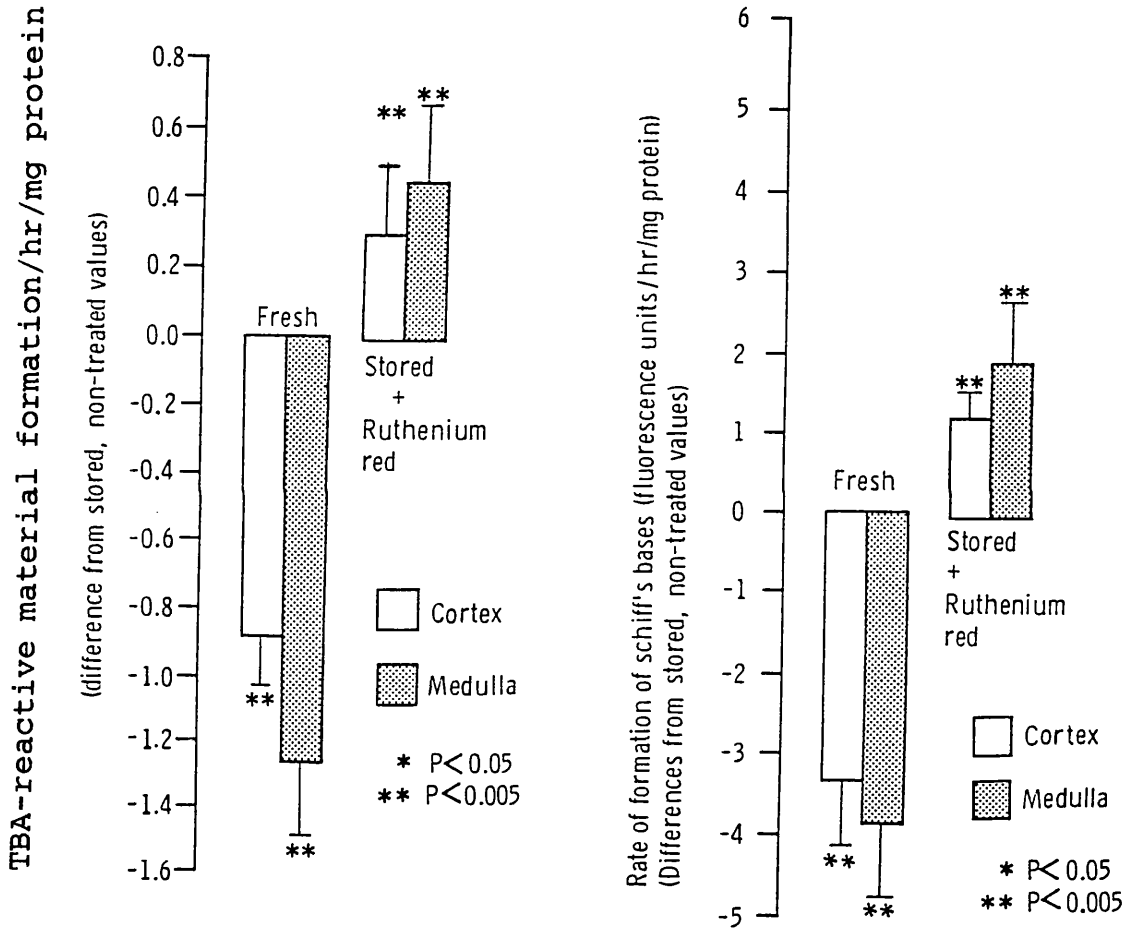


Values are expressed as the mean difference from stored, nontreated kidneys ± 95% confidence interval.

FIGURE 17: The Rate of Formation of TBA-Reactive Material and Schiff Bases in Homogenates of Cortex and Medulla from Kidneys Flushed and Stored for 24 Hours at 0°C in Isotonic Saline Solution Containing Ruthenium Red (5μM).

Section of kidney	Formation of lipid peroxidation products mean + SD (difference from stored, nontreated values)	
	SB Change in fluorescence intensity/hr /mg protein	TBARs /hr /mg protein
cortex	1.254 ± 0.203	0.321 ± 0.243
medulla	1.959 ± 0.886	0.431 ± 0.313

FIGURE 17: The Rate of Formation of TBA-Reactive Material and Schiff Bases in Homogenates of Cortex and Medulla from Kidneys Flushed and Stored for 24 Hours at 0°C in Isotonic Saline Solution Containing Ruthenium Red (5μM).



Values are expressed as the mean difference from stored, nontreated kidneys ± 95% confidence interval.

for 24 hours (Figure 17), compared to stored non-treated controls. It could be concluded from these results that removal of excess cytosolic calcium by mitochondria constituted an important protective mechanism during hypothermic storage. It would therefore seem probable that the mitochondrial ATPase was still able to function even though ATP synthesis was much reduced and that inhibition of this uptake mechanism resulted in an increase in oxidative damage to membranes.

The Effect of Dibucaine on Lipid Peroxidation in Rabbit Kidneys Stored for Either 72 Hours in HCA or 24 Hours in Saline at $0^{o}C$

It was hypothesised that one possible mechanism by which increased cytosolic calcium levels may potentiate free radical damage during storage is the activation of calcium-dependent phospholipases. Dibucaine competitively inhibits calcium-dependent phospholipase A2 which removes predominantly unsaturated fatty acids from the membrane (Reinhart et al, 1984). Addition of this agent to the flush and storage solution significantly ($P<0.05$) decreased both markers of lipid peroxidation in cortex and medulla of kidneys stored in either HCA for 72 hours (Figure 18), or saline for 24 hours (Figure 19) when compared to stored non-treated controls. The addition of dibucaine directly to homogenates of kidney stored in HCA for 72 hours and saline for 24 hours had no effect on lipid peroxidation (Table 4).

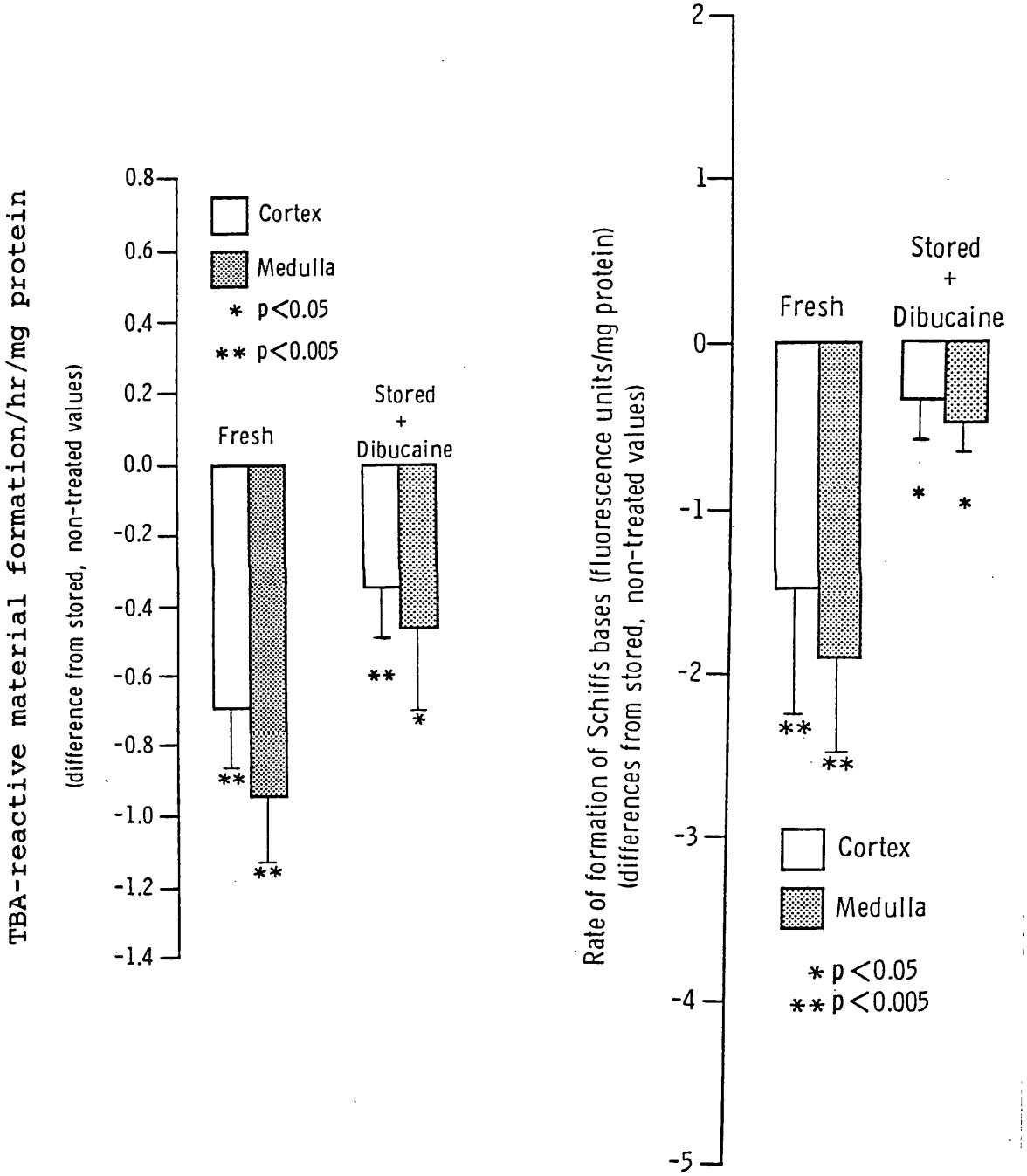
TABLE 4: The Effect of Dibucaine (250μM) on Lipid Peroxidation in Homogenates of Kidneys after Storage

Experiment	Section of kidney	Formation of lipid peroxidation products mean ± 95% confidence interval			
n=6		SB Change in fluorescence intensity/hr /mg protein		TBARs /hr /mg protein	
250μM dibucaine added to the homogenate after storage in HCA	cortex	1.69 ± 0.68	1.91 ± 0.66	1.15 ± 0.25	0.95 ± 0.27
	medulla	1.36 ± 0.71	1.13 ± 0.23	1.41 ± 0.38	1.30 ± 0.36
250μM dibucaine added to the homogenate after storage in saline	cortex	1.11 ± 0.55	1.46 ± 0.58	1.09 ± 0.17	1.14 ± 0.22
	medulla	1.52 ± 0.66	1.21 ± 0.58	1.75 ± 0.38	1.56 ± 0.3

FIGURE 18: The Rate of Formation of TBA-Reactive Material and Schiff Bases in Homogenates of Cortex and Medulla from Kidneys Flushed and Stored for 72 Hours at 0°C in Hypertonic Citrate Solution Containing Dibucaine (250μM).

Section of kidney	Formation of lipid peroxidation products mean + SD (difference from stored, nontreated values)	
	SB Change in fluorescence intensity/hr /mg protein	TBARs /hr /mg protein
cortex	-0.345 ± 0.309	-0.355 ± 0.174
medulla	-0.480 ± 0.210	-0.446 ± 0.262

FIGURE 18: The Rate of Formation of TBA-Reactive Material and Schiff Bases in Homogenates of Cortex and Medulla from Kidneys Flushed and Stored for 72 Hours at 0°C in Hypertonic Citrate Solution Containing Dibucaine (250μM).

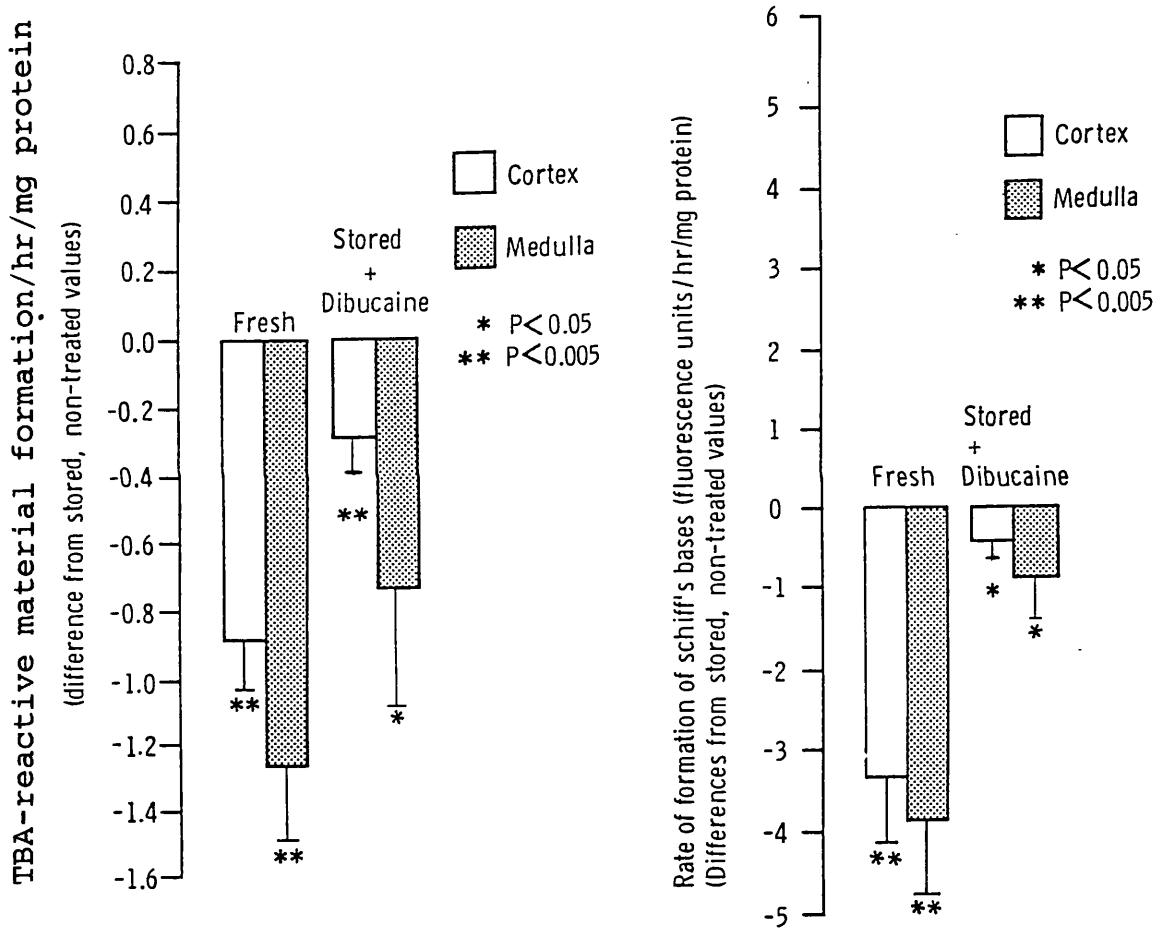


Values are expressed as the mean difference from stored, nontreated kidneys ± 95% confidence interval.

FIGURE 19: The Rate of Formation of TBA-reactive Material and Schiff Bases in Homogenates of Cortex and Medulla from Kidneys Flushed and Stored for 24 Hours at 0°C in Isotonic Saline Solution Containing Dibucaine (250μM).

Section of kidney	Formation of lipid peroxidation products mean + SD (difference from stored, nontreated values)	
	SB Change in fluorescence intensity/hr /mg protein	TBARs /hr /mg protein
cortex	-0.370 ± 0.246	-0.241 ± 0.129
medulla	-0.844 ± 0.493	-0.727 ± 0.413

FIGURE 19: The Rate of Formation of TBA-reactive Material and Schiff Bases in Homogenates of Cortex and Medulla from Kidneys Flushed and Stored for 24 Hours at 0°C in Isotonic Saline Solution Containing Dibucaine (250μM).



Values are expressed as the mean difference from stored, nontreated kidneys ± 95% confidence interval.

Thus this agent at a concentration of 250μM was not exerting any direct antioxidant effect in the homogenate alone. It would appear, therefore, that inhibition of calcium-dependent phospholipase A2 resulted in reduced membrane peroxidation in kidneys following cold storage.

The Effect of Allopurinol on Lipid Peroxidation in Rabbit Kidneys Stored for 24 Hours in Saline at 0°C

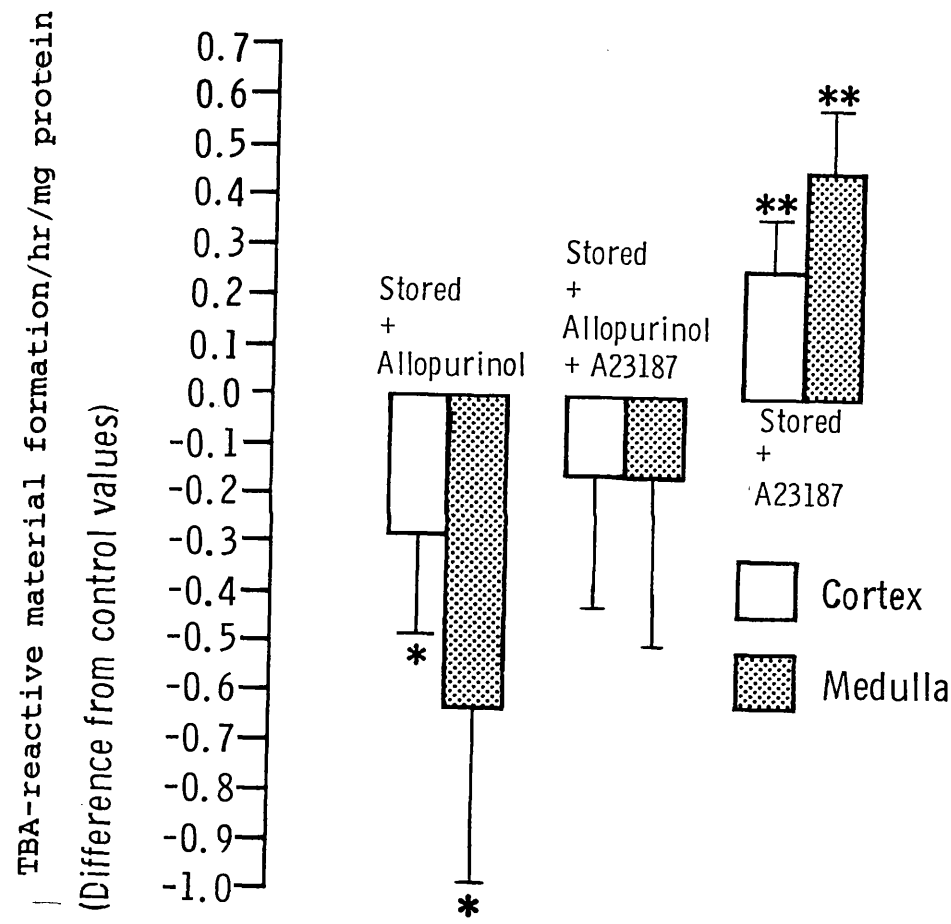
Increased cytosolic calcium levels may enhance free radical production and thence lipid peroxidation by activating proteases which convert the enzyme xanthine dehydrogenase to xanthine oxidase (Roy and McCord, 1983). This is likely to be most important during reperfusion; after exposure to molecular oxygen, this form of the enzyme converts hypoxanthine, a product of adenine nucleotide degradation which accumulates during ischaemia, to xanthine and the superoxide anion ($O_2^{\bullet -}$) is produced. In the presence of transition metal catalysts, $O_2^{\bullet -}$ can be converted to highly reactive species such as the hydroxyl radical ($OH^{\bullet}$) and iron-complexed molecules which then initiate lipid peroxidation.

It was found that addition of the xanthine oxidase inhibitor allopurinol to saline flush and storage solution significantly inhibited lipid peroxidation ($P<0.05$) compared to stored, non-treated controls, and was even able to inhibit the significant ($P<0.005$) rises observed when A23187 was added to the system (Figure 20). This provided some

FIGURE 20: The Rate of Formation of TBA-Reactive Material in Homogenates of Cortex and Medulla from Kidneys Flushed and Stored for 24 Hours at 0°C in Isotonic Saline Solution Containing Allopurinol (5mM) or Allopurinol (5mM) + A23187 (10μM).

Experiment	Section of kidney	Formation of lipid peroxidation products mean + SD (difference from stored, nontreated values)
		TBARs /hr /mg protein
Stored + allopurinol	cortex	-0.290 ± 0.242
	medulla	-0.640 ± 0.491
Stored + allopurinol + A23187	cortex	-0.170 ± 0.483
	medulla	-0.171 ± 0.483
Stored + A23187	cortex	0.248 ± 0.128
	medulla	0.451 ± 0.188

FIGURE 20: The Rate of Formation of TBA-Reactive Material in Homogenates of Cortex and Medulla from Kidneys Flushed and Stored for 24 Hours at 0°C in Isotonic Saline Solution Containing Allopurinol (5mM) or Allopurinol (5mM) + A23187 (10μM).



Values are expressed as the mean difference from stored, nontreated kidneys ± 95% confidence interval.

* P<0.05, ** P<0.005

circumstantial evidence that increased cytosolic calcium levels and xanthine oxidase are important mediators of lipid peroxidation in this model.

The effect of the xanthine oxidase inhibitor allopurinol on rabbit kidneys flushed and stored in saline solution for 24 hours was initially investigated. However, these studies were not extended in this thesis.

CHAPTER 4: FREE FATTY ACID ACCUMULATION IN COLD STORED RABBIT KIDNEYS

One possible mechanism by which calcium ions could potentiate oxidative damage is through the activation of phospholipases. In the previous chapter, it was demonstrated that addition of the phospholipase A2 inhibitor dibucaine to the flush and storage solutions decreased markers of lipid peroxidation following cold storage of rabbit kidneys for 72 hours in HCA and 24 hours in saline. It has also been shown that phospholipase A2 activity and markers of lipid peroxidation increase during ischaemia of rat small intestinal mucosa (Otamiri et al, 1987). Free fatty acids (FFA), released from cellular membranes as a result of phospholipase action, are known to accumulate during periods of ischaemia in several other models (Rehncrona et al, 1982; Shui et al, 1983; Nemoto et al, 1987). Such accumulation could be detrimental to cells both because of the detergent-like properties of FFAs and because they may be implicated in causing further ATP loss and mitochondrial injury following anoxia, for example, in the myocardium (Piper and Das, 1986).

The residual lysophosphatides remaining in the membrane following phospholipase action also increase during ischaemia and are themselves damaging species, potentially deleterious to cell function (Shaikh and Das, 1981). In particular, the disruption of membranes following

phospholipid hydrolysis may render them more susceptible to attack by free radicals (Ungemach, 1985).

In view of these earlier findings, the FFA status of rabbit kidneys following cold storage in a poor storage solution (saline) and the clinically approved hypertonic citrate solution (HCA) was investigated by gas liquid chromatography. In addition, in an attempt to gain further insight into the role of calcium and phospholipase A2 in FFA accumulation and membrane peroxidation, the effects of adding the calcium ionophore A23187 and the phospholipase A2 inhibitor dibucaine to HCA during the storage period were examined.

Fresh as well as treated, stored kidneys were paired against non-treated kidneys from the same rabbit to eliminate variation between animals in the experiments in this chapter. A Student's paired t-test was carried out in each case to determine significance. Six determinations were carried out in all experiments except where stated otherwise.

FFA Extraction and Analysis in Freeze-Clamped Samples Compared with Sample Homogenates

Accurate and valid measurement of FFAs is complicated by the possibility that hydrolysis of membrane phospholipids to free fatty acids and lysophosphatides may increase both during handling (Rouser et al, 1968) and through careless extraction and homogenisation (Nelson, 1969). This is due to activation of phospholipases during these procedures which results in an artifactual elevation in the products of phospholipid hydrolysis. Rabbit kidney cortex and medulla were either homogenised in PBS buffer to form a 5% (w/v) homogenate, 1ml of which was subjected to FFA extraction, or were quickly clamped under liquid nitrogen, ground to a powder and then immediately subjected to FFA extraction to avoid phospholipase activation. Table 5 shows amounts of FFA in cortex and medulla of fresh saline-flushed rabbit kidneys following either homogenization or crushing under liquid nitrogen. Amounts of the unsaturated FFAs oleic ($C_{18:1}$), linoleic ($C_{18:2}$) and arachidonic acid ($C_{20:4}$) were lower in freeze-clamped samples compared to samples which were homogenised. This was highly significant in the case of $C_{20:4}$. The levels of the FFA $C_{16:0}$ were significantly higher in freeze-clamped samples when compared to homogenized samples and the reason for this is not known. These results therefore demonstrate that levels of unsaturated FFAs are augmented when the organ is homogenised prior to FFA extraction. This observation is probably

TABLE 5: FFA Analysis of Rabbit Kidneys Flushed with Saline and either Homogenised or Crushed under Liquid Nitrogen before Extraction

FFA	Section of kidney	FFA (nmol/g tissue) mean ± S.D.		P value
		Homogenised	Crushed under liquid nitrogen	
$C_{16:0}$	cortex	81.89±22.96	149.30±81.50	0.036*
	medulla	78.24±15.55	156.04±82.14	0.059
$C_{18:0}$	cortex	143.24±41.39	181.52±93.50	0.294
	medulla	134.85±35.33	185.12±75.31	0.110
$C_{18:1}$	cortex	16.53±11.34	9.43±10.22	0.179
	medulla	16.05±8.98	9.03±6.64	0.07
$C_{18:2}$	cortex	26.69±17.87	14.22±1.11	0.097
	medulla	20.90±13.06	11.86±6.53	0.093
$C_{20:4}$	cortex	11.50±6.32	1.12±2.15	0.0008*
	medulla	10.59±7.55	1.76±1.26	0.0022*

* Homogenised samples significantly ($P<0.05$) different from those crushed under liquid nitrogen. n=8

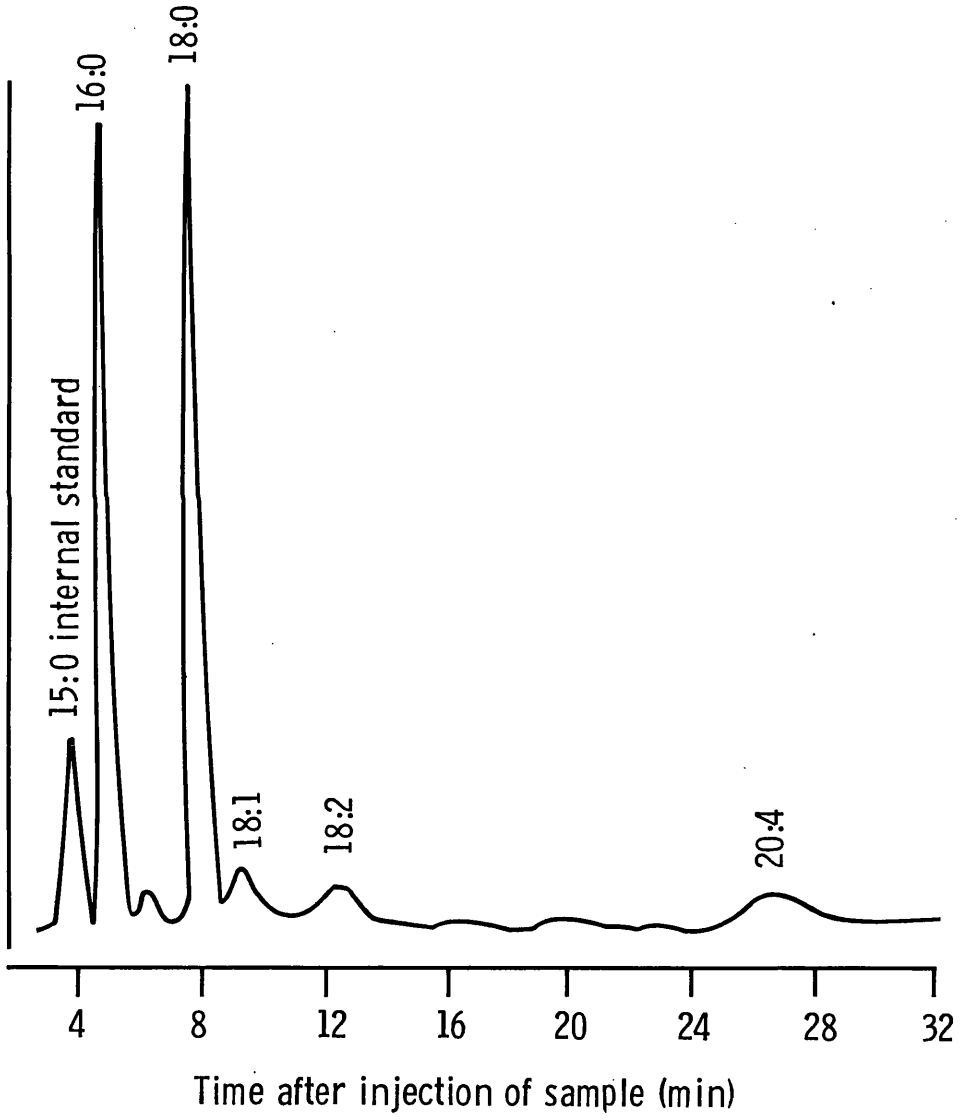
attributable to the homogenisation process itself giving rise to phospholipase activation. By freezing tissue in liquid nitrogen, crushing to a powder while still frozen and then extracting FFAs immediately it would seem possible to minimise this artifactual elevation and so provide a more accurate method of measuring FFAs.

FFA Analysis of Rabbit Kidneys Following 24 Hours Storage in Saline at 0^{o}C

Rabbit kidneys were flushed with ice-cold saline, placed in sterile beakers containing 60ml of saline and stored at 0^{o}C for 24 hours. Fresh control kidneys from the same animal were obtained under identical conditions except that the organs were flushed but not stored. Kidneys were rapidly dissected into cortex and medulla, immediately freeze-clamped and analysed for FFA status (as described in Free Fatty Acid Analysis, Chapter 2). Figure 21 shows a typical glc trace of FFAs in fresh rabbit kidney cortex. Large proportions of the saturated FFAs palmitic ($C_{16:0}$) and stearic ($C_{18:0}$) were evident with smaller amounts of the unsaturated FFAs oleic ($C_{18:1}$), linoleic ($C_{18:2}$) and arachidonic acid ($C_{20:4}$). A similar trace of FFAs was observed in the medulla.

Following the storage period, there were significant ($P<0.05$) increases in the levels of unsaturated free fatty acids oleic ($C_{18:1}$), linoleic ($C_{18:2}$) and arachidonic acid ($C_{20:4}$) compared to unstored controls in both the cortex

FIGURE 21: Separation of a Mixture of Free Fatty Acids (Following Esterification) from Fresh Rabbit Kidney Cortex by Gas-Liquid Chromatography.

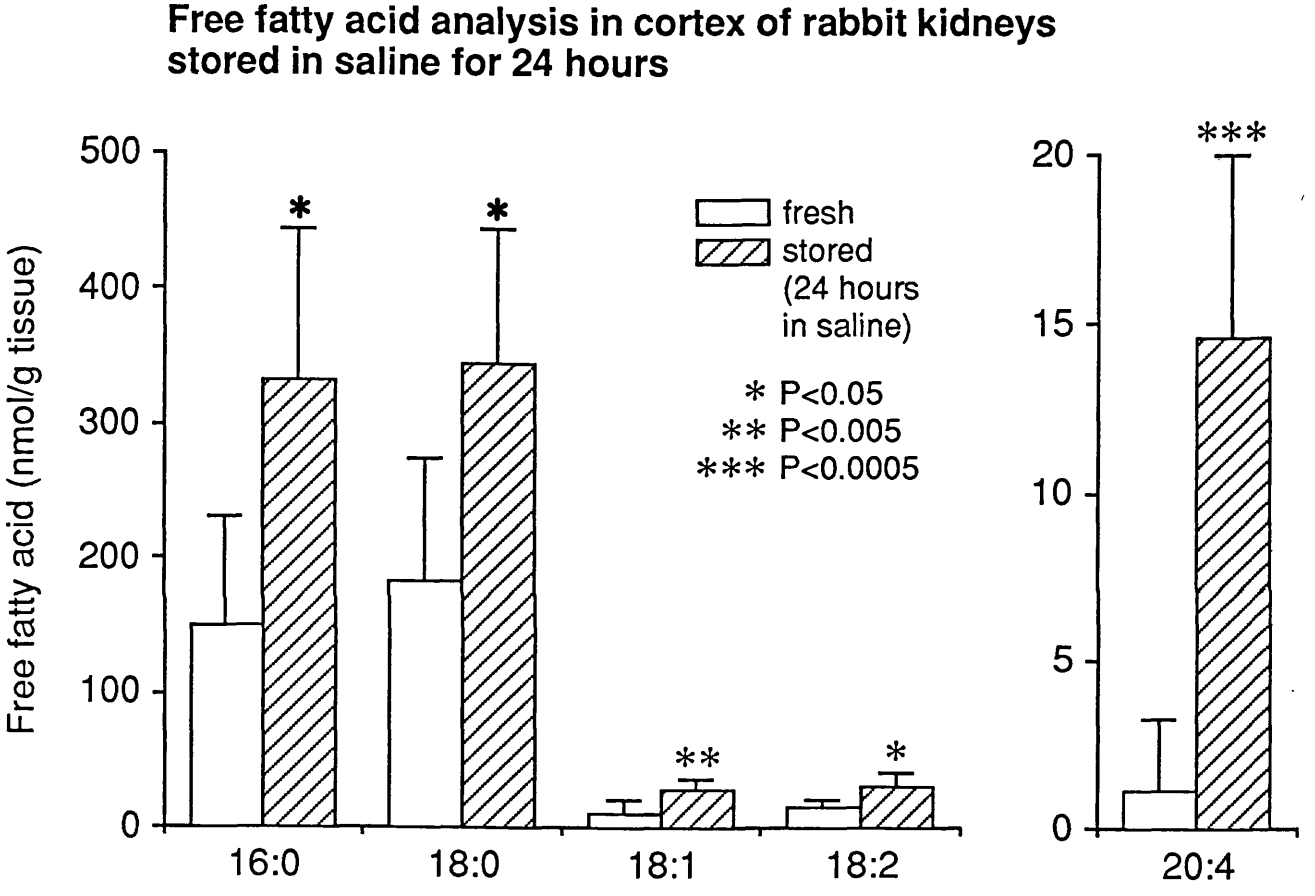


(Figure 22) and medulla (Figure 23). Significant increases were also observed in the level of the saturated FFAs palmitic ($C_{16:0}$) and stearic acid ($C_{18:0}$) in the cortex following cold storage in saline (Figure 24) but although there were increases in these FFAs in the medulla they were not statistically significant (Figure 23). The relative amounts of FFA are represented in Figure 24 for cortex and medulla, stored and non-stored. Following the storage period, the proportion of free fatty acids which were unsaturated increased both in the cortex (from 6.7% of the total FFA in control kidneys to 9.6% in stored organs) and the medulla (6.2% control to 8.9% in stored kidneys). It can be concluded that following storage in saline an increased accumulation of FFAs and particularly unsaturated FFAs is evident in both cortex and medulla.

FFA Analysis of Rabbit Kidneys Following 72 Hours Storage in HCA at $0^{o}C$

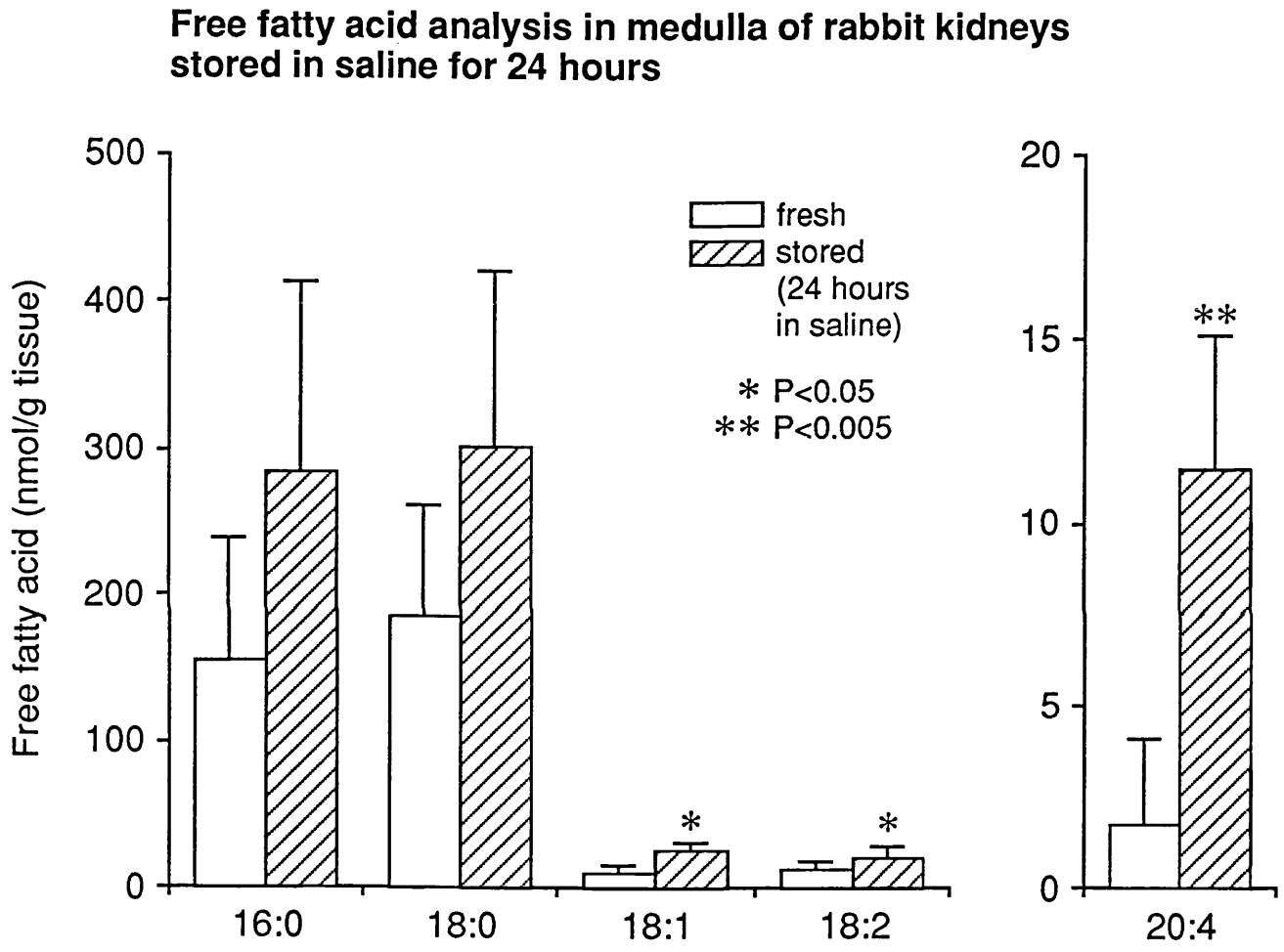
Rabbit kidneys were flushed with ice-cold HCA, placed in sterile beakers containing 60ml of HCA and stored at $0^{o}C$ for 72 hours. Fresh control kidneys from the same animal were obtained under identical conditions except that the organs were flushed but not stored. Kidneys were dissected into cortex and medulla, freeze-clamped, ground to a powder under liquid nitrogen and analysed for FFA status. Following the storage period, there were significant ($P<0.05$) increases in the unsaturated free fatty acids

FIGURE 22: FFA Analysis of Cortex from Rabbit Kidneys Stored in Saline at 0°C for 24 Hours.



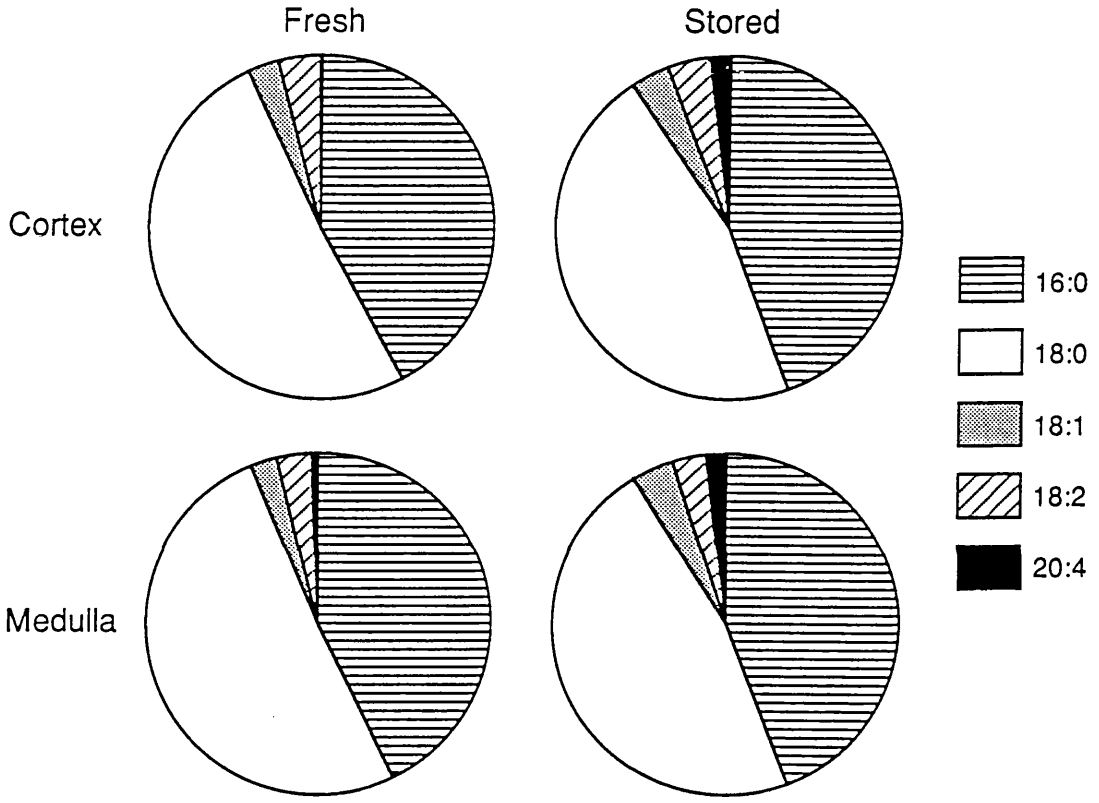
Values represent mean ± S.D. where n=6.

FIGURE 23: FFA Analysis of Medulla from Rabbit Kidneys Stored in Saline at 0°C for 24 Hours.



Values represent mean ± S.D. where n=6.

FIGURE 24: The Relative Amounts of FFAs in The Cortex and Medulla before and after Storage of Kidneys for 24 Hours at $0^{o}C$ in Saline.

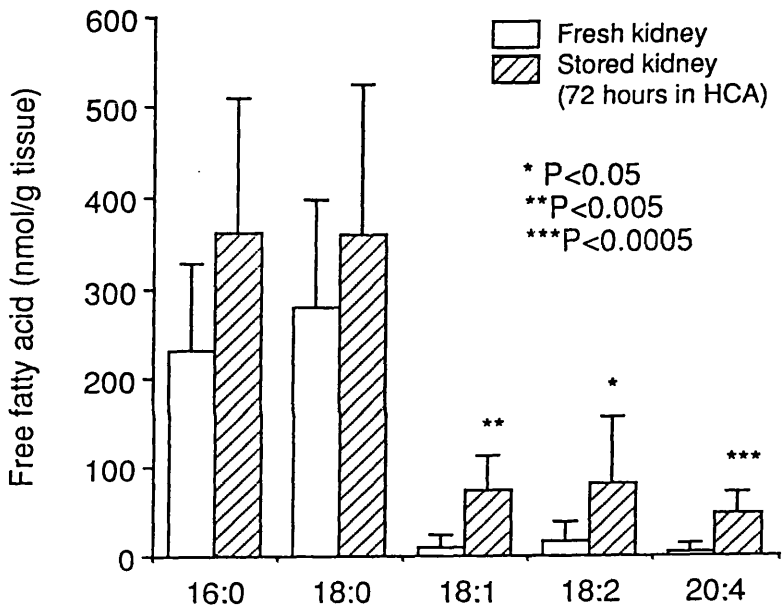


oleic ($C_{18:1}$), linoleic acid ($C_{18:2}$) and arachidonic acid ($C_{20:4}$) compared to unstored controls in both the cortex (Figure 25) and medulla (Figure 26). However, no such increases were observed in the saturated free fatty acids palmitic ($C_{16:0}$) and stearic acid ($C_{18:0}$) following cold storage in HCA in either part of the kidney (Figures 25 and 26). The relative amounts of FFA are represented in Figure 27 for cortex and medulla, stored and unstored. Following the storage period, the proportion of free fatty acids which were unsaturated increased both in the cortex (from 5.8% of the total FFA in control kidneys to 22.2% in stored organs) and the medulla (2.3% control to 13.0% in stored kidneys). It can be concluded that following cold storage in HCA, an increased accumulation of unsaturated FFAs is evident. HCA, although superior as a storage solution compared to saline, fails to protect against an increase in FFA accumulation.

The Effect of Adding A23187 to the HCA Flush and Storage Solution on FFA Content of Kidneys Stored for 72 Hours at 0°C

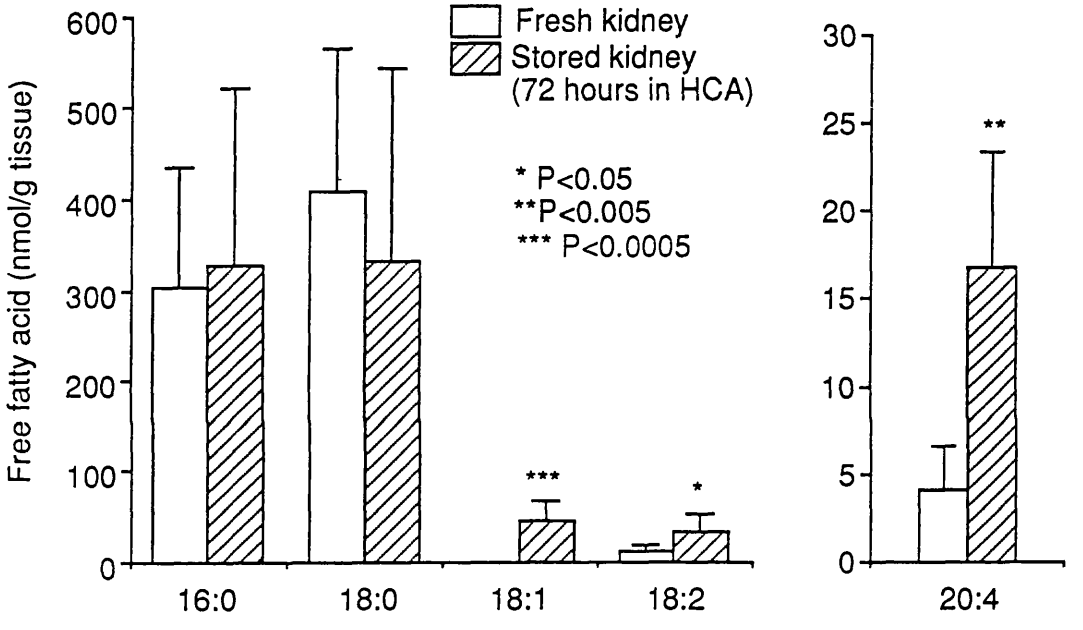
In order to investigate the role for cytosolic calcium in the accumulation of FFAs, the calcium ionophore A23187 was used to deliberately raise calcium levels in stored kidneys. Addition of A23187 (10μM) to the HCA solution used to flush out and store kidneys for 72 hour led to a significant increase ($P<0.05$) in the levels of free arachidonic acid in both the cortex and medulla compared to

FIGURE 25: FFA Analysis of Cortex from Rabbit Kidneys Stored in HCA at 0°C for 72 Hours.



Values represent mean ± S.D. where n=6.

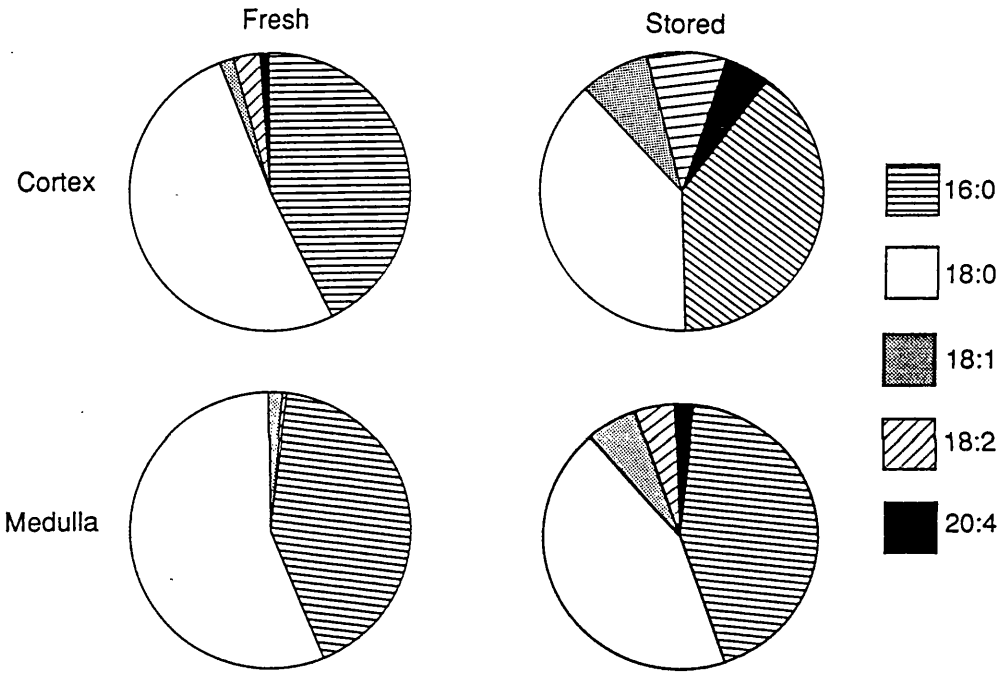
FIGURE 26: FFA Analysis of Medulla from Rabbit Kidneys Stored in HCA at 0°C for 72 Hours.



In fresh samples of kidney medulla the FFA $C_{18:1}$ was not detectable.

Values represent mean ± S.D. where n=6.

FIGURE 27: The Relative Amounts of FFAs in the Cortex and Medulla before and after Storage of Kidneys for 72 Hours at $0^{o}C$ in HCA.

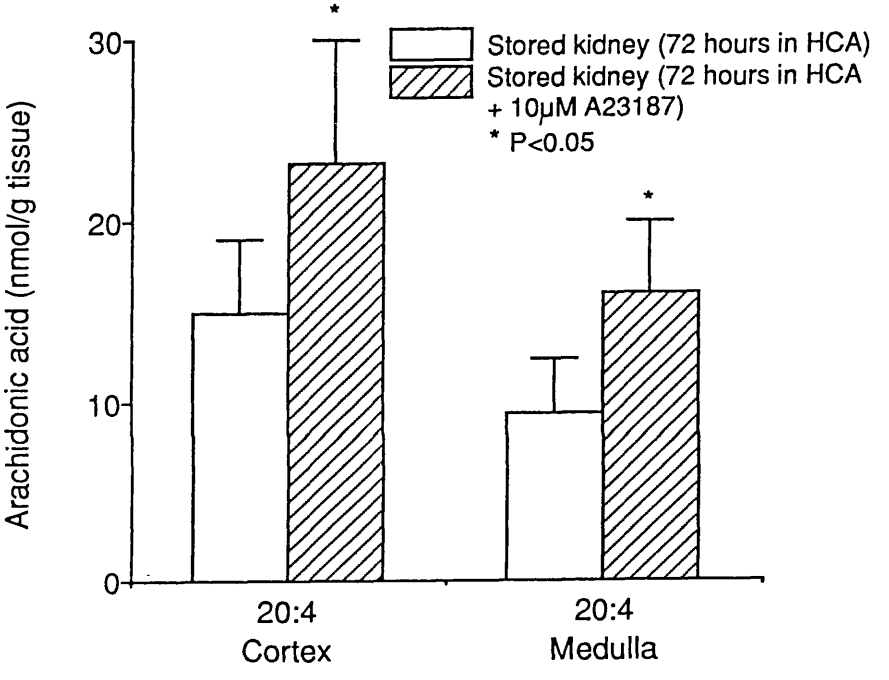


the already elevated levels in the stored untreated controls (Figure 28). A23187 had no significant effect on the levels of free $C_{16:0}$, $C_{18:0}$, $C_{18:1}$ and $C_{18:2}$ following the storage period (Table 6). Addition of A23187 (10μM) to the HCA solution used to flush and store kidneys prior to 72 hour storage led to a significant increase in TBARs (see Figure 14 in Chapter 3) in cortex and medulla ($P<0.05$) and the formation of Schiff bases (see Figure 14 in Chapter 3) in both cortex ($P<0.005$) and medulla ($P<0.05$). It would appear from these results that an increase in cytosolic calcium evoked by A23187 resulted in an increased accumulation of free arachidonic acid during cold storage of kidneys in HCA for 72 hours and also increased the organ's susceptibility to peroxidation.

The Effect of Adding Dibucaine to the HCA Flush and Storage Solutions on FFA Content of Kidneys Stored for 72 Hours at $0^{o}C$

In order to investigate the role of phospholipase A2 in the accumulation of FFAs, the calcium-dependent phospholipase A2 inhibitor dibucaine was used. Addition of dibucaine (250μM) to the HCA solution used to flush out and store kidneys prior to 72 hour storage led to a significant decrease ($P<0.05$) in the level of free $C_{20:4}$ in both the renal cortex and medulla compared to stored, untreated controls (Figure 29). However, dibucaine had no significant effect on the levels of free $C_{16:0}$, $C_{18:0}$, $C_{18:1}$

FIGURE 28: Free Arachidonic Acid Analysis of Cortex and Medulla from Rabbit Kidneys Stored for 72 Hours at 0°C in HCA Containing A23187 (10μM).



Values represent mean ± S.D. where n=6.

TABLE 6: FFA Analysis of Rabbit Kidneys Following 72 Hours Storage at 0°C in HCA with 10μM A23187

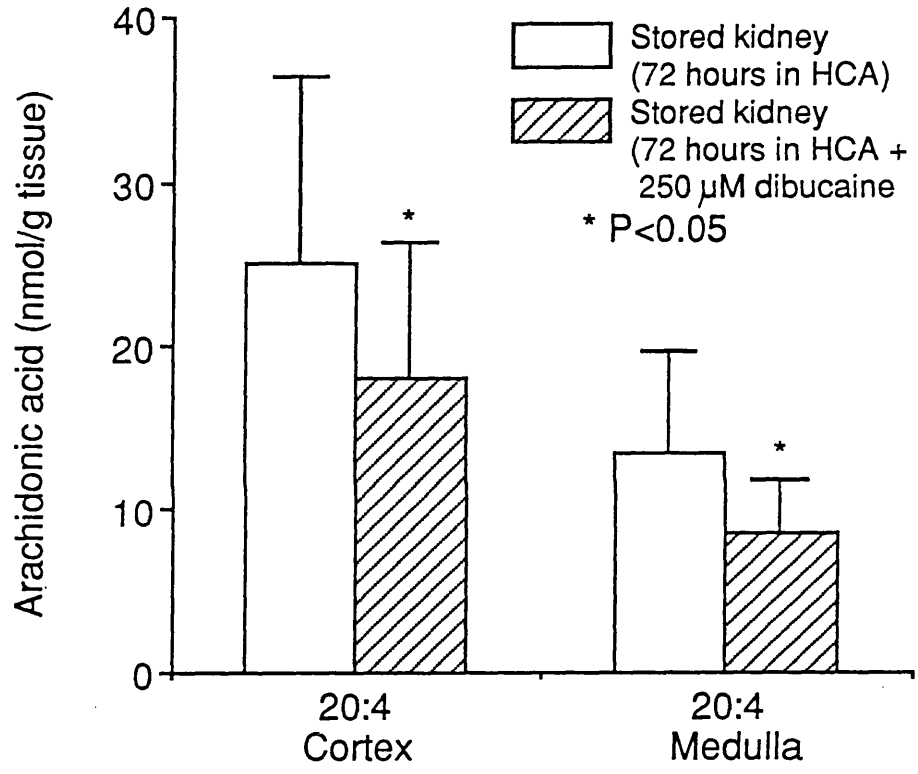
FFA	Section of kidney	Control (stored, untreated) nmol/g wet weight	Treated (stored +A23187) nmol/g wet weight
$C_{16:0}$	cortex	455.29 ±135.03	495.26 ±120.89
	medulla	452.37 ±115.04	473.33 ±185.24
$C_{18:0}$	cortex	599.34 ±155.55	675.36 ±167.85
	medulla	653.39 ±195.53	646.35 ±257.04
$C_{18:1}$	cortex	45.10 ±10.18	50.00 ±13.72
	medulla	34.96 ±9.74	35.84 ±15.05
$C_{18:2}$	cortex	39.67 ±8.91	53.04 ±16.05
	medulla	26.74 ±4.46	24.96 ±8.02

Values represent mean ± S.D. where n=6.

and $C_{18:2}$ following storage (Table 7). Addition of dibucaine (250μM) to the HCA solution used to flush out and store kidneys for 72 hours led to a significant decrease in TBARs (see Figure 18 in Chapter 3) in cortex ($P<0.005$) and medulla ($P<0.05$) and in the formation of Schiff bases (see Figure 18 in Chapter 3) in both cortex ($P<0.05$) and medulla ($P<0.005$). This data suggest the involvement of a calcium-dependent phospholipase A2 in the increased accumulation of arachidonic acid following storage. Inhibition of phospholipase A2 by dibucaine not only decreased free arachidonic acid accumulation, but also decreased the tissue's susceptibility to peroxidation.

The effects of the phospholipase A2 inhibitor dibucaine and the calcium ionophore A23187 on the FFA status of rabbit kidneys following 72 hours cold storage were significant in altering the rate of accumulation of the FFA arachidonic acid. However, these effects were relatively small when compared to the changes observed in markers of lipid peroxidation (Figures 19 and 20) in this model. This suggests that although calcium-dependent activation of phospholipase A2 may be at least in part responsible for the potentiation of lipid peroxidation, other calcium-dependent mechanisms are also likely to be important.

FIGURE 29: Free Arachidonic Acid Analysis of Cortex and Medulla from Rabbit Kidneys Stored for 72 Hours at 0°C in HCA Containing Dibucaine (250μM).



Values represent mean ± S.D. where n=6.

TABLE 7: FFA Analysis of Rabbit Kidneys Following 72 Hours Storage at 0°C in HCA containing 250μM Dibucaine

FFA	Section of kidney	Control (stored, untreated) nmol/g wet weight	Treated (stored +dibucaine)
$C_{16:0}$	cortex	565.94 ±139.90	538.16 ±138.93
	medulla	498.67 ±154.04	473.32 ±178.41
$C_{18:0}$	cortex	803.66 ±208.71	744.34 ±190.26
	medulla	726.76 ±223.65	703.48 ±235.52
$C_{18:1}$	cortex	43.37 ±7.52	39.38 ±8.85
	medulla	27.44 ±7.97	28.76 ±9.29
$C_{18:2}$	cortex	47.24 ±9.8	34.76 ±19.61
	medulla	25.85 ±8.91	27.63 ±8.47

Values represent mean ± S.D. where n=6.

CHAPTER 5: THE EFFECT OF LIPID PEROXIDATION AND HYPOXIA/REOXYGENATION ON PHOSPHATIDYLINOSITOL TURNOVER IN RABBIT KIDNEY SLICES

It is now well establised that certain agonists (e.g. hormones and neurotransmitters) evoke a secondary messenger response through the specific hydrolysis of membrane phosphoinositides (Berridge, 1984). Agonists may induce a conformational change in the receptor, which in turn perturbs the membrane sufficiently to make phosphatidylinositol-4,5,-bisphosphate (PIP_2) accessible to a PIP_2-specific phosphodiesterase which is thought to be controlled by coupling of receptors to the enzyme through a GTP-binding protein (G-protein) (Gomperts, 1983; Gilman, 1984). Cleavage of PIP_2 by this phosphodiesterase generates two second messenger products; inositol trisphosphate (IP_3) and diacylglycerol (DAG) which initiate cellular responses. IP_3 mobilizes calcium from intracellular stores and DAG stimulates phosphorylation of protein kinase C (PKc), a process which requires phospholipids and calcium for maximum activity. The inositol phosphate cycle converts IP_3 to inositol-1,4,-bisphosphate (IP_2) and then to inositol-1-phosphate (IP_1) (Downes et al, 1982) which is finally converted to free inositol by inositol-1-phosphatase. This enzyme can be markedly inhibited by lithium (Hallcher and Sherman, 1980; Berridge et al, 1982) leading to accumulation of these water soluble inositol phosphates.

Activation of α_1-adrenoceptors leads to phosphoinositide hydrolysis to produce secondary messengers which lead to a variety of responses in the cell (Berridge, 1984). In the kidney, binding of agonists to α_1-adrenoceptors stimulates a variety of responses. Dianzani et al (1989) have shown that an aldehydic product of lipid peroxidation 4-hydroxynonenal (HNE), can provoke the breakdown of PIP_2 possibly through the interaction of HNE with the regulatory G-protein. It is therefore possible that lipid peroxidation might augment PIP_2 breakdown and so release secondary messengers.

In view of previous findings (Chapter 3) that ischaemia renders kidneys more susceptible to lipid peroxidation, experiments were carried out to investigate the possibility that ischaemia may lead to altered PI turnover which, by causing an imbalance in intracellular signalling, may contribute to physiological deterioration of transplanted organs.

Myo-(2-^{3}H)-Inositol Uptake: An Assessment in Three Different Models

Determination of PIP_2 breakdown by methods used in this investigation involves the incorporation into the cell and then into the membrane of the radiolabelled precursor myo-(2-^{3}H)-inositol. It is therefore important to choose a model in which the uptake of this precursor is efficient. Three models were investigated initially: isolated cultured hepatocytes; rabbit kidney in vivo; and rabbit kidney cortical slices. In each case ^{3}H-inositol was administered and the amount of incorporated myo-(2-^{3}H)-inositol and radiolabelled water-soluble products assayed by hplc (see Materials and Methods). Figure 30 shows hplc analysis obtained when hepatocytes were incubated with ^{3}H-inositol. Prior to hplc analysis, adenine nucleotides were added as markers which were detected by u.v. absorption and facilitated the identification of the inositol phosphate peaks (See Analysis of Radiolabelled Inositol Phosphates). As previously reported (Irvine et al, 1985), inositol itself eluted very close to the solvent front, glycerphosphoinositol (GPI) soon after, IP_1 eluted slightly after AMP, IP_2 after ADP and IP_3 after ATP. IP_1 was also identified by comparison of its retention time against a known standard. The peak inositol count was 1.5×10^{3} c.p.m. in hepatocytes. However, as shown in Figures 31 and 32, the level of radioactivity from the incorporated myo-(^{3}H)-inositol was greater in both the cortex and medulla of

FIGURE 30: Hplc Analysis of Inositol Phosphates in Rat Hepatocytes Following Incubation with Myo-(2-^{3}H)-Inositol.

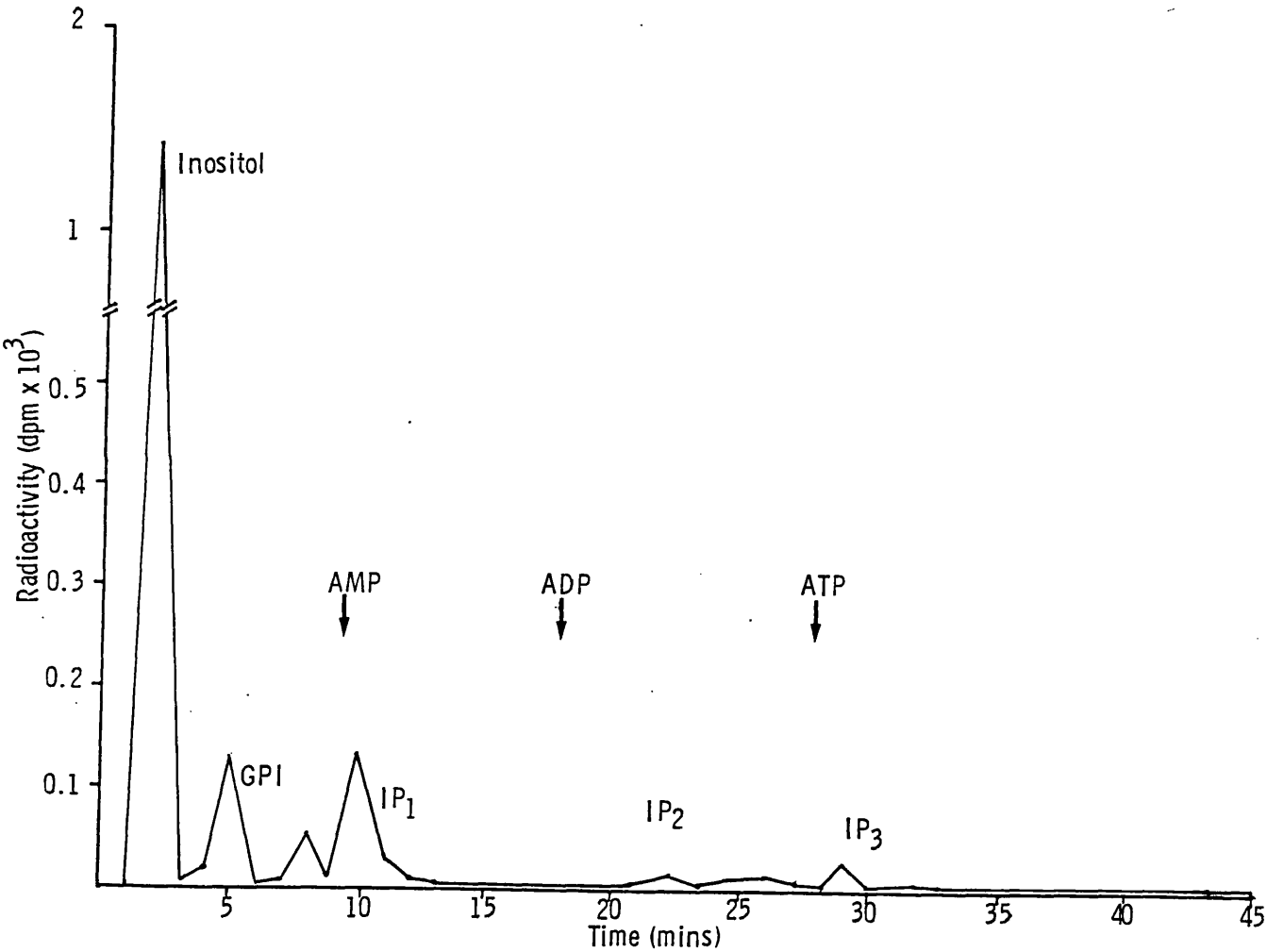


FIGURE 31: Hplc Analysis of Inositol Phosphates in Rabbit Kidney Cortex Following i.p. Injection of Myo-(2-^{3}H)-Inositol into the Rabbit.

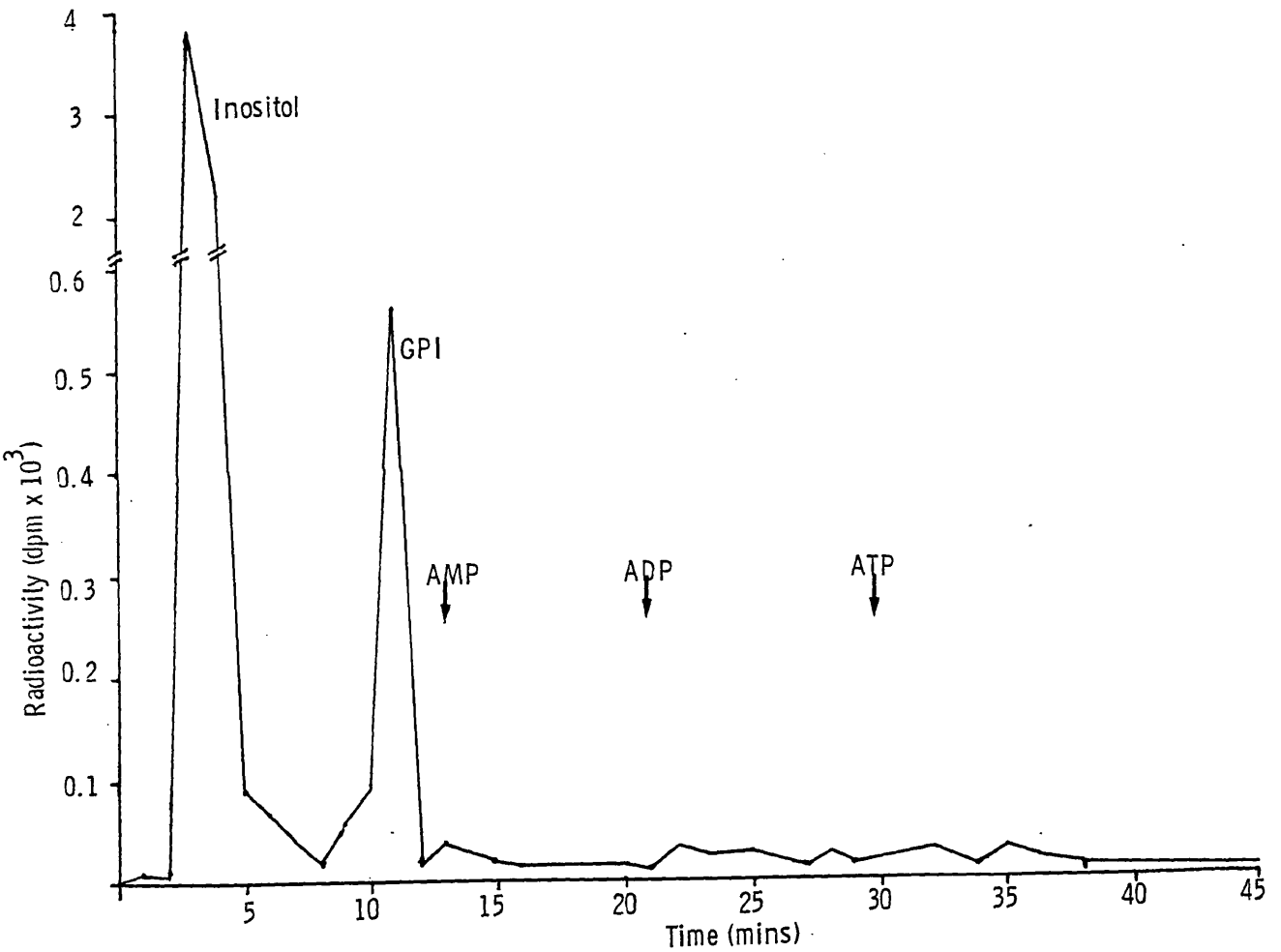


FIGURE 32: Hplc Analysis of Inositol Phosphates in Rabbit Kidney Medulla Following i.p. Injection of Myo-(2-^{3}H)-Inositol into the Rabbit.

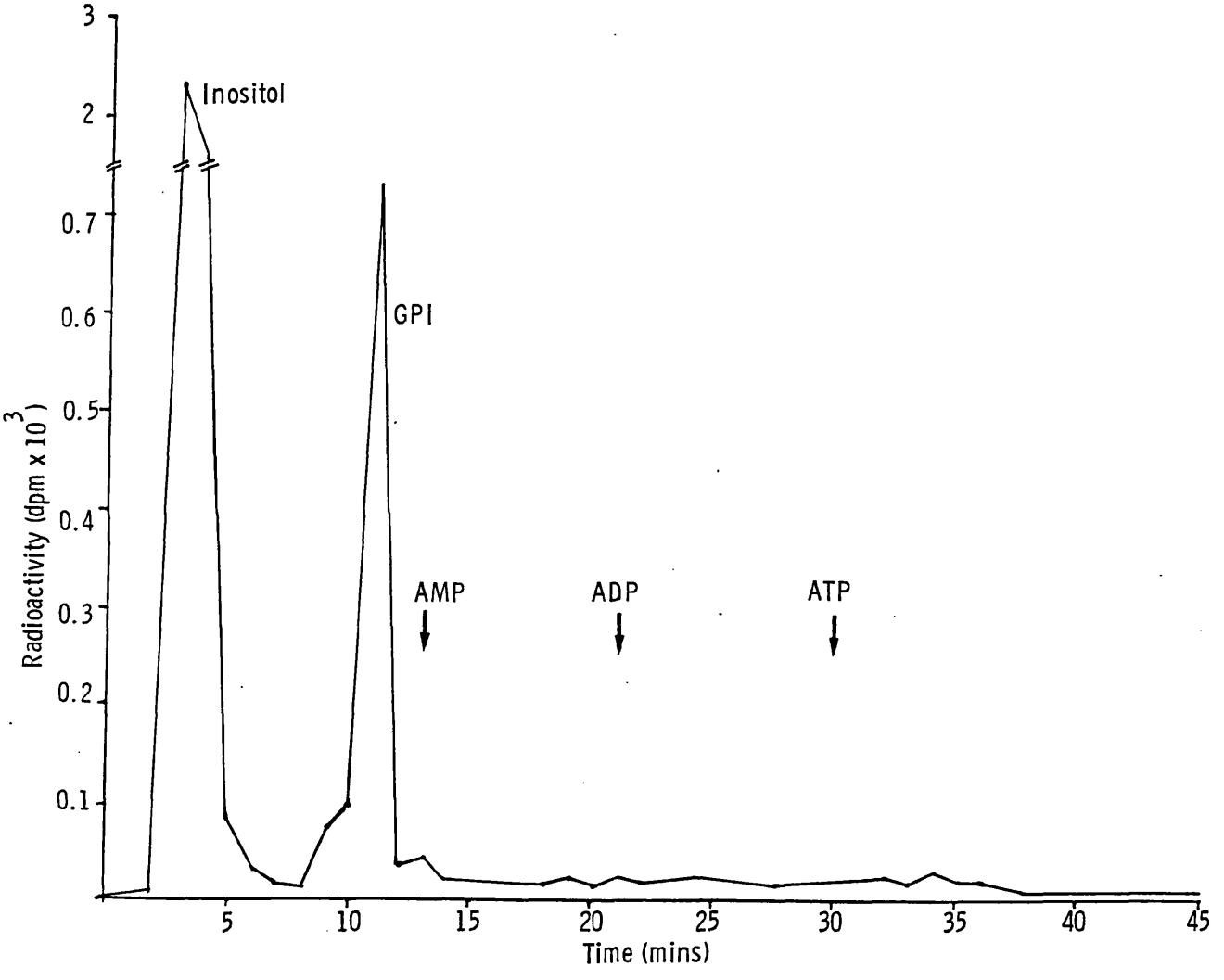
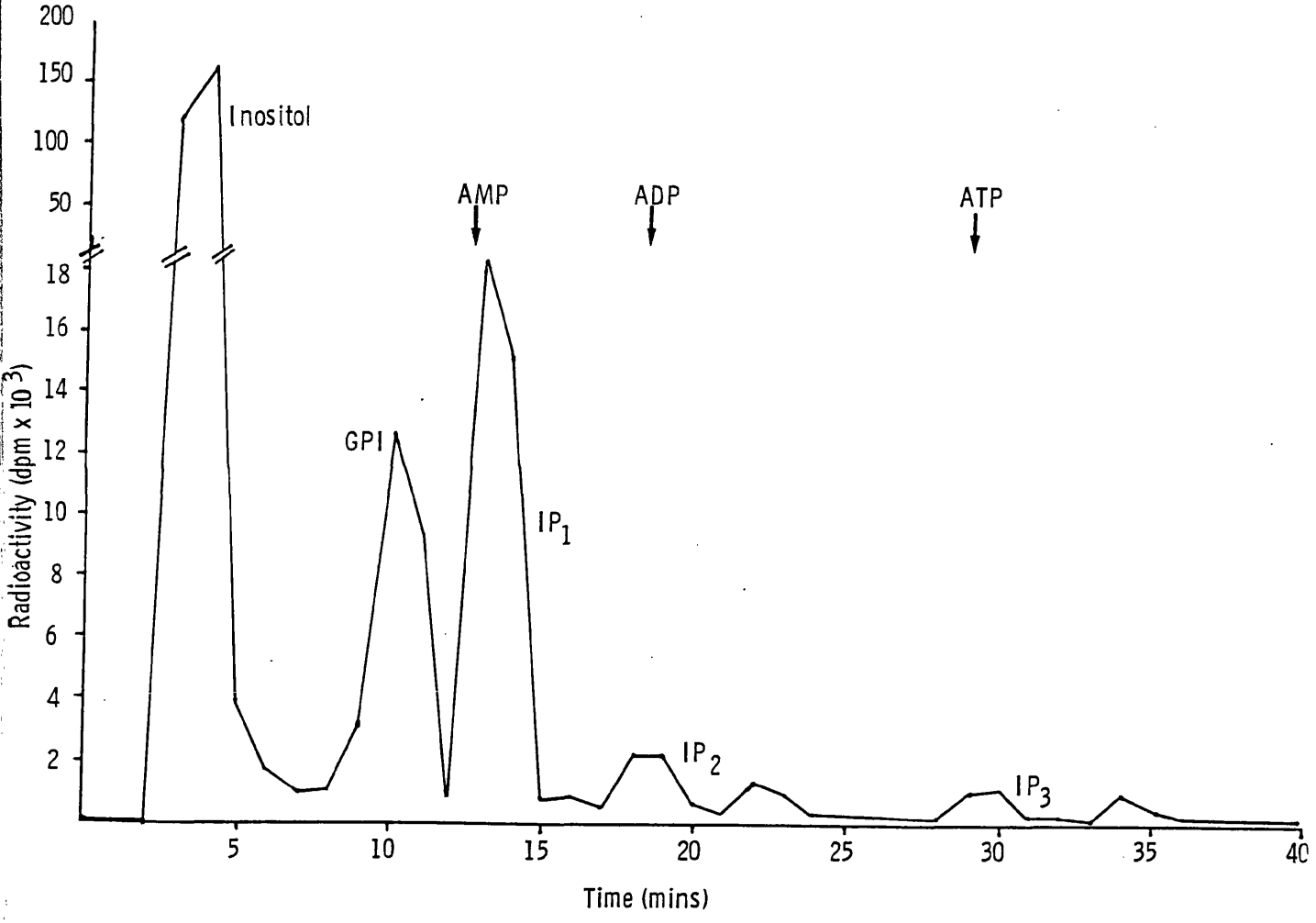


FIGURE 33: Hplc Analysis of Inositol Phosphates in Rabbit Kidney Cortical Slices Following Incubation with Myo-(2-^{3}H)-Inositol.

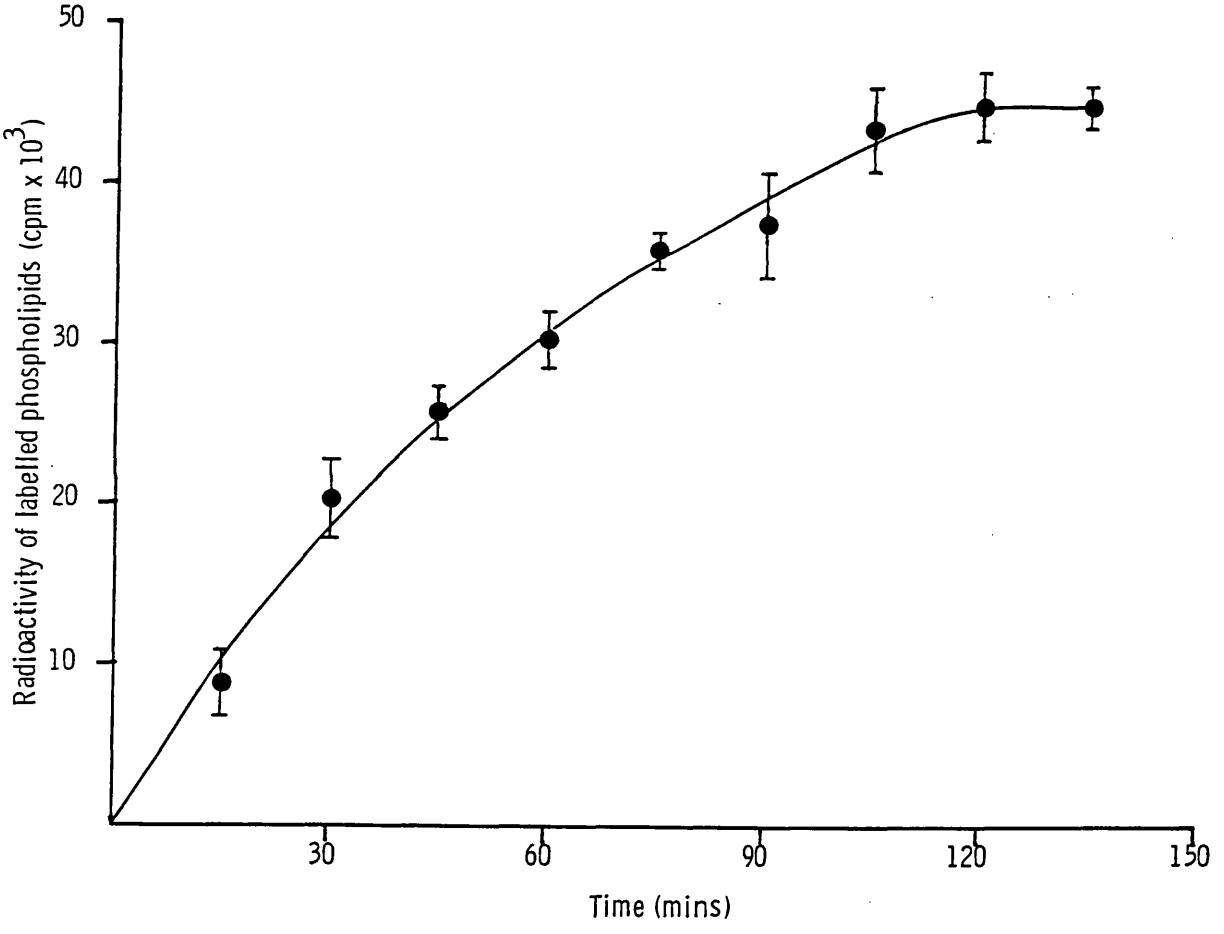


rabbit kidneys following i.p. administration of myo-(2-^{3}H)-inositol to rabbits, although the radioactivity from the inositol phosphates appeared to be similar in both these models. It is therefore possible that incorporation of the label from the cytosol into the membrane and subsequently into the soluble inositol phosphates was relatively greater in hepatocytes than in the rabbit kidney in vivo model. Figure 33 shows hplc analysis of radiolabelled inositol and inositol phosphates following incubation of rabbit kidney cortical slices with myo-(2-^{3}H)-inositol. A much greater amount of radioactivity was observed in the incorporated inositol and inositol phosphate fractions indicating a considerably better uptake of the label in this model. All three inositol phosphates could be easily identified and there was an additional peak which eluted before AMP and this was likely to be glycerophosphoinositol as suggested by Berridge (1986). Rabbit kidney cortical slices were therefore chosen for further experimentation.

Incorporation of Myo-(2-^{3}H)-Inositol into the Membranes of Rabbit Kidney Cortical Slices

In addition to establishing that uptake of myo-(2-^{3}H)-inositol into cells was satisfactory, it is also important to verify that the label was incorporated into the membrane to form radiolabelled PIP_2. A time course was carried out in which rabbit kidney cortical slices were incubated with myo-(2-^{3}H)-inositol for 135 minutes. At 15

FIGURE 34: Incorporation of Myo-(2-^{3}H)-Inositol into the Membranes of Rabbit Kidney Cortical Slices.

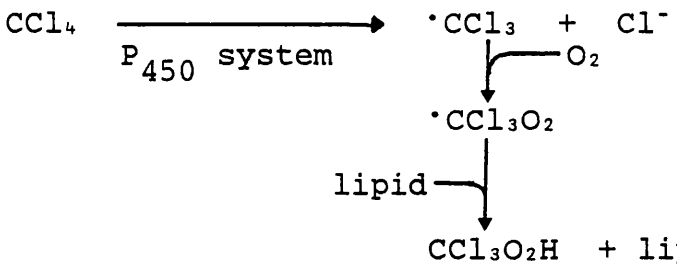


Values are the means ± 95% confidence limits of 3 separate determinations.

minute intervals, aliquots were removed and the lipid fraction extracted into chloroform:methanol (2:1) (see Methods). Figure 34 shows the amount (mean ± 95% confidence intervals) of radioactivity in the lipid phase of the slices over the 135 minutes. Incorporation of the label into the membrane was time-dependent reaching a plateau after 120 minutes. However, sufficient incorporation took place after 60 minutes to allow examination of changes in the accumulation of inositol phophates and hence this was the time chosen for the pre-incubation of rabbit kidney cortical slices with myo-(2-^{3}H)-inositol in subsequent experiments.

Membrane Peroxidation of Rabbit Kidney Slices using Ferrous Ions and Carbon Tetrachloride

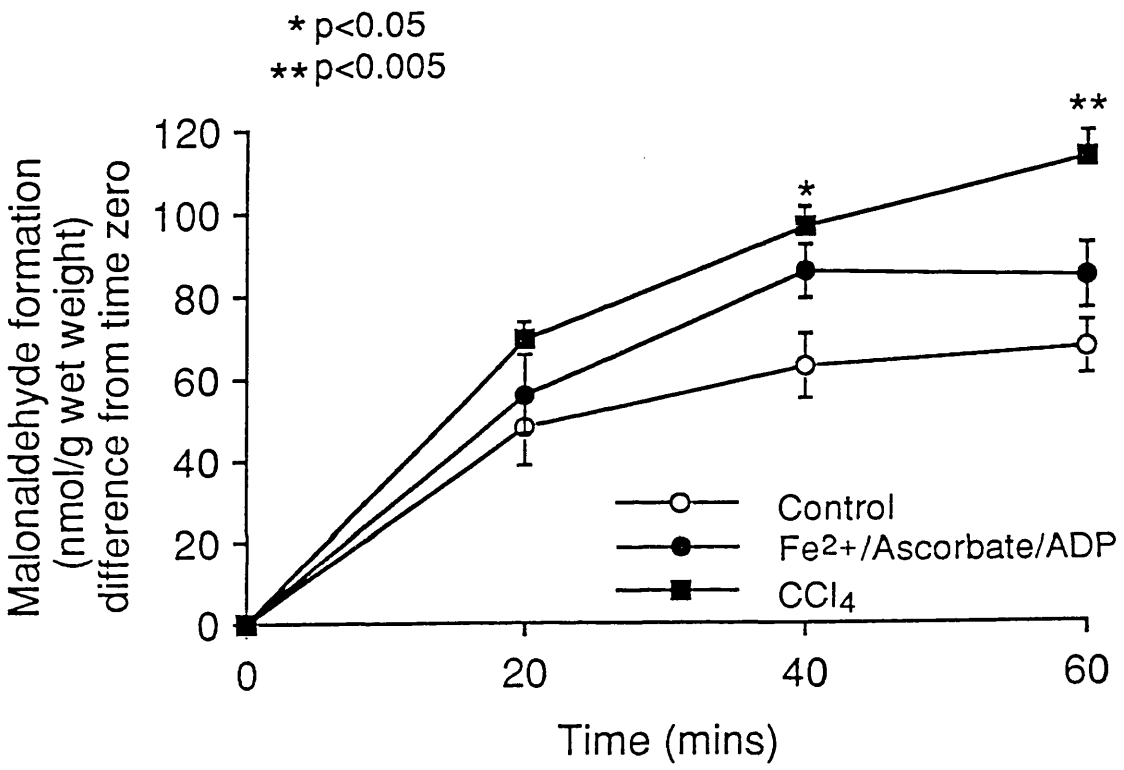
In order to investigate the effect of lipid peroxidation on PIP_2 breakdown it was necessary to develop a system for producing membrane peroxidation in rabbit kidney slices. Two systems were investigated; carbon tetrachloride (CCl_4) and iron-ADP in the presence of ascorbate. CCl_4 is metabolised by the cytochrome P_{450} system to give the trichloromethyl radical ($CCl_3^{\bullet}$) (Slater, 1984), which can react with molecular O_2 to form the more reactive species, the trichloromethyl peroxy radical ($CCl_3O_2^{\bullet}$) (Packer, et al, 1978). This is a likely candidate for causing membrane peroxidation:



Iron in the presence of O_2 and a reducing agent such as ascorbate which reduces Fe(III) ions to Fe(II), is effective at initiating lipid peroxidation in liver microsomes (Orrenius, Dallner and Ernster, 1964). ADP is added to this system as a ligand for Fe^{2+} to form a more catalytic iron complex.

TBARs were measured as a marker of peroxidation and increased during the incubation of rabbit kidney slices in KRB (25% v/v) over 60 minutes (Figure 35). Incubation of the same slices with 50µM $FeSO_4$, 4mM ADP and 1mM ascorbate resulted in an elevation in lipid peroxidation compared to the non-treated slices, although this did not reach significance following 60 minutes of incubation (Figure 35). Incubation of slices in the presence of CCl_4 (5µl in 12ml aliquots of slices) resulted in a greater elevation in lipid peroxidaton when compared to slices incubated with Fe^{2+} and control non-treated slices. The extent of lipid peroxidation was significantly higher than control levels at 40 minutes ($P<0.05$) and 60 minutes ($P<0.005$) of incubation. These results indicated that CCl_4 was an effective initiator of lipid peroxidation in this model.

FIGURE 35: Lipid Peroxidation in Rabbit Kidney Slices Exposed to Either Fe^{2+}/Ascorbate/ADP or CCl_4.

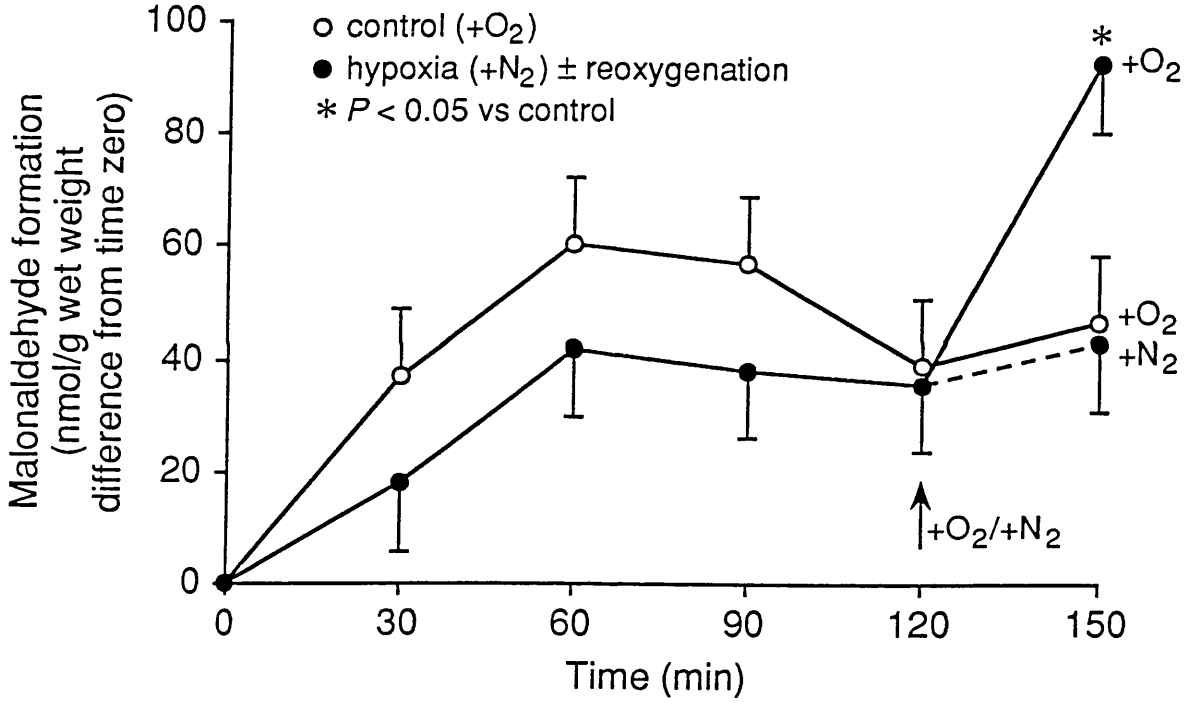


Values are the means ± 95% confidence limits of 6 separate determinations. Lipid peroxidation was measured as TBA-reactive material and expressed as malonaldehyde formation.

The Effect of Hypoxia/Reoxygenation on Lipid Peroxidation in Rabbit Kidney Slices

Rabbit kidney slices were rendered hypoxic by gassing with N_2 for at least 5 minutes to ensure that most of the O_2 was removed. Control slices were gassed with 95% O_2 : 5% CO_2. Rabbit kidney slices were then incubated at 37°C in the presence of O_2 or N_2 in stoppered flasks for 120 minutes. TBARs were measured as a marker of peroxidation. The level of lipid peroxidation increased during the first 60 minutes of incubation in the presence of O_2, levelled off and then decreased slightly between 90 and 120 minutes. This may be due to a decrease in the O_2 tension in the stoppered flasks. Lipid peroxidation was also evident in hypoxic slices but at a slower rate than that of oxygenated samples ($P<0.002$ comparing overall mean of control vs overall mean of hypoxic slices up to 120 minutes of incubation) and also levelled off after 60 minutes (Figure 36). Following 120 minute incubation, the oxygenated slices and half of the hypoxic slices were reoxygenated with 95% O_2:5% CO_2 and incubated at 37°C for a further 30 minutes. The remaining hypoxic slices were incubated at 37°C in the presence of N_2 for 30 minutes. Following this further incubation period, TBARs were seen to increase significantly ($P<0.001$) in the hypoxic/reoxygenated slices when compared to control oxygenated slices (Figure 36). No increase was observed in hypoxic slices maintained under N_2. These results clearly show that while there is little change in

FIGURE 36: Lipid Peroxidation in Kidney Cortical Slices Incubated at 37°C in KRB for 120 Minutes under an Atmosphere of N_2 (●) or O_2 (O) Followed by Regassing with Either O_2 (———) or N_2 (- - - -) and Aerobic Incubation for a Further 30 Minutes.



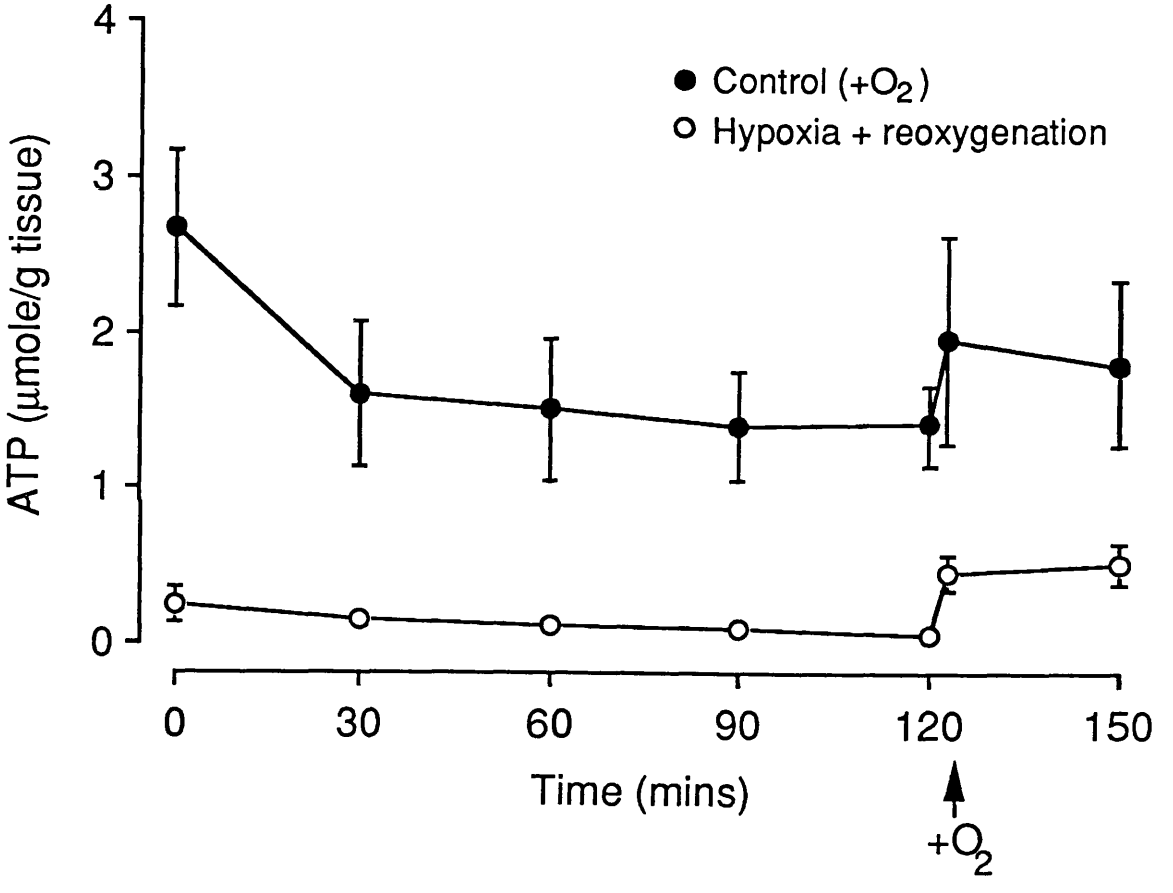
Values are the means ± 95% confidence limits of 6 separate determinations. Lipid peroxidation was measured as TBA-reactive material and expressed as malonaldehyde formation.

lipid peroxidation when slices are made hypoxic, a period of hypoxia followed by reoxygenation leads to a burst in oxidative damage to membranes at the point of reoxygenation.

ATP Determination in Rabbit Kidney Slices During Hypoxia/Reoxygenation

ATP levels were measured in rabbit kidney cortical slices (25% suspension in KRB) at 30 minute intervals throughout the 120 minutes of hypoxia and following reoxygenation. It can clearly be seen that at the onset of the hypoxic period the ATP levels dropped dramatically compared to control, oxygenated samples ($P<0.005$) (Figure 37). These low levels were maintained throughout the hypoxic period. Control ATP levels dropped significantly over the first 30 minutes of incubation and then reached a plateau over the next 90 minutes. The initial high value may have been the result of the burst of oxygen which was introduced at time zero. Upon reoxygenation of the hypoxic slices, ATP levels increased slightly but remained much lower than control levels. Control ATP levels were also raised to some extent. It can be concluded that ATP levels become depleted very rapidly at the onset of hypoxia and the ability of cells to produce ATP is considerably reduced if not totally prevented. Following reoxygenation of slices, after 120 minutes hypoxia, ATP recovers to a certain extent but is still low compared to oxygenated samples. This may have been due to impairment of the mitochondria

FIGURE 37: ATP Levels in Rabbit Kidney Cortical Slices During Hypoxia/Reoxygenation.



Values represent mean ± 95% confidence intervals of six determinations. * P<0.005.

during the hypoxic period or at the point of reoxygenation which led to a decline in the ability of these organelles to provide ATP.

Efficacy of the Simple Two Step Chromatography for Determining PIP_2 Breakdown

In order to simplify the quantitation of PIP_2 breakdown, instead of hplc, a simple two step method was employed using small anion-exchange columns and using two buffers to elute first a fraction containing inositol and glycerophosphoinositol (GPI) (fraction 1) and secondly a fraction containing inositol phosphates (fraction 2). The ratio of inositol phosphate products (fraction 2) : inositol and glycerophosphoinositol (fraction 1) was calculated and taken as a measure of PIP_2 breakdown (PIP_2 breakdown ratio) (see Materials and Methods). It was important to establish that this simple two step chromatography technique (using Dowex X8 (formate form) resin) separated these 2 fractions with accuracy. To assess the efficacy of this method, each fraction was analysed by hplc as described in Methods. Figure 38 shows hplc analysis of fraction 1. Inositol and GPI were present but inositol phosphates were not evident. Figure 39 shows hplc analysis of fraction 2. Inositol phosphates only were present. Hence, it can be concluded that good separation of these 2 fractions by this simple method can be achieved.

FIGURE 38: Hplc Analysis of Fraction 1 Showing Inositol and Glycerophosphoinositol Only.

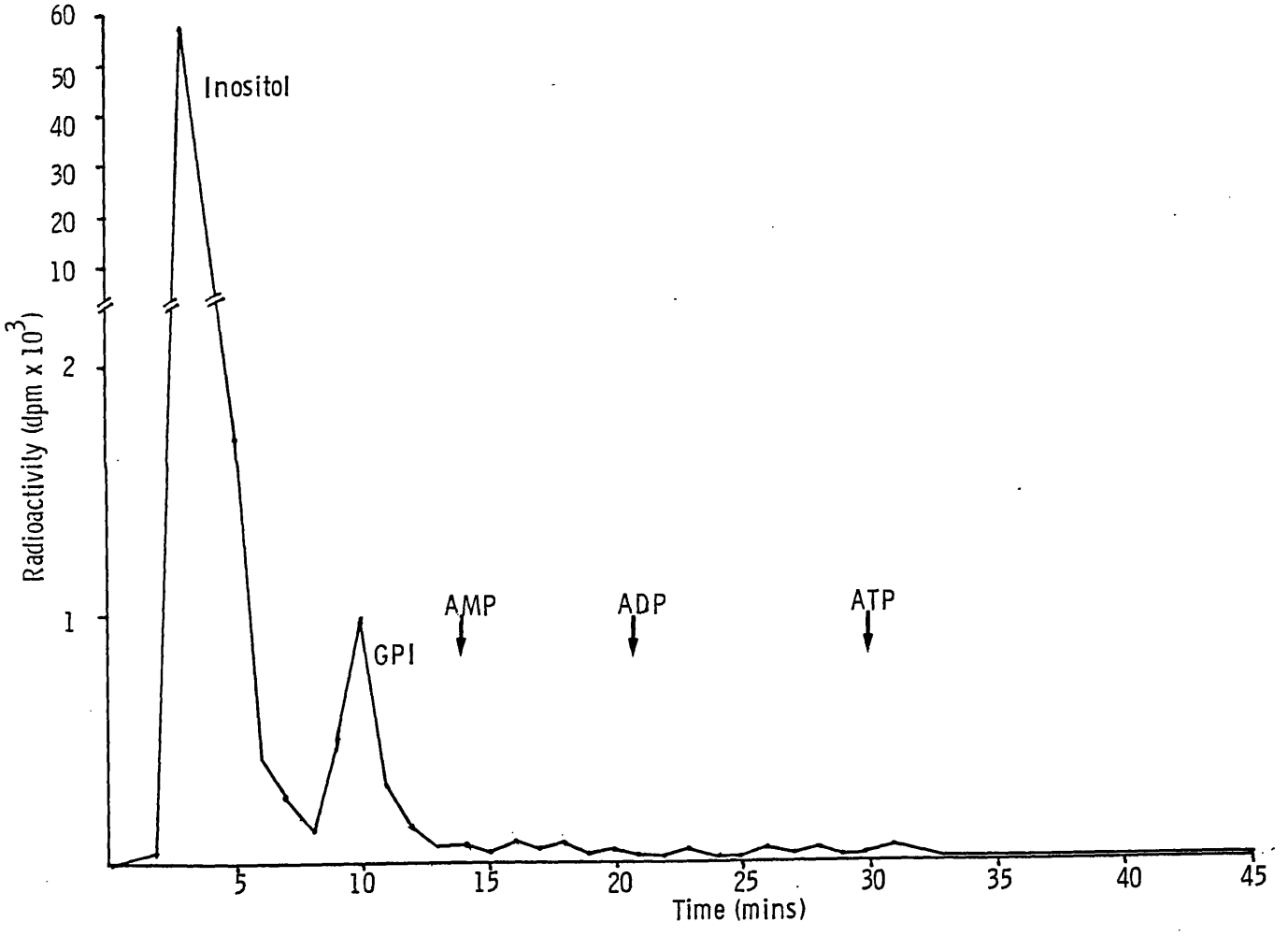
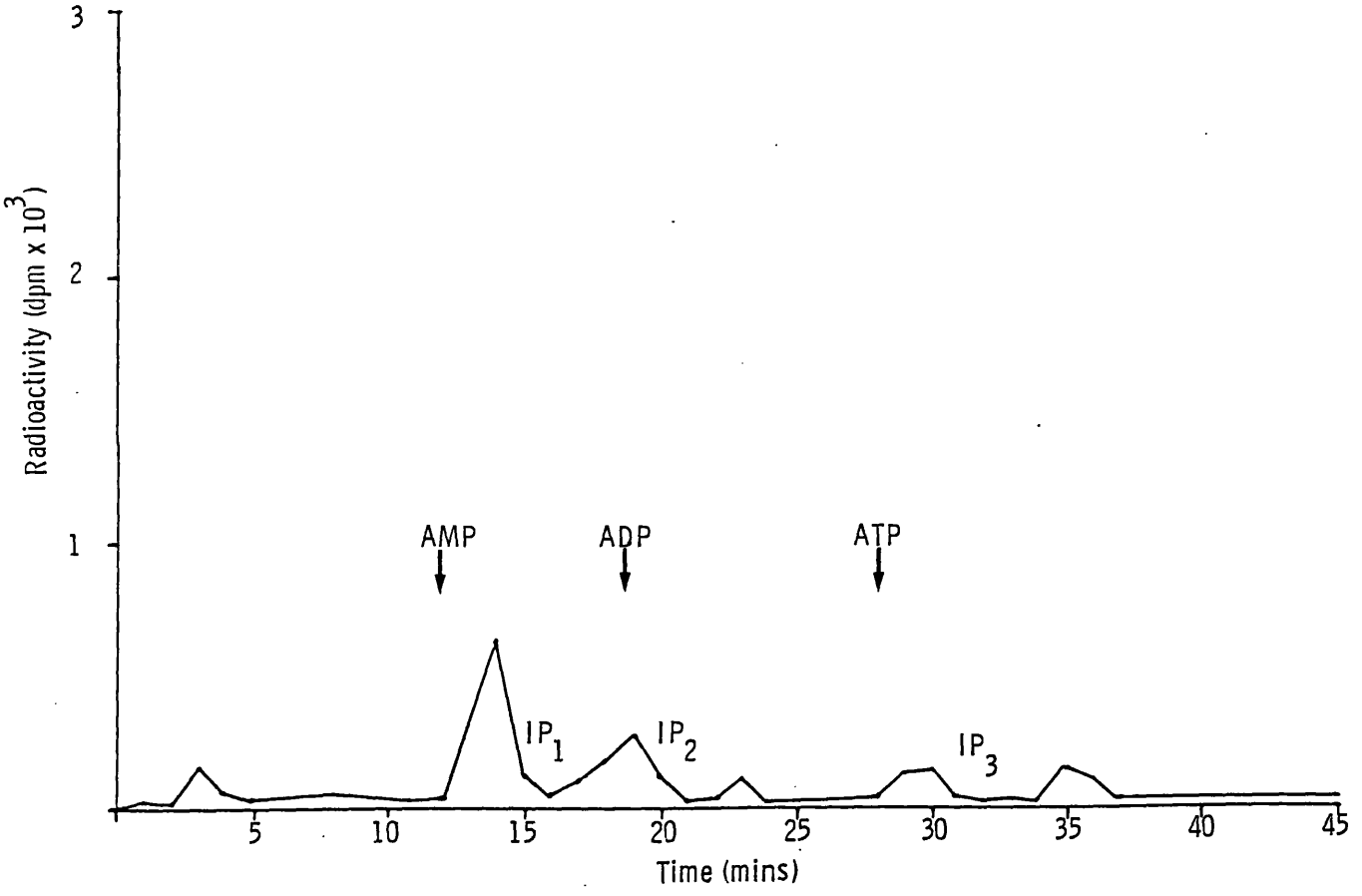


FIGURE 39: Hplc Analysis of Fraction 2 Showing Inositol Phosphates Only.



The Effect of CCl_4 on PIP_2 Breakdown in Rabbit Kidney Cortical Slices

Following hypoxia there is a marked increase in lipid peroxidation in kidney cortical slices at the time of reoxygenation. It is possible that alterations in the membrane itself or certain intermediates produced during lipid peroxidation may alter activities of membrane proteins such as the G-protein and phosphodiesterase (PDE) which may lead to altered PIP_2 breakdown. In order to investigate the effects of lipid peroxidation and calcium on PIP_2 breakdown, rabbit kidney cortical slices were incubated for 60 minutes in either KRB or calcium-free KRB containing EGTA (10mM) and peroxidation initiated by CCl_4 (See Membrane Peroxidation of Rabbit Kidney Slices using Fe^{2+} and CCl_4).

Statistical analysis was carried out using analysis of variance (ANOVA) in this experiment with the PIP_2 breakdown ratio used as the dependent variable. Since slices from each rabbit (n=8) were analysed for PIP_2 breakdown at all the combinations of agent (CCl_4 or none), chemicals (calcium or EGTA) and time points (initially and at 20, 40 and 60 minutes), the rabbits were regarded as 'blocks' and agent, chemicals and time points as within 'block factors' (Armitage and Berry, 1987). The residuals from the ANOVA were checked for Normal distribution using the Shapiro-Wilks W test (Royston, 1983), and for equal variances in the agent, chemical and time point groups using the Schweder test (Schweder, 1981). When constructing confidence

intervals and/or comparing the means of groups using t-tests the appropriate mean square error from the ANOVA was used as the variance estimate.

The ANOVA results are summarised in Table 8 and the mean ratio for all combinations (calcium/EGTA ± CCl_4) for each time point are shown in Figure 40. Following 60 minutes of incubation in the presence of CCl_4 and calcium (Figure 40), there was an increase in PIP_2 breakdown when compared to controls (slices in the presence of calcium only). However, this increase was not significant (P=0.125). In the presence of EGTA, CCl_4 had no effect. In order to look at the effect of calcium on PIP_2 breakdown, the data was pooled to form two groups: all slices in the presence of calcium and all slices in the presence of EGTA (n=16). Figure 41 gives the mean ratio at each time point for slices incubated in calcium or EGTA. A significant increase in PIP_2 breakdown was observed in the slices incubated in the presence of calcium compared to those incubated with EGTA at 60 minutes only (Figure 41). From this data it would seem that CCl_4 may have a slight but non-significant effect on PIP_2 breakdown in rabbit kidney cortical slices. In this model, PIP_2 breakdown appears to be stimulated in the presence of calcium, whereas EGTA appears to be effective in slowing this process.

TABLE 8: ANOVA Results for the Effect of CCl_4 on PIP_2 Breakdown in Rabbit Kidney Cortical Slices

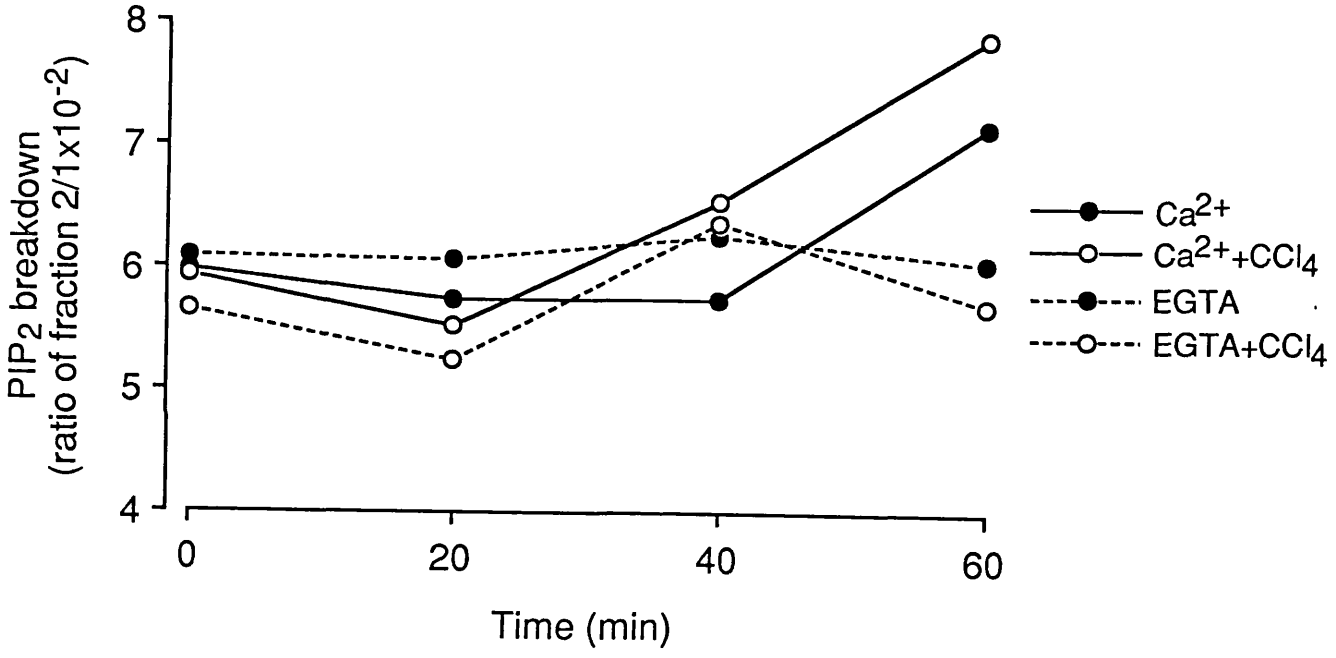
Source	F	P
Time	1.47	0.251
CCl_4	0.00	0.999
Calcium/EGTA	2.76	0.141
Time x CCl_4	1.15	0.352
Time x calcium/EGTA	4.16	0.019
CCl_4 x calcium/EGTA	3.03	0.125
Time x CCl_4 x calcium/EGTA	0.11	0.952

FIGURE 40: Phosphatidylinositol Breakdown in Kidney Cortical Slices Incubated at 37°C in KRB Containing Calcium (2.5mM) + CCl_4 or EGTA (10mM) + CCl_4 for 60 Minutes.

Experiment	PIP_2 breakdown F(2)/F(1) mean + SD			
	Time (mins)			
	0	20	40	60
+ calcium	5.984 ±2.941	5.739 ±2.311	5.750 ±2.053	7.151 ±1.461
+ calcium + CCl_4	5.922 ±2.896	5.588 ±2.176	6.532 ±2.293	7.864 ±1.380
+ EGTA	6.070 ±2.435	5.982 ±2.314	6.264 ±2.296	5.911 ±1.255
+ EGTA + CCl_4	5.649 ±2.317	5.216 ±1.725	6.372 ±2.437	5.655 ±1.586

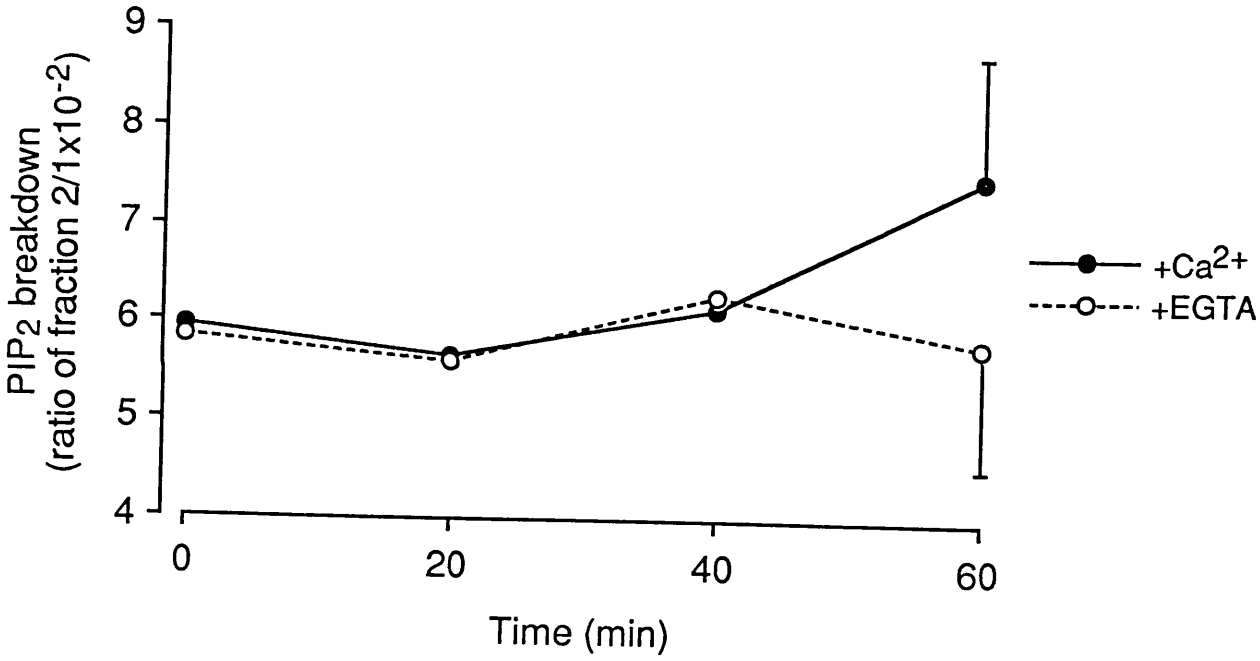
Values represent the means of 8 separate determinations performed in duplicate. PIP_2 breakdown was determined by Dowex chromatography as described in methods.

FIGURE 40: Phosphatidylinositol Breakdown in Kidney Cortical Slices Incubated at 37°C in KRB Containing Calcium (2.5mM) ± CCl_4 or EGTA (10mM) ± CCl_4 for 60 Minutes.



Values represent the means of 8 separate determinations performed in duplicate. PIP_2 breakdown was determined by Dowex chromatography as described in methods.

FIGURE 41: Phosphatidylinositol Breakdown in Kidney Cortical Slices Incubated at 37°C in KRB Containing Calcium (2.5mM) or EGTA (10mM) for 60 Minutes.



Values represent the pooled means of 16 seperate (8 -CCl_4 and 8 +CCl_4) determinations performed in duplicate. PIP_2 breakdown was determined by Dowex chromatography as described in methods.

The Effect of Hypoxia/Reoxygenation on PIP_2 Breakdown in Rabbit Kidney Cortical Slices

Many changes occur in an ischaemic organ which 'prime' the organ for a further increase in damage when molecular oxygen is reintroduced. Chapters 3 and 4 have indicated that during ischaemia an alteration in calcium homeostasis occurs which appears to be an important mediator of oxidative damage in tissues when they are reperfused. In the kidney, increased hydrolysis of PIP_2 to generate the two second messengers IP_3 and DAG would lead to amplification in certain cellular responses some of which are observed in the ischaemia/reperfusion situation, for example, renal vasoconstriction (Schmitz et al, 1981; Smyth et al, 1984; Cooper and Malik, 1985). I have hypothesised that changes occurring during ischaemia may affect PIP_2 breakdown leading to alterations which are detrimental to the organ when it is reoxygenated.

In order to investigate the effects of hypoxia/reoxygenation and calcium on PIP_2 breakdown, rabbit kidney cortical slices were incubated in either KRB or calcium-free KRB containing EGTA (10mM), exposed to hypoxic conditions (N_2 gassing) for 120 minutes and then reoxygenated for a further 30 minutes (see Methods).

Statistical analysis was performed using analysis of variance (ANOVA) with the PIP_2 ratio used as the dependent variable. Since slices from each rabbit (n=8) were measured for PIP_2 breakdown at all the combinations of gas (O_2 or

N_2), chemicals (calcium or EGTA) and time points (initially, and 30, 60, 90, 120 minutes, then with added oxygen, and at 150 minutes with added oxygen and nitrogen), the rabbits were regarded as 'blocks' and gas, chemicals and time points as within 'block factors' (Armitage and Berry, 1987). The residuals from the ANOVA were checked for Normal distribution using the Shapiro-Wilks W test (Royston, 1983), and for equal variances in the atmosphere, chemical and time point groups using the Schweder test (Schweder, 1981). When constructing confidence intervals and/or comparing the means of groups using t tests the appropriate mean square error from the ANOVA was used as the variance estimate. When examining changes over time, orthogonal contrasts for linear and quadratic trend were used to assess whether any relationship existed and, if it did, if there was any departure from linearity (Fleiss, 1986).

The ANOVA results are summarised in Table 9, and the mean ratio for all O_2/N_2 and calcium/EGTA combinations for each time point are shown in Figure 42.

Statistical analysis showed the following:

(1) The most important result was the significant interaction between time point, O_2/N_2 and calcium/EGTA ($P=0.007$). This indicated that to examine the O_2/N_2 and calcium/EGTA difference each time point needed to be considered separately.

(2) There also appeared to be some overall difference between calcium and EGTA ($P=0.019$), and an interaction

TABLE 9: ANOVA Results for the Effect of Hypoxia/Reoxygenation on PIP_2 Breakdown in Rabbit Kidney Cortical Slices

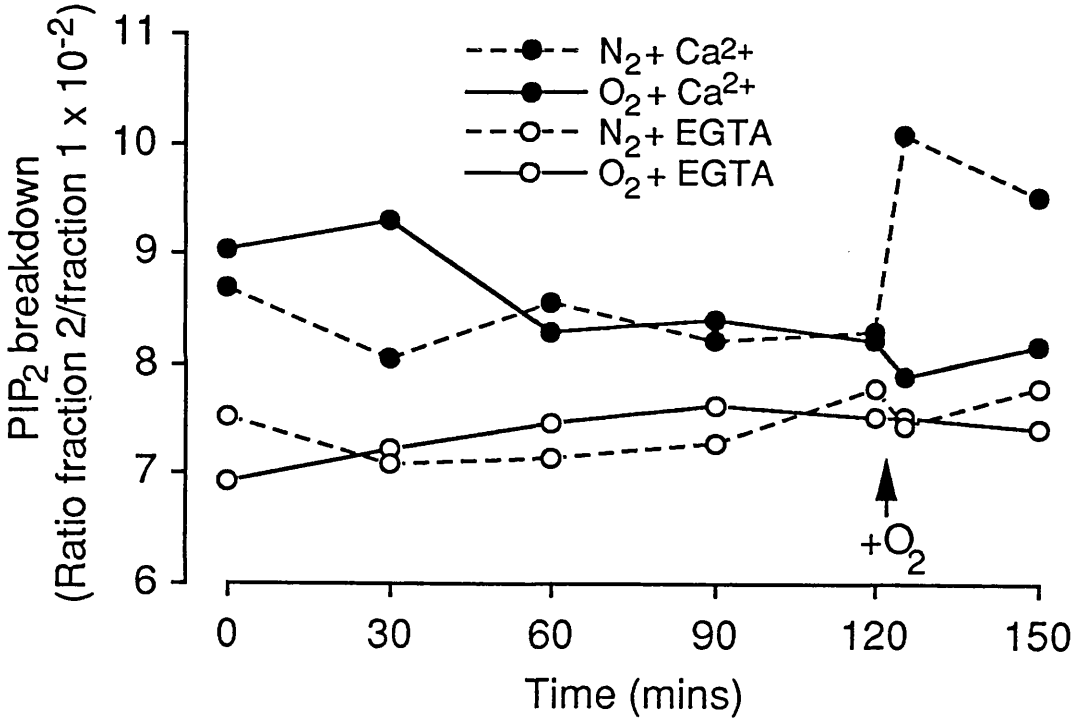
Source	F	P
Time	1.11	0.374
O_2/N_2	0.57	0.477
Calcium/EGTA	9.22	0.019
Time x O_2/N_2	2.95	0.017
Time x calcium/EGTA	0.86	0.546
O_2/N_2 x calcium/EGTA	0.93	0.366
Time x O_2/N x calcium/EGTA	3.52	0.007

FIGURE 42: Phosphatidylinositol Breakdown in Kidney Cortical Slices Incubated at 37°C in KRB Containing Calcium (2.5mM) or EGTA (10mM) for 120 min under an Atmosphere of N_2 (- - - -) or O_2 (————) Followed by Reoxygenation and Aerobic Incubation for a Further 30 Minutes.

Time (mins0	PIP_2 breakdown F(2)/F(1) mean + SD			
	Experiment			
	N_2 $+Ca^{2+}$	O_2 $+Ca^{2+}$	N_2 +EGTA	O_2 +EGTA
0	8.684 ±1.855	9.045 ±1.908	7.527 ±1.763	6.926 ±1.331
30	8.668 ±1.982	9.291 ±1.884	7.093 ±1.643	7.237 ±1.364
60	8.558 ±2.119	8.305 ±1.728	7.153 ±1.225	7.471 ±1.748
90	8.199 ±1.317	8.432 ±1.314	7.276 ±1.752	7.613 ±1.748
120	8.288 ±1.272	8.207 ±1.486	7.795 ±1.720	7.512 ±1.045
120 + O_2	10.070 ±2.681	7.965 ±2.038	7.447 ±1.751	7.529 ±1.091
150 + O_2	9.420 ±1.137	8.162 ±1.857	7.718 ±1.243	7.420 ±1.137

Values represent the means of 8 separate determinations performed in duplicate. PIP_2 breakdown was determined by Dowex chromatography as described in methods.

FIGURE 42: Phosphatidylinositol Breakdown in Kidney Cortical Slices Incubated at 37°C in KRB Containing Calcium (2.5mM) or EGTA (10mM) for 120 min under an Atmosphere of N_2 (- - - -) or O_2 (————) Followed by Reoxygenation and Aerobic Incubation for a Further 30 Minutes.



Values represent the means of 8 separate determinations performed in duplicate. PIP_2 breakdown was determined by Dowex chromatography as described in methods.

between time point and O_2/N_2 (P=0.017) which implied that the O_2/N_2 difference varied according to the time points. However, these two results must be interpreted in the light of point 1 above.

(3) Overall there were no differences between the time points (P=0.374) and no difference between O_2 and N_2 (P=0.477), but again these results have to be interpreted in the light of 1 and 2 above.

(4) Result 2 essentially means that when comparing O_2 and N_2 or calcium with EGTA then all four combinations of chemicals and gases need to be considered at each time point separately. Averaging one factor (say O_2/N_2) over the levels of the other (say calcium/EGTA) could give misleading results.

Table 10 shows the mean PIP_2 ratio at 120 minutes and immediately after reoxygenation, the mean change and P value for comparison of the 120 minute time point and following the addition of oxygen. There was a highly significant increase in PIP_2 breakdown following the reoxygenation of hypoxic slices in the presence of calcium (P=0.0002). This effect was maintained following the further 30 minutes incubation in oxygen (i.e. no significant fall in value (P=0.21)). No real changes were observed in controls or when EGTA was present. An overall difference was observed between slices incubated with either calcium or EGTA (P=0.019) such that, in the presence of calcium, PIP_2 breakdown was greater indicating that this

TABLE 10: Change in PIP_2 Breakdown in Rabbit Kidney Cortical Slices Following the Introduction of O_2 at 120 Minutes

	O_2 + Ca^{++}	O_2 + EGTA	N_2 + Ca^{++}	N_2 + EGTA
	Phosphatidylinositol breakdown (ratio fraction 2/fraction 1 x 10^{-2})			
120 min pre-O_2 mean ratio	8.21	7.51	8.29	7.80
120 min post-O_2 mean ratio	7.97	7.53	10.07	7.45
mean change	-0.24	+0.02	+1.78	+0.35
95% CI for change	-1.11,0.63	-0.85,0.89	0.91,2.65	-1.22,0.52
P-value*	0.58	0.97	0.0002	0.43

Values are mean and 95% confidence intervals (CI) of 8 seperate determinations performed in duplicate.

* P-Value for t-test comparing pre-O_2 and post-O_2 mean rates.

process was calcium-dependent.

In summary, PIP_2 breakdown remained unchanged during the period of hypoxia but was seen to increase in hypoxic slices in the presence of calcium following reoxygenation, and this increase was sustained over a further 30 minute incubation. PIP_2 breakdown was always greater in slices incubated in the presence of calcium compared to those incubated in EGTA, indicating that, in this model, PIP_2 breakdown is stimulated by calcium. These findings provide evidence that hypoxia 'primes' a tissue for a burst in the PI secondary messenger system when oxygen is introduced.

NOTE:

Initial values (time 0) for PIP_2 breakdown in rabbit kidney cortical slices (Figures 41 and 42) were observed to be highly variable ranging from approximately 6 to 9 (ratio of fraction 2:1 x 10^{-2}). This indicates a variability in the basal rate of PI turnover occuring in kidney cortex from different rabbits.

CHAPTER 6

DISCUSSION

The Relationship Between Lipid Peroxidation and Altered Calcium Homeostasis in Cold Ischaemic Rabbit Kidneys

Physiological deterioration of organs as a result of hypothermic storage is likely to be the result of a number of adverse biochemical processes associated with ischaemia. It has previously been demonstrated that the susceptibility to lipid peroxidation initiated by free radicals is high following storage of organs in a poor preservation medium (saline) and much less when organs are stored in a good medium such as HCA (Green et al, 1986a). This investigation extends those studies by demonstrating that altered calcium homeostasis plays a significant role in mediating free radical-induced damage.

Experiments in this study involved the storage of rabbit kidneys in two storage media; isotonic saline (a poor storage medium) and the clinically approved hypertonic citrate solution (HCA) (a good storage medium). Table 11 compares the constituents of both these solutions. In HCA, mannitol is used as an impermeant to prevent hypoxic-hypothermic cell swelling by increasing solution osmolality to 400 mosmol/kg. Citrate is an important slowly permeating anion and buffer. It chelates magnesium to give a stable complex without risk of precipitation, and its buffering

TABLE 11: The Composition of Isotonic Saline Solution (NaCl 0.9%) and Hypertonic Citrate Solution

ISOTONIC SALINE		HYPERTONIC CITRATE	
Na^+	154mM	Na^+	83mM
Cl^-	154mM	K^+	80mM
		Mg^{2+}	40mM
		SO_4^{2-}	40mM
		Citrate	55mM
		Mannitol	186mM

capacity maintains the pH at a steady level. Saline, on the other hand has no such beneficial constituents and cannot protect against cellular efflux of potassium and magnesium and the corresponding influx of sodium, chloride and water which causes the cells to swell (Belzer and Southard, 1988). Rabbit kidneys flushed and stored with this solution can rarely survive 16 hours of refrigeration (Green and Pegg, 1973). Saline was examined in these studies to provide a direct comparison with the effective HCA solution.

Free radical-mediated lipid peroxidation was determined by the measurement of TBAR material and Schiff bases. The TBA test is one of the oldest and most frequently used tests for measuring the peroxidation of biological lipids, especially membranes. A range of short-chain aldehydes including malonaldehyde (MDA) are produced during peroxidation and can react in the TBA test to generate a coloured product. In acidic solution the products absorbs light at 532nm and fluoresces at 553nm, and are readily extractable into organic solvents such as butan-1-ol (Halliwell and Gutteridge, 1985). However, although the test is calibrated with MDA and results are expressed in terms of MDA, it detects other compounds as well. Prostaglandin endoperoxides formed via cyclooxygenase, such as PGG_2 and PGH_2 and some of their derivatives, are also TBA-positive (Hayaishi and Shimizu, 1982). Hence the term TBA-reactive material (TBARs) is used to describe the level of lipid peroxidation determined with this test. The

decomposition of peroxides to malonaldehyde (MDA), via peroxy and alkoxy radicals, requires traces of transition metal catalysts, notably iron salts, and so the presence of iron contamination from reagents may affect the assay.

The reaction of carbonyl compounds, such as malonaldehyde, with side chain amino groups of proteins, free amino acids or even nucleic acid bases produces products known as Schiff bases (SB) (Halliwell and Gutteridge, 1985). Lipid-soluble fluorescent SB can be easily extracted from biological material for analysis. SB fluorescence is a sensitive method for measuring lipid peroxidation and has the advantage over the TBA-test in that no incubation at 95^{o}C is required. Both of these methods provide a measurement of a late stage in the peroxidation process. It has been demonstrated that indomethacin (an inhibitor of cyclooxygenase) significantly decreased TBARs in medulla of rabbit kidneys but had little effect on SB measurements (Gower et al, 1987). This indicates that although some of the products of prostaglandin synthesis are TBA-positive, they are unlikely to contribute significantly to the formation of SB markers of lipid peroxidation.

Both TBARs and SB determinations should only be considered as a measure of the susceptibility of the kidney to peroxidative damage, since the production of these markers was measured during incubation of the homogenates for 60 minutes at 37^{o}C in open vessels. This period of incubation may be considered as an <u>in vitro</u> simulation of

reoxygenation. Indeed, little difference in markers of lipid peroxiation was found between control and ischaemic kidneys before in vitro incubation, indicating that ischaemia does not itself initiate an increase in free radical production. When considered together therefore, the evidence obtained from measuring these markers of lipid peroxidation provides good, if somewhat circumstantial, evidence for the involvement of free radicals in ischaemia and reperfusion damage.

Extracellular fluid is rich in calcium ions (10^{-3}M) and there is a highly regulated gradient across the cell membrane, which is poorly permeable to calcium, such that cytosolic calcium levels are maintained at low levels (10^{-7}M). Normally calcium may only enter cells via specific voltage operated channels or receptor operated channels and is extruded from the cell by a sodium/calcium exchange mechanism and ATP-driven pumps. If this permeability barrier is breached, for example by a calcium ionophore such as A23187, then any extracellular free calcium present will flood into the cell. Increased cytosolic calcium levels are associated with many biochemical changes as well as altered cellular morphology such as blebbing and many studies have demonstrated a close temporal association between elevated cell calcium levels, severe histological damage and cell death, strongly suggesting a causal connection between these events (Schanne et al, 1979).

One important consequence of raised cytosolic calcium levels is the activation of phospholipases (Wong and Cheung, 1979; Harris and Cooper, 1982) which remove fatty acids, particularly arachidonic acid, from membranes. In addition, calcium regulates a number of proteases of which the proteolytic conversion of xanthine dehydrogenase to xanthine oxidase may be important during ischaemia (Roy and McCord, 1983). Accumulation of xanthine oxidase can later increase generation of free-radical species during reperfusion because of the interaction between breakdown products of adenine nucleotide metabolism and incoming molecular oxygen. Thus altered calcium homoestasis and the production of reactive free radicals could act synergistically to bring about cell death.

In this study, addition of the voltage-operated channel blocking agent verapamil to the saline flush solution resulted in significant inhibition of the raised levels of lipid peroxidation observed in both the cortex and medulla of organs subjected to 24 hours cold storage. Since addition of verapamil directly to kidney homogenates had no effect on lipid peroxidation, it can be concluded that the protective effect of this agent was due to its ability to prevent influx of extracellular calcium into the cytosol of intact kidney cells during the storage period. This effect of verapamil on saline-stored organs suggests that a significant amount of extracellular calcium remains in the tissue after flushing with a storage solution; this is not

surprising considering that a single-passage vascular flush is unlikely to gain access to all the extracellular spaces. Deliberately raising the concentration of extracellular calcium by addition of $CaCl_2$ (1mM) to the saline flush solution resulted in further elevations in the extent of lipid peroxidation following the storage period. This demonstrated that the extent of oxidative damage was dependent on the concentration of extracellular calcium when kidneys were stored in saline.

Verapamil had no effect on the extent of oxidative damage to kidneys following storage in HCA for 72 hours which demonstrated a highly specific difference between the way kidneys stored in HCA and saline respond to extracellular calcium. It is possible that protection against extracellular calcium entry by HCA was due to the ability of the large excess of citrate (55mM) in this medium to chelate free calcium ions. Citrate was orginally added to HCA as a substrate for 'basal' renal respiration during organ storage (Ross et al, 1979). It also acts as a buffer and a slowly permeating anion, entering the cell only very slowly and thus reducing cell swelling. Measurement by an ion-specific electrode, of free calcium concentrations in HCA to which various amounts of calcium had been added confirmed the very effective buffering capacity of this medium. Thus entry of free calcium ions through VOCs will be prevented or at least slowed during the storage period and it is likely that the addition of $CaCl_2$

(1mM) had no effect on peroxidative damage as it was sequestered by the citrate leaving only very low amounts (approximately 10μM) of free cations in the extracellular medium. Alternatively changes in cellular ionic gradients and membrane potential in kidneys stored in saline may have resulted in the activation of VOCs, whereas, with the superior ionic balance of HCA such an activation may have been prevented during the ischaemic period.

It is interesting that Shapiro and coworkers (1985) have demonstrated that addition of verapamil (2.5μM) to Collin's C_2 flush solution, which does not contain a calcium-buffering system such as citrate, significantly improved inulin clearance, urine flow rate and sodium reabsorption in kidneys after 8 hours of simple storage at $0^{o}C$ followed by isolated perfusion (Shapiro et al, 1985). Since addition of verapamil to the perfusate after the ischaemic period had no effect, it was concluded that verapamil protected the organ during the ischaemic period itself. Other experiments in which verapamil reduced warm ischaemic damage did not attempt to determine whether it worked primarily by preventing calcium influx into cells or through its potent diuretic, vasodilator or antiplatelet activities (Malis et al, 1983; Burke et al, 1985; Gingrich et al, 1985).

Intracellular organelles are also important in maintaining calcium homeostasis. They contain over 90% of intracellular calcium and provide 'sinks' which sequester

excess calcium from the cytosol. Lipid peroxidation of mitochondria (Masini et al, 1985) and the endoplasmic reticulum (Bellomo et al, 1985) impairs the calcium buffering capacity of these structures. Membranes, including those of intracellular organelles can also be rendered permeable to calcium by the ionophore A23187, a carboxylic acid antibiotic (Reed and Lardy, 1972) which will therefore lead to increased cytosolic calcium levels. The effect of this agent was therefore studied in this model of renal ischaemia. Addition of A23187 to both the HCA and saline flush and storage solutions resulted in significantly increased levels of lipid peroxidation in both cortex and medulla over and above the already elevated levels observed following storage of organs in HCA or saline alone. This therefore suggested that detrimental redistribution of intracellular calcium occurring during cold storage, even in the absence of extracellular free calcium ions, as in the case of organs stored in HCA, can significantly mediate oxidative damage to the tissue.

There are several ways in which periods of warm and cold ischaemia may bring about altered calcium homeostasis. Depletion of O_2, accompanied by falling ATP levels will reduce the cellular ability to extrude calcium by ATP-dependent pumps, as well as the ability of organelles to sequester calcium by energy-dependent shuttles, especially at low temperatures (Fuller, 1987). In addition, a falling pH will decrease the affinity of the 10μM of calcium

normally complexed to soluble substances such as ATP, citrate and glutamate and may release calcium as free ions (Fuller, 1987). The movement of other ions is also seriously altered during ischaemia and may induce pathological fluxes in calcium. In particular, during storage in saline, cells would exchange much of their intracellular potassium for the sodium present in high concentrations outside the cell. Having entered the cell, sodium may then exchange with calcium in mitochondria by a passive mechanism and would result in increased calcium levels in the cytosol. In addition, as the temperature of cells falls during cold ischaemia, membrane fluidity is diminished and molecular packing faults developing in the phospholipid bilayer could allow abnormal calcium movements to occur. Swelling of organs which can occur during storage in saline may also contribute to changes in membrane integrity.

It has previously been demonstrated that cytosolic calcium levels increase in cultured kidney cells as a result of anoxia and that these levels correlate well with falling ATP concentrations (Snowdowne et al, 1985); in that study, it was concluded that rising cytosolic calcium was mostly due to release of calcium from intracellular depots, particularly mitochondria (Snowdowne et al, 1985). In another study, cellular calcium in kidney cortex slices incubated at 4^{o}C increased by 50% after one hour of hypoxia, suggesting that calcium overload commences soon after

depletion of energy supplies in tubular cells (Trump et al, 1974). Other studies have demonstrated that there is considerable uptake of calcium into cells from the extracellular space during reoxygenation following anoxia and much of this is accumulated by respiring mitochondria (Arnold et al, 1986; Humes, 1986).

The importance of mitochondria in modulating calcium homeostasis and free radical mediated damage was assessed in cold-stored rabbit kidneys by investigating the effects of ruthenium red which inhibits mitochondrial calcium transport. Whole kidneys which were exposed to ruthenium red for the duration of the storage period exhibited significantly elevated levels of lipid peroxidation products in both the cortex and medulla following storage in HCA or in saline solutions. This may have been a consequence of inhibiting mitochondrial calcium uptake, a process which utilizes electrochemical gradients across the mitochondrial membrane and which takes precedence over oxidative phosphorylation (Humes, 1986). Thus with the onset of ischaemia, mitochondria would divert declining energy resources to buffer initial rises in cytosolic calcium levels. In the presence of ruthenium red, cytosolic calcium levels would rise, unchecked by mitochondria, and could trigger increased oxidative damage outside the organelle. This suggests that mitochondrial calcium uptake was still functioning to some extent under these conditions. During ischaemia, oxidative phosphorylation in a tissue is much

reduced due to the shortage of molecular O_2. The storage solutions used in these experiments were exposed to air and consequently were likely to contain dissolved oxygen, especially as they were maintained at $0^{o}C$ which increases O_2 solubility. It is plausible that this small amount of O_2 was accessible to cells by diffusion allowing them to engage in some oxidative phosphorylation, ATP production and mitochondrial calcium uptake, although at a much reduced rate. Hence, even during ischaemia, and particularly in the initial stages of hypoxia, mitochondria may be important in calcium regulation by active sequestration.

Alternatively, one pathway of mitochondrial calcium efflux can be blocked by ruthenium red which may therefore result in the accumulation of abnormally high levels of calcium ions in these organelles. Oxygen derived free radical damage to mitochondria <u>in vitro</u> is markedly enhanced by addition of calcium to the system which leads to increased membrane permeability and decreased ability to synthesise ATP (Malis and Bonventre, 1986). Thus following organ storage, calcium-rich mitochondria would be particularly prone to oxidative damage by free radicals produced on reperfusion and ATP synthesis would be diminished, a situation aggravated still further by calcium overload activating calcium-ATPases and consuming valuable ATP resources (Peng et al, 1982).

In conclusion, the data presented strongly suggests that calcium redistribution takes place during cold storage

of kidneys and this results in subsequent increased oxidative damage to the organs upon reperfusion. Since addition of free radical scavengers and iron chelators to the flush solution is also effective at reducing levels of lipid peroxidation in stored rabbit kidneys (Green et al, 1986b), and administration of superoxide dismutase (an enzyme which scavenges $O_2^{\bullet -}$ radicals) or allopurinol (a xanthine oxidase inhibitor) to the recipient has been reported to improve post-storage function of porcine kidneys (Koyama, et al, 1985), it is likely that altered calcium homeostasis during the ischaemic peroid acts synergistically with oxygen-derived free radicals produced during reperfusion to produce cellular and vascular damage. It has been demonstrated that prevention of extracellular calcium entry either by specific blocking agents such as verapamil or buffering systems such as citrate is an important consideration in the design of a good storage solution. This is in accordance with another report which showed that while 0.5mM calcium in the University of Wisconsin solution is optimal for maintaining the structural integrity of perfused dog kidneys, increased organ viability requires control of intracellular calcium by the use of the calmodulin inhibitor chlorpromazine (McAnulty et al, 1987). In contrast calcium levels of up to 0.8mM have been reported to increase the viability of hepatocytes stored in Euro-Collins solution without any precautions being taken against a rise in intracellular calcium levels (Umeshita et al,

1988). It was further demonstrated in the present study that intracellular calcium redistribution during storage of organs in HCA leads to adverse increases in oxidative damage. Administration to the donor (if considered ethical) and incorporation in the flush solutions of agents which modulate calcium movements, inhibit free radical production or scavenge reactive free radical intermediates, would be logical candidates for a pharmacological strategy. A better understanding of the underlying mechanisms which cause damage would provide a superior basis for devising such a strategy.

Possible Mechanisms by which Calcium may Potentiate Lipid Peroxidation

There are several mechanisms by which increased cytosolic calcium levels may potentiate free radical damage to membranes. Increased cytosolic calcium levels may enhance free radical production and thence lipid peroxidation by activating proteases which convert the enzyme xanthine dehydrogenase to xanthine oxidase (Roy and McCord, 1983). This is likely to be most important during reperfusion; after exposure to molecular oxygen, this form of the enzyme converts hypoxanthine, a product of adenine nucleotide degradation which accumulates during ischaemia, to xanthine, and the superoxide anion ($O_2^{\cdot -}$) is produced.

In the presence of transition metal catalysts, $O_2^{\bullet -}$ can be converted to highly reactive species such as the hydroxyl radical ($OH^{\bullet}$) and iron-complexed molecules which then initiate lipid peroxidation. The finding in the present experiments that allopurinol added to the saline flush and storage solution inhibited lipid peroxidation and was even able to inhibit the highly significant rises observed when the calcium ionophore A23187 was added to the system, provided some circumstantial evidence that calcium and xanthine oxidase are important mediators of lipid peroxidation. In the past it has been assumed that allopurinol added to a cold flush solution would not be accessible to intracellular compartments or have enough time to modulate cell metabolism. However, this present data suggests that allopurinol might well prove useful if added to flush solutions for clinical use; indeed allopurinol is a constituent of the recently-formulated UW storage solution (Belzer and Southard, 1988).

Allopurinol has been claimed to improve the post-storage function of porcine kidneys when administered to the recipient animal (Koyama et al, 1985) and to protect ischaemic rat kidneys (Baker et al, 1985) if given to the recipient after warm ischaemia but before reperfusion. In other studies, however neither in dogs (Murdock and Cho, 1975) nor in rats did allopurinol prove beneficial in renal ischaemia. More recently, it was shown to prevent erythrocyte accumulation otherwise observed after ischaemia

(Hansson et al, 1982) but had little effect on subsequent renal function (Hansson et al, 1986). In terms of function and survival after ischaemic insult, the value of allopurinol is still in question.

Phospholipases are activated by calcium and remove fatty acids from membranes by a process which does not require oxygen or energy and could therefore occur during the storage period at low temperatures. Results from these studies suggest that phospholipase activation during ischaemia can significantly mediate lipid peroxidation on subsequent reperfusion. Phospholipase activity was determined by measuring free fatty acid accumulation in stored organs. Accurate and valid measurement of FFAs is complicated by the possibility that hydrolysis of membrane phospholipids to free fatty acids and lysophosphatides, may increase both during handling (Rouser et al, 1968) and through careless extraction and homogenization (Nelson, 1969). This is due to activation of phospholipases during these procedures which results in an artifactual elevation in the products of phospholipid hydrolysis. In the present study, rabbit kidney cortex and medulla were quickly clamped under liquid nitrogen, ground to a powder and then immediately subjected to FFA extraction to avoid phospholipase activation. Kidneys subjected to FFA analysis after simple homogenization had elevated levels of the unsaturated FFAs oleic ($C_{18:1}$), linoleic ($C_{18:2}$) and arachidonic acid ($C_{20:4}$), compared to kidneys clamped under

liquid nitrogen and then assessed for FFA status. A decrease in the level of palmitic acid ($C_{16:0}$) was also observed. These results suggested that the process of homogenization caused artifactual elevations in unsaturated FFAs probably through activation of phospholipases. The reason for the decrease in $C_{16:0}$ remains unclear. It is possible that homogenization also inactivates phospholipases responsible for the release of the fatty acid. Losses in FFAs during the extraction and methylation procedures were corrected for by the addition of an internal standard, pentadecanoic acid ($C_{15:0}$).

Hypertonic citrate solution has been used successfully in clinical renal preservation for several years. Developed by Ross, Marshall and Escott, (1976), it is hypertonic with respect to plasma and contains 186mM mannitol which permeates cell membranes slowly, thus preventing cell swelling. This study has demonstrated that HCA protects stored rabbit kidneys against influx of extracellular calcium but does not appear to reduce damage mediated by rises in cytosolic calcium caused through altered intracellular calcium homeostasis. The 55mM citrate present in HCA may be involved in calcium chelation and thus prevent or at least slow pathological elevations in intracellular calcium during cold ischaemia.

An increase in unsaturated FFA accumulation was evident in kidneys following both 24 hour cold storage in saline or 72 hours cold storage in hypertonic citrate solution with

a greater proportion of unsaturates being evident in both regions of the kidney following storage. Although HCA benefits organs during storage and is superior to other flush solutions for preservation of rabbit kidneys, it has been shown that less than 50% of rabbit kidneys stored for 72 hours in HCA prove life-supporting after transplantation (Gower et al, 1989a). The present study demonstrates that HCA provides little or no protection against the accumulation of unsaturated FFA during the ischaemic period and this may therefore be a contributing factor in the eventual failure of these organs.

Both FFAs and lysophosphatides products display detergent-like properties and are deleterious to cell function. High FFA concentrations are damaging to ischaemic mitochondria (Piper and Das, 1986) and result in oedema in brain tissues (Chan and Fishman, 1978). Lysophosphatides within the membrane are normally maintained at very low levels. In sufficient quantities, however, they can alter membrane properties such as fluidity and permeability (Weltzem, 1979) and may leave the membrane more susceptible to free radical attack.

The phospholipase A2 inhibitor dibucaine competes with calcium for the site of activation on the enzyme (Reinhart et al, 1984). Addition of dibucaine to the HCA solution led to a significant reduction in the accumulation of free arachidonic acid in both cortex and medulla of rabbit kidneys following 72 hour storage. This observation

indicated the presence of a calcium-dependent phospholipase A2 in rabbit kidneys responsible for the hydrolysis of phospholipids leading to arachidonic acid release and was further evidence for intracellular calcium redistribution under these conditions. As dibucaine did not affect the levels of other FFAs, it is probable that release of these during renal ischaemia was by another mechanism, possibly hydrolysis by calcium-independent phospholipases. In the present study, inhibition of phospholipase A2 by dibucaine significantly decreased but did not abolish arachidonic acid accumulation during storage. It could be postulated that while the hydrolysis of phospholipids by phospholipases, particularly the release of arachidonic acid, is a major cause of free fatty acid accumulation during storage, so too was the blockade of β-oxidation of FFAs and their re-incorporation back into phospholipids due to shortage of ATP during ischaemia (Rehncrona et al, 1982).

A variety of phospholipases have now been identified and membrane-bound phospholipases show an absolute requirement for calcium (Van den Bosch, 1980). Addition of calcium has been shown to augment arachidonic acid release 20-fold in human platelet membranes (Derksen and Cohen, 1975). The calcium ionophore A23187 is an excellent experimental tool which facilitates the passage of calcium ions across membranes producing an overall increase in cytosolic calcium (Reed and Lardy, 1972). A23187 in human platelets, for example, in the presence of EGTA, releases

calcium from internal stores which induces the release of 46% of the total phospholipid arachidonic acid content in 1 min (Pickett et al, 1977). Addition of A23187 to HCA during the storage of rabbit kidneys for 72 hours in the present study, was found to significantly stimulate the release of arachidonic acid. In contrast, A23187 had no effect on free $C_{16:0}$, $C_{18:0}$, $C_{18:1}$ and $C_{18:2}$ release following storage thus providing further evidence for calcium-independent mechanisms mediating the release of FFAs other than arachidonic acid. It would appear, therefore, that an increase in cytosolic calcium possibly through the activation of a calcium-dependent phospholipase may be responsible for the accumulation of arachidonic acid in the cortex and medulla of rabbit kidneys following ischaemic insult.

The highly significant increase observed in free arachidonic acid levels is potentially of great pathophysiological importance. Release of arachidonic acid through hydrolysis of phospholipids is the rate limiting step in the cyclo-oxygenase and lipoxygenase pathways (Isakson et al, 1978). Higher concentrations of free arachidonic acid may therefore lead to a burst in the production of eicosanoids: prostaglandins and thromboxanes via cyclo-oxygenase and leukotrienes via lipoxygenase respectively when the organ is reperfused. Upsetting the delicate eicosanoid balance between vasoconstriction and vasodilation may have important consequences in the

endothelial lining of the vascular bed (Schlondorff and Ardaillou, 1986). This has been demonstrated experimentally in perfused rabbit lungs when addition of exogenous arachidonic acid, A23187 and phospholipase A2 to the perfusate increased pulmonary vascular resistance (Littner et al, 1986). Prostaglandin synthesis is dependent upon the concentration of hydroperoxides formed as a result of lipid peroxidation and an increase inhibits prostacyclin synthesis. Increased rates of lipid peroxidation upon reperfusion may therefore result in a decrease in the potent vasodilator prostacyclin and a concomitant elevation in the formation of thromboxanes which mediate vasoconstriction, a situation known to occur following ischaemic insult (Lelcuk et al, 1985a; 1985b; Schmitz et al, 1985). In addition, inhibition of cyclo-oxygenase by administering indomethacin to recipient rabbits autografted with cold stored kidneys has been shown to protect against ischaemic damage (Gower et al, 1989a). Such alterations in eicosanoid homeostasis may contribute to the "no-reflow" phenomenon observed when some organs are transplanted.

Inhibition of phospholipase A2 by dibucaine in rabbit kidneys stored either for 72 hours in HCA or 24 hours in saline significantly reduced the rate of lipid peroxidation in both cortex and medulla. Furthermore, an increase in cytosolic calcium resulting from the addition of A23187 increased the rate of lipid peroxidation. These results indicate that an increase in cytosolic calcium and calcium-

dependent activation of phospholipase A2 may be responsible, at least in part, for mediating lipid peroxidation in kidneys following storage.

It is possible that by inhibition of phospholipase A2 the levels of free arachidonic acid were reduced and so the rate of prostaglandin synthesis was much decreased. This effect alone may have been be responsible for the decrease in the TBAR markers of lipid peroxidation as some products of the arachidonic acid cascade exhibit positive TBA-reactivity (Hayaishi and Shimizu, 1982). However the finding that SB markers of lipid peroxidation, which are much less effected by prostaglandin intermediates, correlated well with TBARs strongly suggested that the inhibition of phospholipase A2 did in fact reduce the level of oxidative damage.

Although allopurinol and dibucaine were effective in reducing the level of lipid peroxidation in rabbit kidneys following cold storage in saline, peroxidation was still elevated following storage of these kidneys when compared to fresh organs. In saline stored kidneys, the addition of the calcium channel blocker verapamil, was much more effective, bringing the levels of lipid peroxidation close to the levels observed in fresh kidneys. This suggested that inhibition of xanthine oxidase and phospholipase A2 can reduce only some of the peroxidative damage to kidneys and that other mechanisms of damage exist which rely on increased cytosolic calcium during the storage period.

It does seem unlikely that the dramatic effect of verapamil in saline-stored organs was due to inhibition of VOCs alone. Increases in cytosolic calcium may be facilitated by other mechanisms including entry via the calcium/sodium exchange mechanism and intracellular redistribution (Opie, 1989). It must therefore be considered that verapamil may be exerting its effect by another mode of action. However, addition of this drug to homogenates of stored kidneys had no effect on peroxidative damage in vitro indicating that it is an unlikely antioxidant at the concentrations used and that its mode of action relied on intact cells.

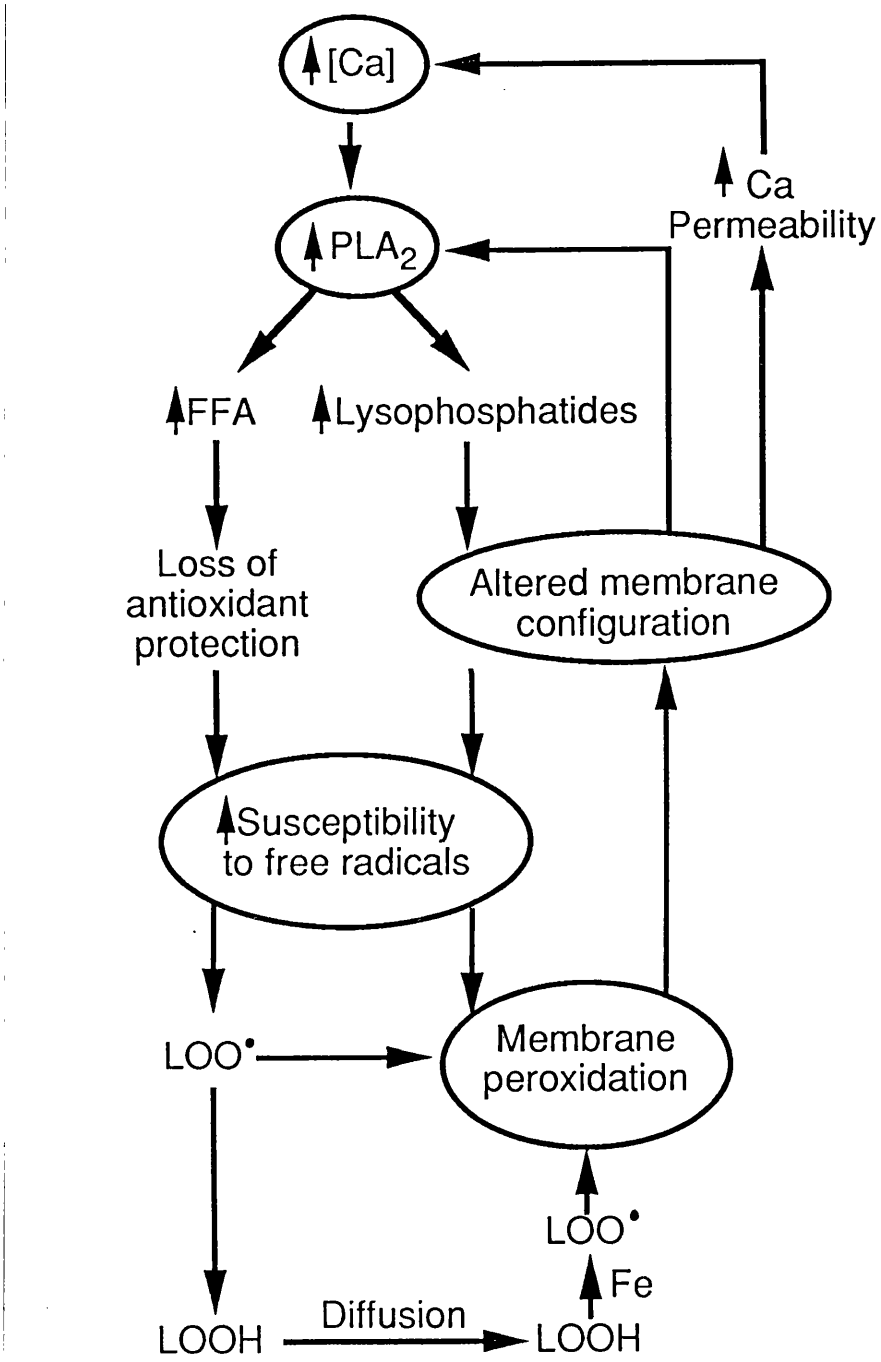
These studies clearly demonstrate a relationship between altered intracellular calcium homeostasis, phospholipase A2 activation and increased damage to lipid membranes in rabbit kidneys subjected to ischaemia and reoxygenation in vitro, what remains unclear however, is the temporal sequence of events with regard to increased calcium and increased lipid peroxidation. Malis and Bonventre (1986) have reported the involvement of a mitochondrial calcium-dependent phospholipase A2 in the potentiation of oxygen free radical injury to renal mitochondria. It was suggested that phospholipase A2 degrades catalytic subunits of the electron transport chain causing severe defects which enhance oxygen-derived free radical-induced mitochondrial membrane permeability. Such effects were mitigated by dibucaine. Damage to mitochondria

through calcium-activated phospholipase A2 and a build-up of reduced components of the electron transport chain during ischaemia may also increase $O_2^{\cdot -}$ production on reoxygenation due to increased leakage of single electrons onto O_2 and this may contribute to the increase in oxidative damage to kidneys observed following cold storage.

Oxygen free radical-induced damage has been reported to be involved in alterations in intracellular calcium homoestasis to isolated rat hepatocytes (Bellomo et al, 1985). The mitochondria and endoplasmic reticulum normally act as internal "sinks", sequestering excess cytosolic calcium. However, oxidative damage leads to depletion of intracellular calcium stores (Bellomo et al, 1985) and also to inhibition of the plasma membrane calcium-extruding system (Nicotera et al, 1985). Furthermore, free radical production induced by exposing hepatocytes to carbon tetrachloride caused an early rise in cytosolic calcium and initiated the activation of phospholipase A2 (Dolak, Glende and Recknagel, 1985). The resulting rise in cytosolic free calcium produces cell blebbing (Jewell et al, 1982) and eventual cell death.

Possible relationships between calcium, phospholipase activity and lipid peroxidation during ischaemia and subsequent reperfusion of kidneys are shown in Figure 43. Early redistribution of intracellular calcium during the ischaemic period leads to activation of phospholipases during the hypoxic period. The released unsaturated FFAs,

FIGURE 43: A Scheme Depicting the Possible Relationships Between Increased Intracellular Calcium Levels, Phospholipase A_2 (PLA_2) Activity, Lipid Peroxidation and Membrane Damage.



FFA-free fatty acids, LOO•-lipid peroxy radical, LOOH-lipid hydroperoxide, Fe-catalytic iron complex.

unprotected by the membrane-bound antioxidant vitamin E, would form excellent targets for free radical attack upon reoxygenation. The resulting alkoxy and peroxy radicals may then initiate peroxidation of membranes directly or may break down to relatively stable hydroperoxides which could diffuse to other sites in the cell and stimulate lipid peroxidation through interaction with catalytic iron complexes which regenerate reactive lipid radicals (Halliwell and Gutteridge, 1984). In addition, the concomitant build-up of residual lysophosphatides in the membrane, due to phospholipase activation, alter fluidity and permeability (Weltzem, 1979) and may render membranes more susceptible to free radical attack (Ungemach, 1985). Peroxidation of membrane lipids results in a loss of the fatty acid content of membranes, and also increases lysophosphatide levels (Ungemach, 1985) and membrane rigidity (Demopoulos et al, 1980).

Previous investigations have established that the activity of rat liver microsomal phospholipase A2 is higher in rigid membranes and decreases when the membrane lipid is fluidized (Momchilova et al, 1986a; 1986b). It appears that the rigidity of the lipid phase facilitates the penetration and binding of the enzyme. Peroxidation of membrane phospholipids increases rigidity (Demopoulos at al, 1980) and hence, increased free radical activity upon reperfusion may lead to further increases in phospholipase A2 activation and the damaging cycle of events shown in Figure 43 may

therefore ensue. This would lead to extensive damage to membranes which would become permeabilised to calcium and hence cytosolic calcium levels would rise further. Highly elevated levels of cytosolic calcium resulting from the above sequence of events (Figure 43) could alter cell morphology causing blebbing (Jewell et al, 1982) and, in conjunction with free radical mediated damage, would lead to irreversible injury to the cell.

In summary, renal ischaemia produces an accumulation of FFAs probably as a result of an increase in the calcium activation of phospholipases and blocked β-oxidation. Although HCA benefits organs during storage and is superior to other flush solutions for preservation of rabbit kidneys, it apparently provides no mechanism for preventing unsaturated FFA accumulation during the ischaemic period. The increased arachidonic acid release is of particular importance and is potentiated by calcium-dependent activation of phopholipase A2. Such effects are likely to mediate, or at least contribute to oxygen free radical damage. It may therefore be possible in future to afford significant protection to stored organs by chelating extracellular calcium, preventing its entry into cells by calcium antagonists and inhibiting phospholipase A2 activity in conjunction with other agents which inhibit adverse free radical-mediated reactions. However, much further work is necessary to evaluate functional benefits resulting from

such control of oxidative damage during storage and reperfusion.

The Effect of Hypoxia and Reoxygenation on Phosphatidylinositol Turnover in Rabbit Kidney Cortical Slices

Agonists that interact with receptors to produce an intracellular calcium signal are also able to induce the hydrolysis of inositol-containing phospholipids (Berridge, 1984). Agonist binding of such a receptor activates a specific phosphodiesterase which cleaves phosphatidyl-inositol-4,5,-bisphosphate (PIP_2). This process is thought to be controlled a GTP-binding protein (G-protein) which couples the receptor to the enzyme (Gomperts, 1983; Gilman, 1984). Hydrolysis of PIP_2 by the phosphodiesterase generates two products: inositol triphosphate (IP_3) and diacylglycerol (DAG) both of which act as secondary messengers. IP_3 is water soluble and binds to a receptor on the ER which leads to the mobilization of calcium and DAG remains in the plane of the membrane and stimulates phosphorylation of protein kinase C (PKc). IP_3 is then converted to inositol-1,4,-bisphosphate (IP_2) by inositol trisphosphatase and then to inositol-1-phosphate (IP_1) by inositol bisphosphatase (Downes et al, 1982) and this is finally converted to free inositol by inositol-1-phosphatase. Inhibition of this enzyme by lithium (Hallcher

and Sherman, 1980; Berridge et al, 1982) leads to accumulation of IP_1, IP_2 and IP_3.

Agonist binding of α_1-adrenoceptors leads to intracellular calcium signalling with the concomitant hydrolysis of phosphoinositides which in turn leads to a variety of cellular responses. (Berridge, 1984). In the kidney, agonist binding of α_1-adrenoceptors evokes many different responses including: increased sodium reabsorption (Osborn et al, 1983; Hesse and Johns, 1984), prostanoid production (Cooper and Malik, 1985), gluconeogenesis (Kessar and Saggerson, 1980; McPherson and Summers, 1982), renal vasoconstriction (Schmitz et al, 1981; Smyth et al, 1984; Cooper and Malik, 1985) and inhibition of renin release (Matsumura et al, 1985).

In order to extend previous findings that ischaemia renders kidneys more susceptible to lipid peroxidation, possibly through altered calcium homeostasis, experiments were carried out to investigate the possibility that hypoxia/reoxygenation may lead to an alteration in intracellular signalling through phosphoinositide hydrolysis which may contribute to reperfusion injury of ischaemic organs.

Rabbit kidney cortical slices were chosen as a model for these experiments because they allowed efficient incorporation of myo-(2-^{3}H)-inositol into the cytosol and subsequent incorporation of the label into the membrane. It was important to pre-incubate the slices to increase the

ATP levels in the tissue in order to facilitate active uptake of the labelled inositol. In order to investigate the effect of lipid peroxidation on PIP_2 breakdown, it was necessary to develop a system for producing membrane peroxidation in rabbit kidney slices. Two systems were considered, the first carbon tetrachloride (CCl_4) and the second iron-ADP in the presence of ascorbate. CCl_4 is metabolised by the cytochrome P_{450} system to give the trichloromethyl radical ($CCl_3^{\bullet}$) (Slater, 1984), which can react with molecular O_2 to form a more reactive species, the trichloromethyl peroxy radical ($CCl_3O_2^{\bullet}$) (Packer, et al, 1978).

Iron in the presence of ADP and ascorbate has been shown to be effective at initiating lipid peroxidation in liver microsomes (Orrenius et al, 1964). However, in rabbit kidney slices exposed to iron, ADP and ascorbate, lipid peroxidation although higher than control values was not significantly so. The increase in TBARs reached a plateau after 40 minutes of incubation which may have been due to the ascorbate becoming oxidised and hence no longer able to reduce Fe(III) ions to Fe(II). CCl_4 was found to be a superior initiator of lipid peroxidation in this model. Furthermore, the requirement of CCl_4 to be metabolically activated meant that by using this compound it could be concluded that lipid peroxidation was taking place intracellularly. The rate of lipid peroxidation was more

linear than with iron, ADP and ascorbate and was still increasing after 60 minutes of incubation.

Pre-labelled slices incubated in a calcium-containing medium, in the presence of CCl_4, appeared to possess an increased PIP_2 breakdown although this was not statistically significant. The effect of CCl_4 on lipid peroxidation in slices appeared to be linear with time, with peroxidation increasing up to 60 minutes of incubation. It seems likely that a longer period of incubation would have resulted in greater peroxidation and hence could have evoked a statistically significant increase in the rate of PIP_2 breakdown in this model.

It has recently been postulated that 4-hydroxynonenal (HNE), an aldehyde produced during lipid peroxidation, stimulates adenylate cyclase, guanylate cyclase and PIP_2 breakdown (Dianzani et al, 1989). Stimulation is evoked by concentrations of HNE in the range of 10^{-6}-10^{-7}M which is physiological within cells. It is thought that HNE acts on the stimulatory component of the regulatory G-protein and so may mediate responses such as renal vasoconstriction. Furthermore, recent work has suggested that the hepatic α_1-receptor is vulnerable to radical stress which may lead to an impairment of hormonally regulated calcium homeostasis in hepatocytes (Pruijin and Bast, 1988). The initiating radicals used in this experiment were thought to either change the membrane micro-environment or the α_1-receptor, or react directly with the receptor possibly with a free

sulfhydryl-group on the receptor protein. The ATP-dependent microsomal calcium sequestration mechanism was also modified, probably by sulfhydryl modification and lipid peroxidation which may contribute to an alteration in calcium homeostasis. Hence, lipid peroxidation might augment PIP_2 breakdown through the regulatory G-protein or the α_1-receptor and so cause uncontrolled cellular responses.

In the presence of EGTA, PIP_2 breakdown was significantly reduced after 60 minutes of incubation which may indicate the calcium-dependency of this process. Previous work has indicated that, although the initial cleavage of PIP_2 by phospholipase C may be stimulated by the receptor binding of an agonist, the resultant elevated calcium levels could then lead to further phospholipase C activation (Wilson et al, 1985). In brain, omission of calcium from the incubation medium can attenuate, and inclusion of calcium chelators can abolish, stimulated inositol lipid turnover (Griffen et al, 1979; Fisher and Agranoff, 1980; 1981). In some circumstances, addition of the calcium ionophore can mimic the stimulatory effect of natural agonists to evoke PI turnover (Fisher and Agranoff, 1981; Kendall and Nahorski, 1984). Hence an increase in cytosolic calcium may in some cases increase the turnover of PI.

In agreement with previous studies on whole rabbit kidneys rendered ischaemic <u>in vivo</u> (Green et al, 1986a),

reoxygenation of cortical slices rendered hypoxic in vitro resulted in an elevated rate of lipid peroxidation. This in vitro system was therefore a good model of reperfusion-induced free radical damage to renal tissue. Levels of lipid peroxidation during the hypoxic period itself were significantly lower than control oxygenated slices which further suggested that free radicals play little role during the hypoxic period itself. It would therefore appear that the hypoxic period 'primes' the tissue for greater free radical damage upon reoxygenation. This is likely to be the result of a number of events occurring both during hypoxia and during reoxygenation including: redistribution of intracellular iron (Gower et al, 1989b) which is involved in the initiation of lipid peroxidation; altered intracellular calcium homeostasis which activates phospholipase A_2 and renders membranes more susceptible to free radical attack; and increased production of oxygen-derived free radicals due to mitochondrial dysfunction and activation of xanthine oxidase (Roy and McCord, 1983).

Measurement of PIP_2 in slices exposed to hypoxia and reoxygenation clearly demonstrated that hydrolysis of phosphatidylinositols to secondary messenger products is activated very rapidly on reoxygenation of renal tissue following a period of hypoxia. This is the first report of such an effect and inhibition by EGTA strongly suggested the involvement of calcium. No changes in PIP_2 breakdown were observed during hypoxia itself. Recently it has been

reported that phospholipase C activity, measured in synaptosomal fractions *in vitro*, is stimulated as a result of ischaemia of brain tissue *in vivo* (Stosznajder, 1989). In that report, metabolism of exogenous phosphatidylinositols added *after* the ischaemic period was measured whereas in this study hydrolysis of endogenous membrane-bound phosphatidylinositols was measured in kidney cortical slices actually during hypoxia and reoxygenation.

It is possible that increased levels of aldehydic products (such as HNE) produced on reoxygenation were responsible for increased PIP_2 hydrolysis. In addition, both lipid peroxidation and high calcium-dependent phospholipase A_2 activity alter membrane configuration (Ungemach, 1985) and this may affect the interaction of phospholipase C with membrane-bound regulatory components or make it more accessible to its substrate PIP_2. Alternatively, altered calcium homeostasis may be involved in other ways. During ischaemia, cytosolic calcium levels may increase due to failure of energy-dependent pumps, although this perturbation did not affect PIP_2 hydrolysis during hypoxia. However, on reoxygenation much more dramatic changes to calcium homeostasis can occur due to loss of calcium-sequestering ability of damaged intracellular organelles and increased permeability of the plasma membrane allowing entry of extracellular calcium. It is possible that highly altered calcium levels on reoxygenation were sufficient to evoke a response of the

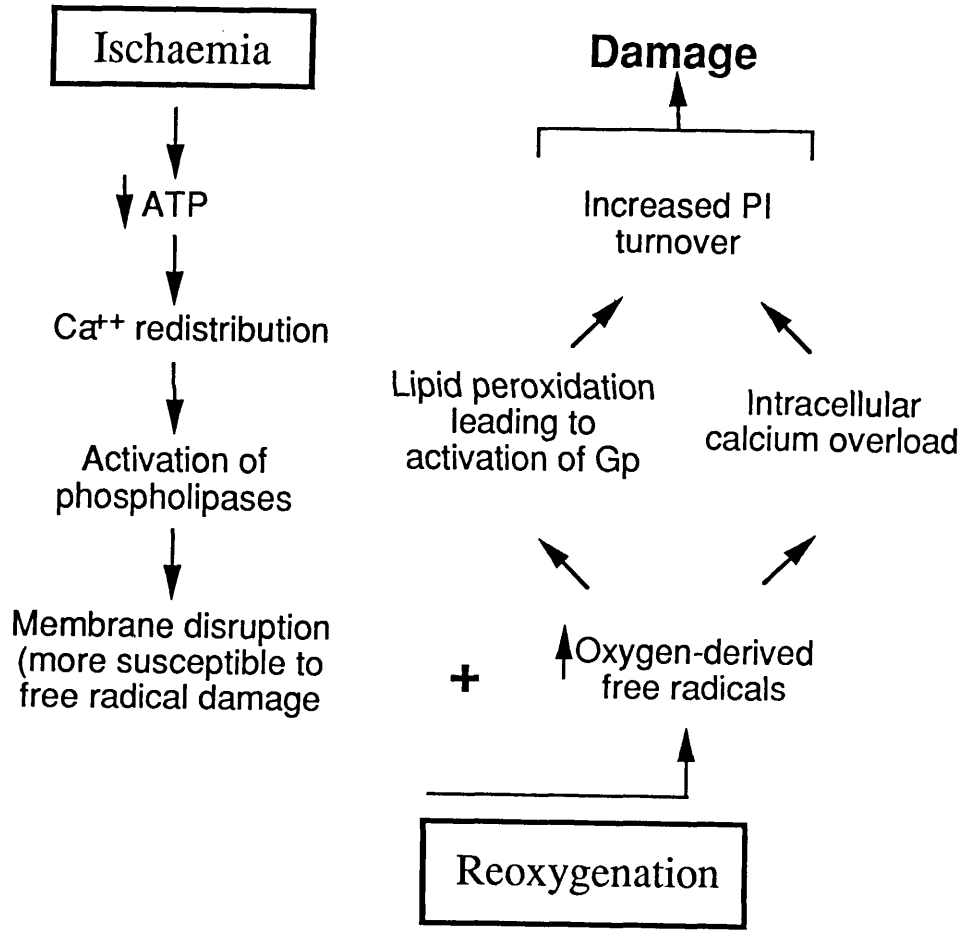
phospholipase C system. Although it is thought that cytosolic calcium rises are important during the hypoxic period, it is unlikely that the phospholipase C is effective during this stage, due to a decreased availability of its substrate PIP_2. ATP levels were seen to be dramatically decreased at the onset of the hypoxic period and so the ATP-dependent conversion of PI to PIP_1 and PIP_1 to PIP_2 would be limited. However, during reoxygenation an increase in ATP levels may create a greater availability of PIP_2 for the formation of second messenger products.

ATP levels became depleted very rapidly at the onset of hypoxia and the ability of cells to produce ATP was considerably reduced if not totally prevented. Following reoxygenation of slices, after 120 minutes of hypoxia, ATP production recovered to a certain extent but was still low compared to oxygenated samples. This may have been due to a loss of ATP precursors which may have been degraded during the hypoxic period, or to impairment of mitochondria during the hypoxic period or at the time of reoxygenation which led in turn to a decline in the ability of these organelles to provide ATP. However, the increase in ATP synthesis following hypoxia was similar to that observed when slices which had been incubated under aerobic conditions were reoxygenated. This demonstrated that while a period of hypoxia compromised the slices, they were still capable of revovery.

Rapid hydrolysis of PIP_2 on reoxygenation following ischaemia would lead to deregulation of receptor mediated function through this intracellular secondary messenger system. One of the consequences of this is likely to be an imbalance in eicosanoid production and vascular disturbances. Prostaglandin (PG) synthesis has been linked to phosphatidylinositol turnover in isolated rat glomeruli (Folkert et al, 1984). Furthermore, PG release has been shown to be stimulated in rat kidneys by angiotensin II through increased PI turnover (Benabe et al, 1982) and in rabbit renal medullary interstitial cells by vasopressin through calcium influx and the activation of calcium-calmodulin phospholipases (Ausiello and Zusman, 1984). DAG may also act as a precursor for the release of arachidonic acid which is then converted to PGs. Alternatively, the IP_3 pathway of PI turnover may provide calcium necessary to activate phospholipase A2 and so increase release of arachidonic acid. Also, DAG binding to protein kinase C may phosphorylate a protein termed lipocortin which normally inhibits phospholipase A2 and hence this would lead to increased release of arachidonic acid (Berridge, 1987).

It is interesting that enoximone, a phosphodiesterase inhibitor, was effective in treating advanced unstable chronic heart failure (often a result of ischaemic heart disease) by increasing vasodilation (Weber et al, 1986). In addition, previous studies have indicated that verapamil may augment renal blood flow and increase glomerular

FIGURE 44: A Scheme Depicting the Sequence of Events during Ischaemia and Reoxygenation Leading to Cellular Damage and Increased PI Turnover



filtration rate (Bell and Lindner, 1984; Steele and Challoner-Hue, 1985), counteract the intrarenal vasoconstriction produced by norepinephrine (Malis et al, 1983) and reverse the renal cortical actions of angiotensin II (Ichikawa et al, 1979). This suggests that verapamil inhibits the cellular responses intiated by PI turnover either by inhibiting calcium influx through VOCs or by another mechanism which may include inhibition of calcium influx through SMOCs.

It therefore seems likely that an increase in PI turnover upon reoxygenation caused by free radicals and/or calcium overload will cause a deregulation in receptor-mediated function (Figure 44) which may contribute to the pathogenesis of renal damage following ischaemia and reperfusion.

General Summary

This thesis has attempted to elucidate some of the underlying mechanisms of biochemical damage associated with renal ischaemia and reperfusion. However it is unlikely that the events described are solely responsible for the deterioration of organs following prolonged periods of ischaemia, but that the complex interaction of a number of factors is the cause.

Evidence has been presented that a redistribution of calcium occurs during the ischaemic period itself. This is likely to be an early event due to the rapid depletion in

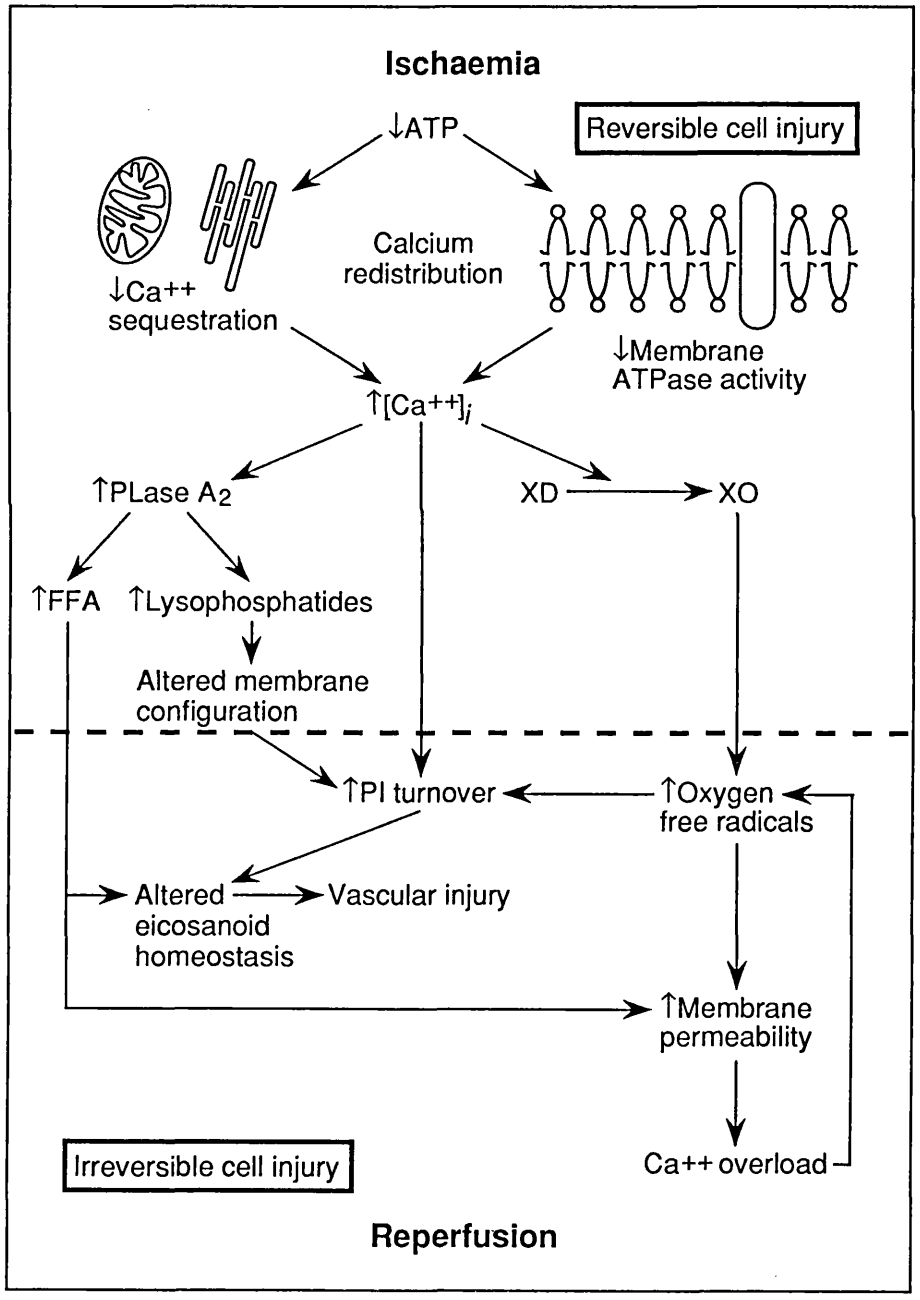
levels of ATP in the tissue and concomitant failure of energy-dependent mechanisms responsible for calcium regulation. While these changes may be considered adverse to normal cell function they are nevertheless reversible within certain limits. Thus upon reperfusion, ATP may be regenerated providing energy for calcium pumps and restoration of calcium homeostasis. Such changes may be viewed as crucial in the 'priming' of tissue for subsequent reperfusion damage. The stimulation of calcium-dependent processes would lead to further disturbances in the cell such as lipid hydrolysis through phospholipase action and proteolysis, for example leading to the formation of xanthine oxidase. These events may then potentiate oxidative damage upon reperfusion of the tissue.

Upon reperfusion a burst of $O_2^{\cdot -}$ production from the incoming O_2 would react with increased levels of catalytic iron (Gower et al, 1989b) to yield much more reactive radical species. Damage to cellular components would ensue including peroxidation of membrane lipids already compromised by increased calcium-dependent phospholipase activity. The resulting loss of integrity of the plasma membrane and intracellular organelles would cause further imbalances in intracellular ion homeostasis including the accumulation of pathological levels of cytosolic free calcium. This cycle of self-perpetuating events may perturb the cell sufficiently to cause irreversible cell injury (Figure 45).

In addition to 'gross' biochemical damage, effects on specific systems may contribute to post-ischaemic organ failure through disturbances in the vasculature of the organ. These include imbalances in eicosanoid production due to calcium-dependent build-up of free arachidonic acid, production of inflammatory mediators such as leukotrienes, release of chemotactic substances with subsequent adhesion and activation of polymorpho-nucleocytes, and derangement of receptor-mediated functions such as the phosphatidylinositol secondary messenger system (Figure 45).

Now that many of the immunological problems associated with organ transplantation can be successfully controlled, retrieval of donor organs in optimum condition is becoming an increasingly important aspect of clinical renal transplantation. Hypothermic storage in special solutions with improved ionic composition has already yielded benefits (Belzer & Southard, 1988). It is envisaged that further advances towards increasing both the number and post-storage viability of transplanted organs will come through a better understanding of the underlying causes of ischaemic/reperfusion damage. A combined pharmacological strategy to prevent, or at least slow, several adverse biochemical changes associated with ischaemia including altered intracellular calcium homeostasis, loss of antioxidant protection and increased capacity for free radical generation, would appear to offer great potential.

FIGURE 45: A Scheme Depicting Possible Relationships Between Oxygen Free Radicals and Alterations in Calcium Homeostasis During Ischaemia and Reperfusion which may Mediate Irreversible Cell Injury



Proposals for Future Work

(1) Studies on kidney function following storage under the conditions described in this thesis including observations of vascular changes and survival assessment on replantation.

(2) Measurement of intracellular free calcium levels during hypoxia and reoxygenation using the fluorescent dye fura2.

(3) Addition of intracellular calcium chelators such as quin-2 to storage solutions in an attempt to reduce calcium-mediated ischaemic damage.

(4) Measurement of membrane fluidity and phospholipase A2 activity in rabbit kidneys during cold ischaemia.

(5) Measurement of levels of xanthine oxidase and xanthine dehydrogenase following manipulation of calcium homeostasis during storage of rabbit kidneys.

(6) Investigation of mechanisms by which PI turnover becomes stimulated upon reoxygenation following hypoxia.

(7) The effects of hypoxia/reoxygenation on DAG production and protein kinase C in rabbit kidney cortical slices.

(8) Free radical measurement during the storage period and

following reperfusion of kidneys by more direct methods such as electron spin resonance spectroscopy.

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LIST OF ABBREVIATIONS

ADP	adenosine diphosphate
AMP	adenosine monophosphate
ARF	acute renal failure
ATN	acute tubular necrosis
ATP	adenosine triphosphate
CI	cold ischaemia
DAG	diacyl glycerol
DEGS	diethylene glycol stearate
DMA	dimethyl acetamide
ER	endoplasmic reticulum
FFA	free fatty acid
GFR	glomerular filtration rate
GLC	gas liquid chromatography
G_p	guanosine nucleotide binding protein
GTP	guanosine triphosphate
H	hydrogen atom
H_2O_2	hydrogen peroxide
HCA	hypertonic citrate solution
HEPES	N_2-hydroxyethyl piperazine-N-ethane sulphonic acid
HPLC	high pressure liquid chromatography
IP_1	inositol 1-phosphate
IP_2	inositol 1,4-bisphosphate
IP_3	inositol 1,4,5,-trisphosphate
KRB	modified Krebs Ringer bicarbonate solution

L· conjugated diene

LO· alkoxy radical

LOO· hydroperoxy radical

LOOH lipid hydroperoxide

MAG monoacyl glycerol

MDA malondialdehyde

NAD nicotinamide adenine dinucleotide

NADH nicotinamide adenine dinucleotide (reduced form)

NZW New Zealand White

$O_2^{\cdot -}$ superoxide anion

O_2^{2-} peroxide ion

OH· hydroxyl radical

PDE phosphodiesterase

PIP_2 phosphatidylinositol 4,5-bisphosphate

PUFAs polyunsaturated fatty acids

ROC receptor operated channel

SB Schiff base

SMOC secondary messenger operated channel

SOD superoxide dismutase

TBAR thiobarbituric acid-reactive

VOC voltage operated channel

WI warm ischaemia

CONTRIBUTION TO PUBLICATIONS

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	Experimental Work	Writing
(1)	100%	100%
(2)	100%	100%
(3)	100%	100%
(4)	100%	100%
(5)	100%	50%
(6)	30%	30%
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FREE FATTY ACID ACCUMULATION FOLLOWING COLD ISCHAEMIA IN RABBIT KIDNEYS AND THE INVOLVEMENT OF A CALCIUM DEPENDENT PHOSPHOLIPASE A_2

L.A. Cotterill*, J.D. Gower, B.J. Fuller+ and C.J. Green
Section of Surgical Research, MRC Clinical Research Centre, Watford Road, Harrow, Middlesex, HA1 3UJ, U.K.
+Academic Department of Surgery, Royal Free Hospital School of Medicine, Pond Street, London, NW3 2QG.
*To whom correspondence should be addressed

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SUMMARY

These experiments were designed to study the possible accumulation of free fatty acids during cold ischaemia of rabbit kidneys. The organs were harvested and immediately flushed with 30ml of cold hypertonic citrate (HCA) solution and stored for 72hr at 0°C. The kidneys were then dissected into cortex and medulla, immediately frozen in liquid nitrogen and free fatty acids were extracted and assayed by gas liquid chromatography. This period of storage resulted in significant ($p<0.05$) increases in free unsaturated fatty acids oleic acid ($C_{18:1}$), linoleic acid ($C_{18:2}$) and arachidonic acid ($C_{20:4}$) in both cortex and medulla. No such increases were observed in the free saturated fatty acids stearic ($C_{16:0}$) or palmitic acid ($C_{18:0}$). Addition of the phospholipase A_2 inhibitor dibucaine to the HCA flush solution significantly ($p<0.05$) reduced the accumulation of free arachidonic acid during cold storage. Addition of the calcium ionophore A23187 to HCA significantly ($p<0.05$) increased the accumulation of free arachidonic acid over the 72hr period of cold ischaemia. These data provide some evidence for the involvement of Ca^{2+} activation of phospholipase A_2 in releasing arachidonic acid from cellular membranes during storage. The resulting accumulation of FFAs may be important in potentiating free radical damage, and physiological deterioration of organs following storage.

KEYWORDS

Cold ischaemic damage, kidney, free fatty acids, calcium, phospholipase A_2.

INTRODUCTION

In the course of renal transplantation, kidneys are subjected to various periods of warm and cold ischaemia and this can result in poor renal function and death of tubular cells soon after engraftment and reperfusion with blood. There is growing evidence that oxygen-derived free radicals are responsible, at least in part, for reperfusion injury (1) and may be involved in ischaemic damage (2). We have shown in previous studies that markers of lipid peroxidation, resulting from oxidative damage, increase in rabbit kidneys following cold storage (3). We have also recently reported evidence for a relationship between altered Ca^{2+} homeostasis and oxidative damage to rabbit kidneys following cold storage (4). Other investigators have implicated a role for Ca^{2+} in the degenerative processes resulting from ischaemia (5); increased cytosolic Ca^{2+} has been observed in isolated cells exposed to anoxia (6) and altered Ca^{2+} homeostasis has been demonstrated in isolated rabbit myocardium subjected to warm ischaemic insults (7).

One possible mechanism by which Ca^{2+} ions could potentiate oxidative damage is through the activation of phospholipases. We have previously demonstrated that a phospholipase A_2 inhibitor, dibucaine, decreases markers of lipid peroxidation (a process initiated by free radicals) following cold storage of rabbit kidneys (8). It has also been shown that phospholipase A_2 activity and markers of lipid peroxidation increase during ischaemia of rat small intestinal mucosa (9). Free fatty acids (FFA), released from cellular membranes as a result of phospholipase action, are known to accumulate during periods of ischaemia in several other models (10, 11, 12, 13). Such accumulation could be detrimental to cells both because of the detergent-like properties of FFAs and because they have already been implicated in causing further increases in ATP loss and mitochondrial injury following anoxia, for example, of myocardial mitochondria (14).

The lysophosphatide residues remaining in the membrane following phospholipase attack also increase during ischaemia and are themselves damaging species, potentially deleterious to cell function (15). The disruption of membranes following phospholipid hydrolysis is likely to render them more susceptible to attack by free radicals (16).

We therefore decided to investigate, by gas liquid chromatography, the FFA status of rabbit kidneys following cold storage in a clinically approved storage medium, hypertonic citrate solution (HCA). In addition, in

an attempt to gain further insight into the role of Ca^{2+} and phospholipase A_2 in FFA accumulation, we have examined the effects of adding the Ca^{2+} ionophore A23187 and the phospholipase A_2 inhibitor dibucaine to HCA during the storage period.

MATERIALS AND METHODS

Chemicals: Dibucaine hydrochloride, A23187 and fatty acid methyl ester standards were purchased from Sigma Chemical Company Ltd., Poole, Dorset. Dimethyl-acetamide (DMA), methyl iodide and pyridine were purchased from BDH Chemicals Ltd., Poole, Dorset.

Operative procedure: Four groups of New Zealand White rabbits (average weight 3kg) were anaesthetised by i.m. injection of 0.2ml/kg fentanyl-fluanisone (Hypnorm), followed by slow i.v. injection of diazepam (Valium 20) at 1.0mg/kg. Frusemide (3mg/kg) and 300i.u./kg of heparin were also administered by i.v. injection. The abdomen was opened by a mid-line incision and the kidneys were dissected free and removed with their vascular pedicle. The renal artery of each kidney was cannulated and the organs were flushed with 30ml of sterile HCA solution already cooled to 4°C. In two groups of kidneys, either dibucaine (250μM) or A23187 (10μM) were incorporated into the flush and storage solutions. The kidneys were placed in sterile beakers containing 60ml of the identical flush solution and stored in ice at 0°C for 72 hours.

Extraction of FFA from kidneys: Extraction and methylation techniques were modified from those described by Allen (17). Kidneys were dissected into cortex and medulla and immediately frozen in liquid nitrogen. Samples were ground to a powder in a pestle and mortar while still frozen. Methanol (6ml), hexane (12.5ml) and 1μg of the internal standard pentadecanoic acid, were added without delay to 80mg of the powdered samples. The mixture was shaken vigorously for 1 min and centrifuged (1000g). The lower phase was removed and the remaining hexane phase was washed twice with 0.1M phosphoric acid (8ml). The hexane layer was removed to another tube and the FFA extracted with 0.5M potassium hydroxide (KOH) solution (40μl). The KOH layer was removed for methylation.

Methyl iodide methylation: N,N-Dimethyl-acetamide (DMA) (100μl) and methyl iodide (60μl) were added to the extracted fatty acids present in the KOH. The mixture was incubated for 10min at 65°C. Pyridine (120μl) was then added followed by a further incubation at 65°C for 10min. After cooling, 0.1M phosphoric acid (1ml) and ethylene chloride (35μl) were added and the fatty acid methyl esters were extracted into ethylene chloride. Samples were washed with distilled water equilibrated with ethylene chloride. The upper phase was removed and the fatty acid methyl esters

were finally extracted in hexane. 2µl was injected into the gas liquid chromatograph (GLC).

Gas-liquid chromatography: GLC was performed with a Pye Unicam series 104 chromatograph connected to a Trivector chromatography computing integrator (Trivector Systems International Ltd., Bedfordshire). A 1.52mx 6mm glass column packed with 10% diethylene glycol stearate (DEGS) on chromosorb WAW 80-100 mesh (Phase Separations Ltd., Clwd) was used. The temperatures of the injector and detector were 250°C and the column was maintained at 190°C. Fatty acid methyl esters were identified on the basis of their retention times compared to known standards of fatty acid methyl esters. The amount of each FFA was expressed as the ratio of its peak area with that of the intenal standard.

Statistical analysis was performed using the Students paired t-test.

RESULTS AND DISCUSSION

Accurate and valid measurement of FFAs is complicated by the possibility that hydrolysis of membrane phospholipids to fatty acids and lysophosphatides may increase both during handling (18) and through careless extraction and homogenisation (19). This is due to activation of phospholipases during these procedures which results in an artifactual elevation in the products of phospholipid hydrolysis. In the present study, rabbit kidney cortex and medulla were quickly clamped under liquid nitrogen, ground to a powder and then immediately subjected to free fatty acid extraction to avoid such artifactual phospholipase activation. Losses in FFAs during the extraction and methylation procedures were corrected for by the addition of an internal standard, pentadecanoic acid ($C_{15:0}$).

Hypertonic citrate solution has been used successfully in clinical renal preservation for several years. Developed by Ross and Marshall (20), it is hypertonic with respect to plasma and contains 186mM mannitol which permeates the cell membrane slowly, thus preventing cell swelling. In previous studies of our own, we have demonstrated that HCA protects stored rabbit kidneys against influx of extracellular Ca^{2+} but apparently fails to reduce damage mediated by altered homeostasis in intracellular Ca^{2+} (7). The 55mM citrate present may be involved in Ca^{2+} chelation and thus prevents or at least slows pathological elevations in intracellular Ca^{2+} during cold ischaemia.

In the present study, following the 72hr storage period, there were significant ($P<0.05$) increases in the free unsaturated fatty acids oleic acid ($C_{18:1}$), linoleic acid ($C_{18:2}$) and arachidonic acid ($C_{20:4}$) in both cortex (Fig 1) and medulla (Fig 2) as compared with unstored controls. No such increases were observed in the saturated fatty acids stearic acid

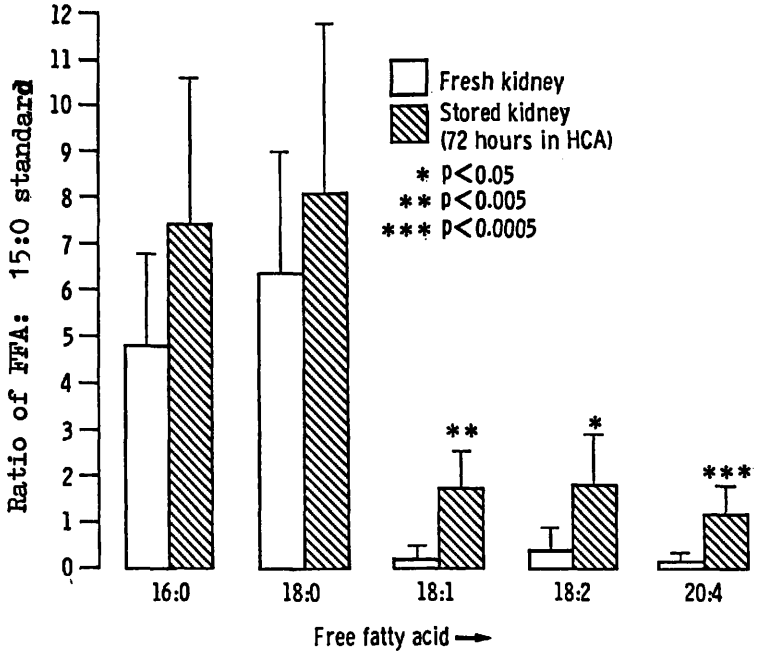

Figure (1): FFA analysis in cortex of rabbit kidneys stored for 72 hours in HCA. Values represent mean ± S.D. of 6 determinations

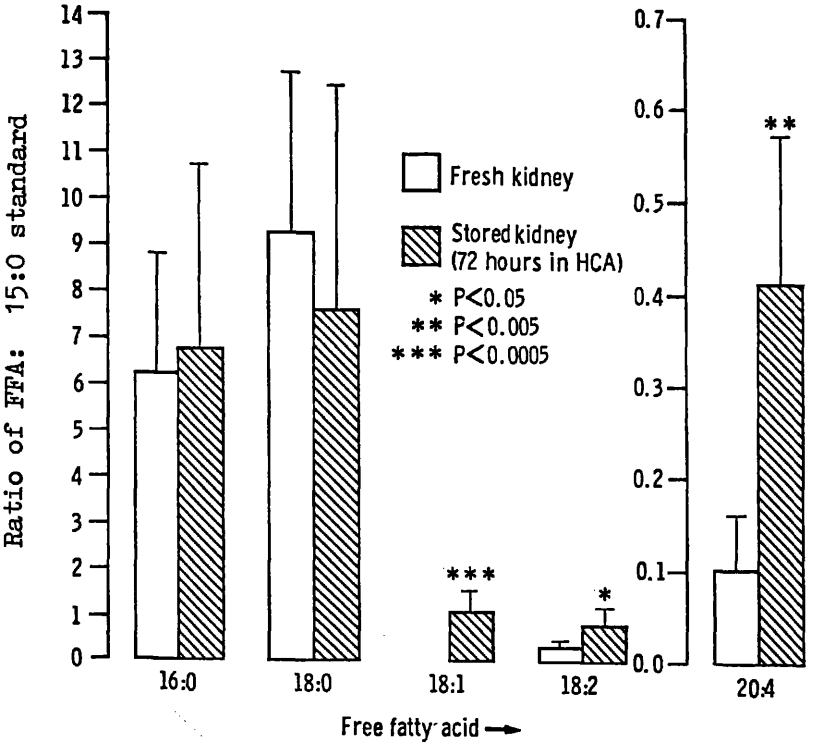

Figure (2): FFA analysis in medulla of rabbit kidneys stored for 72 hours in HCA. Values represent mean ± S.D. of 6 determinations

($C_{16:0}$) or palmitic acid ($C_{18:0}$) in either cortex or medulla (Figs 1 and 2). Thus a greater proportion of unsaturates are evident in both regions of the kidney following storage. Although HCA benefits organs during storage and is superior to other flush solutions for preservation of rabbit kidneys, in our experience only 50% of rabbit kidneys stored for 72 hours in HCA prove life-supporting after transplantation. The present study demonstrates that HCA provides little or no protection against the accumulation of unsaturated FFA during ischaemic period and this may therefore be a contributing factor in the failure of these organs.

The phospholipase A_2 inhibitor dibucaine competes with Ca^{2+} for the site of activation on the enzyme. In these experiments, addition of the phospholipase A_2 inhibitor dibucaine (250μM) to the HCA solution used to flush kidneys prior to 72 hour storage led to a significant decrease in the levels of free $C_{20:4}$ in both the kidney cortex and medulla compared to stored, untreated controls (Fig 3). Dibucaine had no effect on the levels of $C_{16:0}$, $C_{18:0}$, $C_{18:1}$ or $C_{18:2}$ following storage. This observation indicates the presence of a Ca^{2+}-dependent phospholipase A_2 in rabbit kidneys responsible for the hydrolysis of phospholipids leading to $C_{20:4}$ release. It is probable that release of other free fatty acids following ischaemia of rabbit kidneys is the result of another mechanism, possibly hydrolysis by Ca^{2+}-independent phospholipases. It is also possible that blockade of fatty acid esterification due to shortage of ATP during ischaemia is a contributory cause in producing FFA accumulation. In the present study, inhibition of phospholipase A_2 by dibucaine significantly decreased but did not abolish $C_{20:4}$ accumulation during storage. It could be postulated that, although the hydrolysis of phospholipids by phospholipases particularly in the release of $C_{20:4}$ is a major cause of FFA accumulation during storage, so too is the blockade of β oxidation.

A variety of phospholipases have now been identified. Membrane-bound phospholipases show an absolute requirement for Ca^{2+} (21). Addition of Ca^{2+} augments arachidonic acid release by 20-fold in human platelet membranes (22). The Ca^{2+} ionophore A23187 is an excellent experimental tool which facilitates the passage of Ca^{2+} ions across membranes producing an overall increase in cytosolic calcium (23). A23187 added to human platelets, for example, in the presence of EGTA, releases Ca^{2+} from internal stores which induces the release of 46% of the total phospholipid $C_{20:4}$ content within 1 min (24).

In the present study, addition of A23187 (10μM) to the HCA solution used to flush kidneys prior to 72 hour storage led to a significant increase in the levels of free $C_{20:4}$ in both the cortex and medulla

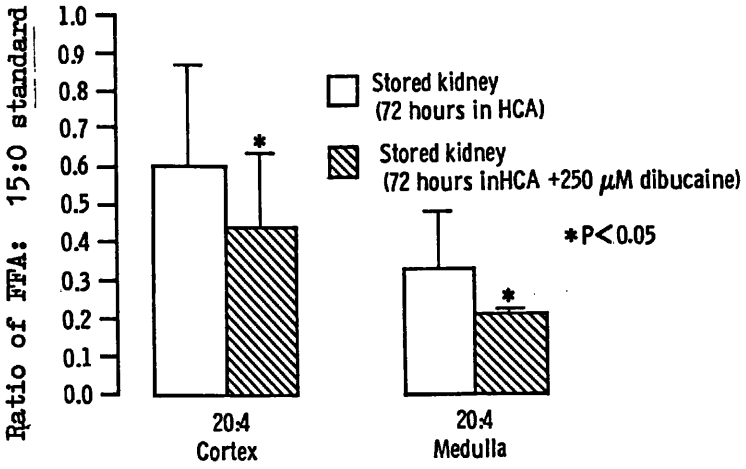

Figure (3): The effect of adding dibucaine (250μM) on the accumulation of free arachidonic acid ($C_{20:4}$) in the cortex and medulla of rabbit kidneys stored for 72 hours in HCA

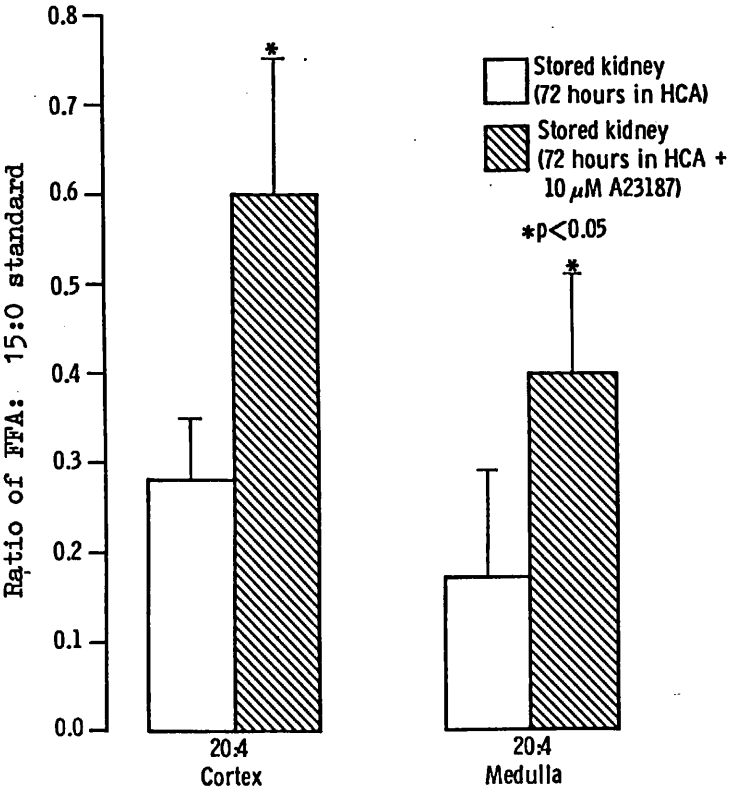

Figure (4): The effect of adding A23187 (10μM) on the accumulation of free arachidonic acid ($C_{20:}$ in the cortex and medulla of rabbit kidneys stored for 72 hours in HCA.

compared to stored untreated controls (Fig 4). A23187 had no effect on the levels of $C_{16:0}$, $C_{18:0}$, $C_{18:1}$ or $C_{18:2}$ following the storage period, providing further evidence for Ca^{2+}-independent mechanisms mediating the release of FFAs other than arachidonic acid. It would appear, therefore, that an increase in cytosolic Ca^{2+} possibly through the activation of a Ca^{2+}-dependent phospholipase is responsible, at least in part for the accumulation of $C_{20:4}$ in the cortex and medulla of rabbit kidneys following ischaemic insult.

In our previous studies, we have provided evidence for phospholipase A_2 activation in mediating oxidative damage following cold storage of the rabbit kidney (8) as dibucaine reduced the rate of lipid peroxidation in this model. What remains unclear however, is the temporal sequence of events during ischaemia linking increased Ca^{2+} to increased lipid peroxidation. In all probability; it is the rise in cytosolic Ca^{2+} in conjunction with other factors, in particular lipid peroxidation, that leads to the activation of phospholipase A_2 and contributes to the degeneration of cell membranes during ischaemia.

What are the consequences of FFA accumulation? Several investigators have reported an accumulation in FFAs following ischaemia (10, 13, 15). In the present study, a highly significant increase in $C_{20:4}$ was observed (figures 1 and 2). This observation is potentially of great pathophysiological importance. Release of $C_{20:4}$ through hydrolysis of phospholipids is the rate limiting step in the cyclo-oxygenase and lipoxygenase pathways (25). Higher concentrations of free $C_{20:4}$ may therefore lead to a burst in the production of the eicosanoids: prostaglandins and thromboxanes via cyclo-oxygenase and leukotrienes via lipoxygenase respectively. Upsetting the delicate eicosanoid balance between vasoconstriction and vasodilation may have important consequences for the endothelial lining of the vascular bed. This has been demonstrated experimentally for example in perfused rabbit lungs when addition of exogenous arachidonic acid, A23187 and phospholipase A2 to the perfusate increased pulmonary vascular resistance (26). Prostaglandin synthesis is dependent upon the concentration of hydroperoxides formed as a result of lipid peroxidation and an increase inhibits prostacyclin synthesis. The result would be a decrease in the potent vasodilator prostacyclin and elevation in the formation of thromboxanes which mediate vasoconstriction (27, 28, 29). This proposition is supported by another of our own studies in which inhibition of cyclo-oxygenase by administering indomethacin to rabbit recipients autografted with cold stored kidneys protected against oxidative damage (30). Such alterations in eicosanoid homeostasis may

contribute to the "no-reflow" phenomenon observed when damaged organs are transplanted.

Phospholipase A_2 hydrolyses phospholipids releasing free fatty acids into the cytosol leaving lysophosphatides behind in the membrane. Both products are deleterious to cell function displaying detergent-like properties. High FFA concentrations are damaging to ischaemic mitochondria (14) and result in oedema in brain tissues (31). Lysophosphatides within the membrane are normally maintained at very low levels. In sufficient quantities, however, they could alter membrane properties such as fluidity and permeability (32) leaving the membrane more susceptible to free radical attack. Normally, vitamin E protects unsaturated fatty acids in the membrane from peroxidation. Following phospholipase attack, the unprotected FFAs form excellent targets for free radical attack particularly from highly reactive hydroxyl radicals. The resulting fatty acid hydroperoxides lend themselves to the propagation of further free radical reactions.

Calcium-dependent activation of phospholipase A_2 is thought to act synergistically with oxygen free radicals during ischaemia to cause defects in the mitochrondrial electron transport chain and increase mitochondrial membrane permeability (4). Under normal circumstances mitochondria produce large quantities of intracellular free radicals but the cells are protected by endogenous scavengers (33). It seems likely that mitochondrial injury results in further production of these damaging species.

In summary, renal ischaemia produces an accumulation of FFAs probably as a result of increased calcium-activated phospholipases. Although HCA benefits organs during storage and is superior to other flush solutions for preservation of rabbit kidneys, it apparently provides no mechanism for preventing unsaturated FFA accumulation during the ischaemic period. An increase in arachidonic acid release may be potentiated by calcium-dependent activation of phospholipase A_2. Such effects are likely to mediate oxygen free radical damage. Exact mechanisms are yet to be elucidated, but these findings clearly suggest that there may be a role for phospholipase inhibitors in the improvement of organ preservation.

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OXIDATIVE STRESS DURING HYPOTHERMIC STORAGE OF RABBIT KIDNEYS: POSSIBLE MECHANISMS BY WHICH CALCIUM MEDIATES FREE RADICAL DAMAGE

L.A. Cotterill, J.D. Gower, B.J. Fuller* and C.J. Green

Section of Surgical Research
MRC Clinical Research Centre,
Watford Road, Harrow,
Middlesex, HA1 3UJ, U.K.

and

*Academic Department of Surgery,
Royal Free Hospital Medical School,
London, NW3 2PF.

SUMMARY

Rabbit kidneys were harvested and immediately flushed with 30 ml of cold isotonic saline solution and stored for 24 hr at 0°C on ice. They were then divided into cortex and medulla, homogenised, incubated in vitro at 37°C and assayed for thiobarbituric acid-reactive material (TBARs)as a marker of lipid peroxidation. Storage resulted in significant increases in TBARs and this was significantly increased still further if a Ca^{++} ionophore A23187 (10μM) was added to the flush solution. However, if dibucaine, which is an inhibitor of Ca^{++}-dependent phospholipase A_2, was added alone to the flush solution the rise was significantly inhibited. When A23187 (10μM) was added together with dibucaine (250μM) the respective effects were cancelled out. Similarly, allopurinol (5mM), an inhibitor of xanthine oxidase and a radical scavenger, added to the flush inhibited the increase in TBARs whereas allopurinol (5mM) together with A23187 (10μM) essentially vitiated the effect of the A23187 alone. These data provide some evidence that Ca^{++}-dependent phospholipases and Ca^{++}-dependent production of xanthine oxidase are involved in the 'storage damage' syndrome.

KEYWORDS

Cold ischaemic damage, Kidney, Dibucaine, Allopurinol, Calcium, Free radicals.

INTRODUCTION

Kidneys for transplantation are usually subjected to a short period of warm ischaemia between cessation of the blood supply and harvesting followed by varying periods of cold ischaemia whilst they are stored for transport to the recipient. They are then rapidly reperfused with fully oxygenated whole blood as soon as the vascular pedicle is reconstructed. The combination of insults often results in poor renal function and death of tubular cells soon after engraftment. Recovery of adequate organ function if it occurs at all, may take up to several weeks.

Several investigations have provided circumstantial evidence that oxygen-derived free radicals are involved in this damaging sequence (1,2,3,4). These reactive species initiate many damaging processes which include peroxidation of membrane-bound polyunsaturated fatty acids, which are essential components of the phospholipid bilayer, so that functional integrity of plasma and organelle membranes may be lost (5). From our own studies of cold-stored organs using NMR spectroscopy (6), and those of others (7), it is known that during hypoxia adenine nucleotide levels rapidly fall and there is an accumulation of protons such that pH falls to 6.8 within 6 hours of simple refrigeration. This has several direct and indirect consequences: in this reducing intracellular milieu, iron may be released from ferritin and become accessible as a catalyst of free radical reactions; altered Ca^{++} homeostasis leading to elevated intracellular cytosolic Ca^{++} concentrations may cause release of free fatty acids (particularly polyunsaturated acids) from the membrane under the influence of phospholipase A_2; and increased hypoxanthine resulting from breakdown of adenine nucleotides is utilised as a substrate for xanthine oxidase (converted from xanthine dehydrogenase by proteolysis with the catalytic aid of Ca^{++}) to produce superoxide anion and thence hydroxyl radicals - perhaps of greatest import during reperfusion immediately after revascularisation (4).

Raised concentrations of cytoplasmic Ca^{++} have been linked to irreversible cell injury in many systems (8) but whether this is the common denominator <u>initiating</u> cell death or merely a post-mortem <u>result</u> of cell death is difficult to determine. However, some studies do suggest that Ca^{++} must be a prime suspect as trigger for a catastrophic cascade of events. For example, the presence of Ca^{++} potentiated oxygen free radical induced damage to isolated renal mitochondria <u>in vitro</u> (5) and Ca^{++} has also been reported to modulate free radical damage to plasma membranes in isolated hepatocytes (9). As the gradient between high extracellular Ca^{++}

(10^{-3}M) and low cytosolic concentrations (10^{-7}M) is normally maintained by membrane-bound ATP-dependent pumps which remove Ca^{++} from the cell and sequester excess cytosolic Ca^{++} in mitochondria and endoplasmic reticulum, it would not be surprising if this mechanism failed as ATP levels are depleted during ischaemia. Indeed, altered Ca^{++} homeostasis has been demonstrated in tissues subjected to warm ischaemic insults (10) and increased free Ca^{++} levels observed in isolated cells exposed to anoxia (11)

In earlier experiments using cold stored rabbit kidneys we have demonstrated in our in vitro test system that lipid peroxidation markers such as Schiff bases and thiobarbituric acid reactive material (TBAR)increase significantly after cold storage. Furthermore, the degree of biochemical derangement correlates quite closely with the morphological change and physiological dysfunction associated with the particular flush solution and the length of cold ischaemic time (2). We have also previously shown that extracellular Ca^{++} influx and intracellular Ca^{++} redistribution due to mitochondrial dysfunction are important mediators of oxidative damage to rabbit kidneys subjected to cold ischaemia (12).

In the present study, we have extended those experiments to investigate the possibility that Ca^{++} activated phospholipases and proteolysis to yield xanthine oxidase are important during cold ischaemia and in initiating or propagating lipid peroxidation. Rabbit kidneys were flushed and stored for 24 hr at 0°C in isotonic saline which we know from our previous studies to be a poor storage medium and which will result in a significant level of oxidative stress to the kidney within this short storage time (2). To this model system were added (a) dibucaine which inhibits Ca^{++}-activated phospholipase A_2 by competing for Ca^{++} binding sites; (b) A23187 an ionophore which renders membranes permeable to Ca^{++}; (c) dibucaine and A23187 together; (d) allopurinol which inhibits xanthine oxidase activity; and (e) allopurinol and A23187 together.

MATERIALS AND METHODS

Six groups of adult New Zealand White rabbits (average weight 3 kg) were anaesthetised by i.m. injection of fentanyl-fluanisone (Hypnorm) at 0.2ml/kg followed by diazepam at 1.5mg/kg by slow i.v. injection. Oxygen flowing at 2L/min was supplied by face mask throughout the operations. Both kidneys were removed as described previously (3). The renal artery of each kidney was cannulated and the organs flushed either with 30 mls of sterile isotonic 0.9% sodium chloride solution (saline) or saline to which

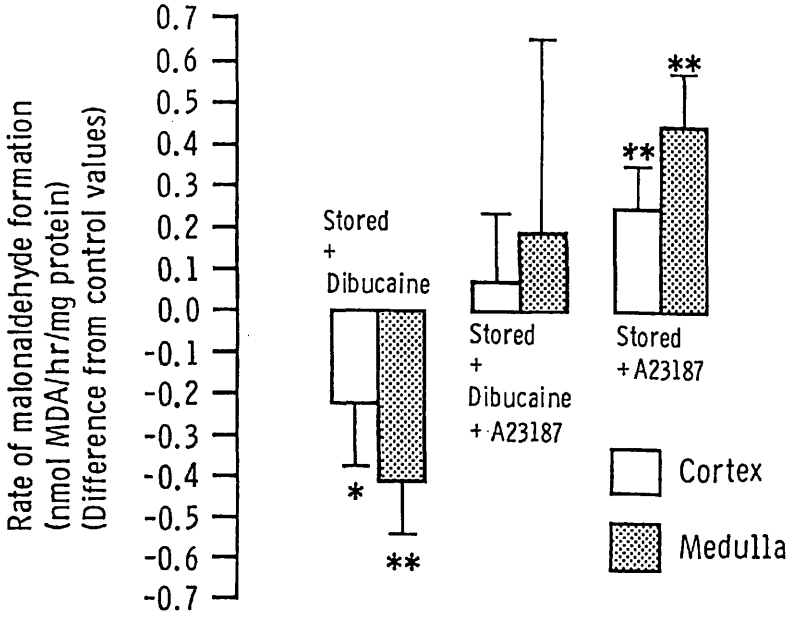

FIGURE 1: The rate of formation of TBA-reactive material in kidney homogenates flushed and stored for 24 hours at $0^{0}C$ in isotonic saline containing dibucaine (250μM). Values are expressed as the mean difference from controls (stored in saline only) ± 95% confidence interval (n=6). Control values: cortex: 1.25 ± 0.81 nmol malonaldehyde/hr/mg protein; medulla: 1.75 ± 1.01 nmol malonaldehyde/hr/mg protein.

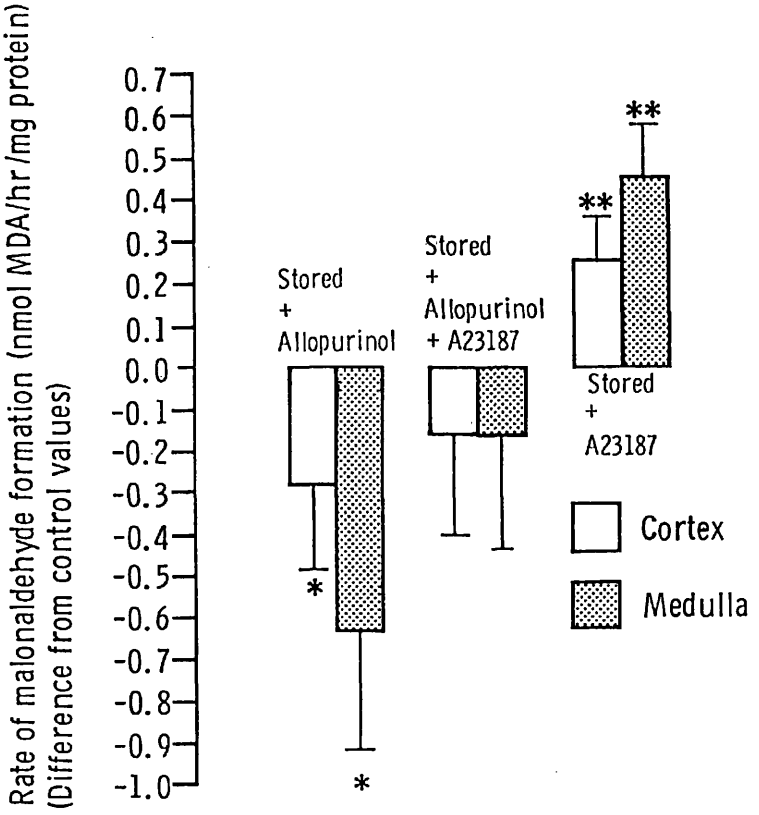

FIGURE 2: The rate of formation of TBA-reactive material in kidney homogenates flushed and stored for 24 hours at $0^{o}C$ in isotonic saline containing allopurinol (5mM) or allopurinol (5mM) and A23187 (10μM). Values are expressed as the mean difference from controls (stored in saline only) ± 95% confidence interval (n=6). Control values: Cortex 1.25 ± 0.81 nmol malonaldehyde/hr/mg protein; medulla: 1.75 ± 1.01 nmol malonaldehyde 1hr/mg protein.

had been added A23187 (10μM), dibucaine (250μM), dibucaine (250μM) plus A23187 (10μM), allopurinol (5mM) or allopurinol (5mM) plus A23187 (10μM). The kidneys were then placed in a beaker containing 60 ml of identical flush solution and stored on ice at 0°C for 24 hr. In each rabbit, one kidney was flushed and stored with the treated saline whilst the contralateral organ was flushed with saline and stored under identical conditions thus providing paired controls.

After 24 hr storage, the kidneys were immediately dissected into cortex and medulla, each of which was then homogenised separately in phosphate-saline buffer (pH7.4) and incubated at 37°C for 60 min in a shaking water bath for the in vitro test described previously (3). The rate of lipid peroxidation was determined by the measurement of thiobarbituric acid reactive material (TBAR) formation, monitoring for a fluorescence maximum at 553 nm when excited at 515 nm (3). Results were corrected for variability in homogenate protein concentration by the method of Lowry (13).

Statistical analysis was performed using the Students paired t-test.

RESULTS AND DISCUSSION

Cold storage of kidneys in isotonic saline solution for 24 hr results in elevated levels of TBARs in both the cortex and medulla when compared with fresh unstored kidneys (3). In these experiments, addition of the calcium ionophore A23187 to the flush solution lead to significant ($p<0.005$) increases (Fig 1) over the stored kidneys; hence, rendering cellular membranes permeable to Ca^{++} had accelerated the rate of lipid peroxidation. Conversely, when dibucaine was added to the flush solution alone, the formation of TBARs was significantly ($p<0.005$) decreased (Fig 1). When kidneys were stored in the presence of both agents, the TBARs were only slightly elevated over untreated stored controls (Fig 1); thus, dibucaine had effectively cancelled out the increase in lipid peroxidation induced by A23187.

In the second set of experiments designed to test the effect of the xanthine oxidase inhibitor allopurinol during cold ischaemia, the data again suggested a link between increased Ca^{++} and increased peroxidation. Whereas addition of allopurinol alone to the saline flush significantly ($p<0.05$) inhibited rises in TBARs and addition of A23187 alone significantly ($p<0.005$) increased TBARs, these effects were virtually cancelled out if the two agents were added to the solution together (Fig 2).

There are several mechanisms by which increased cytosolic Ca^{++} levels

could potentiate free radical damage to cell membranes. Phospholipases are activated by Ca^{++} and remove fatty acids from membranes by a process which does not require oxygen or energy and could therefore function during the storage period at low temperatures. However, both the β-oxidation of fatty acids and their reincorporation back into phospholipids do require ATP and will therefore be diminished during anoxia. Hence, there is likely to be a build-up of free fatty acids which may be potentially damaging per se because of their detergent properties. In particular, an increase in free arachidonic acid could increase enzyme linked peroxidation of this important substrate and hence alter the eicosanoid balance in favour of vasoconstriction and thrombogenesis in the vascular bed. In addition, lysophosphatides (the products of enzymatic degradation of phospholipids) will accumulate in the membrane and disturb its configuration, (14), possibly rendering membrane lipids more susceptible to peroxidation. Consistent with this scenario was the finding in this study that dibucaine, an inhibitor of Ca^{++}-dependent phospholipase A_2, diminished the formation of TBARs resulting from cold storage of rabbit kidneys for 24 hr in saline solution and also prevented the increase in oxidative damage produced by addition of A23187.

Increased cytosolic Ca^{++} levels may also enhance free radical production and thence lipid peroxidation by activating proteases which convert the enzyme xanthine dehydrogenase to xanthine oxidase (15). This is likely to be most important during reperfusion; after exposure to molecular oxygen, this form of the enzyme converts hypoxanthine, a product of adenine nucleotide degradation which accumulates during ischaemia to xanthine, and the superoxide anion ($O_2^{-}\cdot$) is produced. In the presence of transition metal catalysts, $O_2^{-}\cdot$ can be converted to highly reactive species such as hydroxyl radical ($OH\cdot$) and iron-complexed molecules which then initiate lipid peroxidation. The finding in the present experiments that allopurinol added to saline flush inhibited lipid peroxidation and was even able to inhibit the highly significant rises expected when the Ca^{++} ionophore was added to the system, provides some circumstantial evidence that Ca^{++} and xanthine oxidase are important mediators of lipid peroxidation even without reperfusion. In the past it has been assumed that allopurinol added to a cold flush solution would not be accessible to intracellular compartments or have enough time to modulate cell metabolism. However, our present data suggests that allopurinol might well prove useful if added to flush solutions for clinical use, and indeed allopurinol is a constituent of the recently-formulated UW storage solution, which contains

lactobionate and raffinose impermeants to suppress hypothermia-induced cell swelling (16).

Allopurinol has been claimed to improve the post-storage function of porcine kidneys when administered to the recipient animal (17) and to protect ischaemic rat kidneys (18) if given to the recipient after warm ischaemia but before reperfusion. In other studies, however, neither in dogs (19) nor in rats (20) did allopurinol prove beneficial in renal ischaemia. More recently, it was shown to prevent erythrocyte accumulation otherwise observed after ischaemia (21) but had little effect on subsequent renal function (22). In terms of function and survival after ischaemic insult, the value of allopurinol is still in question. In some recent experiments to evaluate it in warm ischaemic kidneys, we found that the agent undoubtedly inhibited rises in markers of lipid peroxidation if administered to the rabbits before reperfusion (unpublished data) but we have not tested its value in terms of subsequent renal function. We know of no previous studies to assess the use of dibucaine in either warm or cold ischaemic organs.

Interest in organ storage has recently been revived with the better results of transplantation worldwide of organs other than kidneys. Meaningful clinical improvements are unlikely until the disturbances in cell biology associated with warm and cold ischaemia are elucidated. From the present and previous studies, it is concluded that Ca^{++} redistribution, which results in increased cytosolic free calcium levels, is an important factor in mediating oxidative membrane damage in cold stored kidneys. It appears likely that elevated Ca^{++} levels and free radicals act synergistically to cause deterioration under cold storage conditions. It may therefore be possible in future to afford significant protection to stored organs by chelating extracellular Ca^{++}, preventing its entry into cells by Ca^{++}-antagonists and inhibiting phospholipase A_2 activity in conjunction with other agents which inhibit free radical production or scavenge free radical intermediates. However, much further work is necessary to evaluate functional benefits resulting from such control of oxidative damage during storage and reperfusion.

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OXIDATIVE DAMAGE TO KIDNEY MEMBRANES DURING COLD ISCHEMIA

EVIDENCE OF A ROLE FOR CALCIUM

L. A. COTTERILL, J. D. GOWER, B. J. FULLER,[1] AND C. J. GREEN

Section of Surgical Research, MRC Clinical Research Centre, Northwick Park Hospital, Harrow, Middlesex; and Academic Department of Surgery, Royal Free Hospital Medical School, London, NW3 2QG, England

Storage of rabbit kidneys at 0°C for periods of 72 hr after flushing with hypertonic citrate solution, or 24 hr when flushed with isotonic saline, resulted in significant increases in Schiff base and thiobarbituric acid-reactive markers of lipid peroxidation in vitro. The extent of lipid peroxidation was not significantly altered by addition of verapamil (100 μM), a Ca^{++} channel blocking agent, or calcium 1 mM ($CaCl_2$) to the HCA storage solution. In contrast, verapamil significantly reduced the extent of lipid peroxidation in kidneys stored in saline solution, and a significant increase in oxidative damage occurred when $CaCl_2$ was added to this storage solution. Thus the extent of lipid peroxidation in kidneys stored in saline was significantly mediated by extracellular Ca^{++}, whereas in HCA this was probably chelated by the large excess of citrate (55 mM) in this medium that prevented, or at least slowed, its entry into the renal cells. Lipid peroxidation was however significantly increased in kidneys stored in both HCA and saline solutions by addition of the calcium ionophore A23187 (10 μM) or the polysaccharide dye ruthenium red (5 μM) that inhibits mitochondrial uptake of Ca^{++}. This strongly suggested that altered intracellular Ca^{++} homeostasis during the storage period played an important role in the development of oxidative damage to kidneys stored in both these media.

Although the techniques available for simple cold storage of kidneys between harvesting and transplantation have improved in the past 10 years, safe preservation of other organs is still limited clinically to a few hours. Even in renal transplantation, immediate primary function is not obtained in a high percentage of kidneys stored for more than 24 hr, and storage damage provides an additional hazard to the nephrotoxicity of cyclosporine (*1*). It is hoped that the introduction of a new washout solution, recently reported (*2*) to provide better protection against cold ischemic damage in several experimental models (*3–5*), will improve the clinical situation; nevertheless, it is still important that the mechanisms of cold storage damage are identified if further improvements are to be made.

Some evidence suggests that following ischemia several harmful processes, including peroxidation of membrane-bound polyunsaturated fatty acids that are essential components of phospholipid bi-layers, are initiated by oxygen-derived free radicals (*6*) and result in the loss of functional integrity of plasma and organelle membranes (*7*). The availability of catalytic iron is thought to be essential to initiate and propagate the chain reactions involved in peroxidation; raised cytosolic Ca^{++} concentrations may be involved in a more indirect way. There is already clear evidence for the role of iron during ischemia and reperfusion injury, and administration of the potent iron-chelating agent desferrioxamine has been shown to inhibit ischemic damage in several tissues (*8–10*). However, the importance of Ca^{++} is less well established; the presence of Ca^{++} potentiates oxygen-derived free radical-induced damage in isolated renal mitochondria in vitro (*7*) and is involved in free radical-mediated damage to plasma membranes, for example in isolated hepatocytes (*11*). Furthermore, investigations in a number of cellular systems have linked irreversible cell injury with an increase in cytosolic Ca^{++} concentrations (*12*). Normally, in intact cells the gradient between relatively high (10^{-3} M) extracellular concentrations and a low (10^{-7} M) cytosolic concentration is maintained by membrane-bound ATP-dependent pumps that remove Ca^{++} from the cytosol and sequester excess Ca^{++} in mitochondria and endoplasmic reticulum. During ischemia on the other hand, ATP levels are rapidly depleted (*13*), and previous reports have demonstrated altered Ca^{++} homeostasis in tissues subjected to warm ischemic insults (*14*) and increased cytosolic Ca^{++} levels in isolated cells exposed to anoxia (*15*).

The present experiments were designed to examine the role of Ca^{++} in mediating oxidative damage to membranes during cold storage of rabbit kidneys and to test the possibility that deliberate manipulation of Ca^{++} homeostasis under these conditions might affect biochemical markers of lipid peroxidation. Rabbit kidneys were flushed and stored at 0°C in solutions containing various agents that affect Ca^{++} movements, and the extent of lipid peroxidation was measured following in vitro incubation of homogenates of the stored kidneys.

In the first set of experiments, rabbit kidneys were flushed with hypertonic citrate (HCA)* solution and stored in this clinically appropriate storage medium for 72 hr. With this solution, 24-hr storage results in no significant increase in markers of lipid peroxidation, but increases do become evident after storage for 72 hr or longer (*16*). In the second set of experiments, rabbit kidneys were flushed with 0.9% NaCl solution (saline) and stored for 24 hr in this poor preservation medium, a period previously shown to produce high levels of lipid peroxidation (*16*).

Verapamil, a Ca^{++}-channel blocker, was added to the solutions with the intention of inhibiting any influx of extracellular Ca^{++} through voltage-operated channels during cold ischemia;

[1] Academic Department of Surgery, Royal Free Hospital Medical School, Pond Street, London, NW3 2QG, England.

* Abbreviations: HCA, hypertonic citrate; SC, Schiff bases; TBAR, thiobarbituric acid reactive material.

$CaCl_2$ (1 mM) was added to the solutions to investigate the importance of extracellular Ca^{++} levels; the ionophore A23187 was used to render both plasma and intracellular organelle membranes permeable to Ca^{++}; and the effect of the polysaccharide dye ruthenium red, which inhibits mitochondrial Ca^{++} uptake, was also investigated. Verapamil was also added direct to the homogenates of kidneys stored in saline or HCA solution, to test the possibility that any changes in fluorescent markers of lipid peroxidation observed after incubation could be due to direct antioxidant properties of traces of this drug remaining in the kidneys from the storage period.

MATERIALS AND METHODS

Materials. Verapamil hydrochloride was purchased from Abbot Laboratories Ltd, Kent, U.K., and the Ca^{++} ionophore A23187 and ruthenium red from Sigma Chemical Co., Ltd., Dorset, U.K. Other chemicals were Analar grade except chloroform and methanol, which were special spectral grade.

Surgical technique. Eleven groups (n=6) of adult New Zealand white rabbits of either sex and weighing 3–3.5 kg were anesthetised by i.m. injection of fentanyl-fluanisone (Hypnorm) at 0.2 ml/kg followed by diazepam at 1.5 mg/kg by slow i.v. injection. Oxygen flowing at 2 L/min was supplied by face mask throughout the operations.

The abdomen was opened along a mid-line incision, and both kidneys were exposed and, together with their vascular pedicle and ureter, were dissected free ready for harvesting. At this stage, a brisk diuresis was induced by injecting the rabbits with 50 ml of isotonic saline and frusemide at 1.0 mg/kg i.v. Both kidneys were removed 10 min later after ensuring that any spasm in the vessels had disappeared and that the organs were well perfused. The renal artery of each kidney was cannulated, and the organs were flushed (constant pressure 100 mm Hg) either with 30 ml of cold (4°C) sterile 0.9% w/v NaCl (saline) or with HCA solution. The kidneys were placed in sterile beakers containing 60 ml of identical flush solution and stored at 0°C surrounded by ice for either 24 hr (saline) or 72 hr (HCA). When kidneys were flushed and then stored in solutions containing verapamil (100 μM), $CaCl_2$ (1 mM), A23187 (10 μM), or ruthenium red (5 μM), the contralateral kidneys were stored under identical conditions except that these agents were not added to the flush solutions, thus providing paired controls. Fresh control kidneys were obtained under identical conditions except that the organs were flushed but not stored.

Biochemical assays. Harvested kidneys were divided into cortex and medulla, each of which were weighed and then homogenized in phosphate saline buffer at pH7.4 and incubated at 37°C in open vessels with mechanical shaking for 60 min. The production of markers of lipid peroxidation in the homogenates was determined as described previously (*17*) by monitoring for lipid soluble Schiff bases (SB) fluorescing at a maxima between 400 and 450 nm when excited at 360 nm and for thiobarbituric acid reactive material (TBAR) fluorescing at 553 nm (excited at 515 nm). The latter determinations were calibrated using malonaldehyde tetraethylacetal. All results were corrected for variability in homogenate protein concentration by the method of Lowry (*18*).

The Ca^{++} chelating ability of the HCA solution itself was determined by adding a range of known amounts of $CaCl_2$ and measuring the resultant "free" Ca^{++} concentration using an ion-selective electrode (Orion model 811) calibrated with calcium-EGTA buffers in the range of 10^{-8}–10^{-4} M.

Statistical analysis was performed using Student's paired *t* test.

Experimental protocol. Each group consisted of 6 rabbits. In groups 2–7, paired controls were provided by flushing the contralateral kidneys with saline or HCA, to which no Ca^{++} mediators had been added, and storing them under identical conditions.

Group 1: Control kidneys were flushed with 30 ml of HCA solution but not stored

Group 2: Kidneys were flushed with 30 ml of HCA solution to which verapamil (100 μM) had been added and were stored for 72 hr at 0°C

Group 3: As in group 2 except that $CaCl_2$ (1 mM) was added to the HCA flush solution instead of verapamil

Group 4: As in group 2 except that A23187 (10 μM) was added to the HCA flush solution instead of verapamil

Group 5: As in group 2 except that ruthenium red (5 μM) was added to the HCA flush solution instead of verapamil

Group 6: Kidneys were flushed with 30 ml of 0.9% w/v NaCl solution to which verapamil (100 μM) had been added and then were stored for 24 hr at 0°C

Group 7: As in group 6 except that $CaCl_2$ (1 mM) was added to the saline solution instead of verapamil

Group 8: As in group 6 except that A23187 (10 μM) was added to the saline flush solution instead of verapamil

Group 9: As in group 6 except that ruthenium red (5 μM) was added to the saline flush solution instead of verapamil

Group 10: Kidneys were flushed with 30 ml of 0.9% w/v NaCl solution and stored for 24 hr at 0°C; verapamil (100 μM) was added in vitro to homogenates of these kidneys after storage

Group 11: Kidneys were flushed with 30 ml of HCA and stored for 72 hr at 0°C; verapamil (100 μM) was added in vitro to homogenates of these kidneys after storage

RESULTS

Cold storage of rabbit kidneys for 72 hr in HCA resulted in significantly ($P<0.005$) elevated levels of SB and TBARs markers of lipid peroxidation in homogenates of both cortex and medulla compared to fresh kidneys (Figs. 1 and 2). The prevention of extracellular calcium entry into kidney cells through

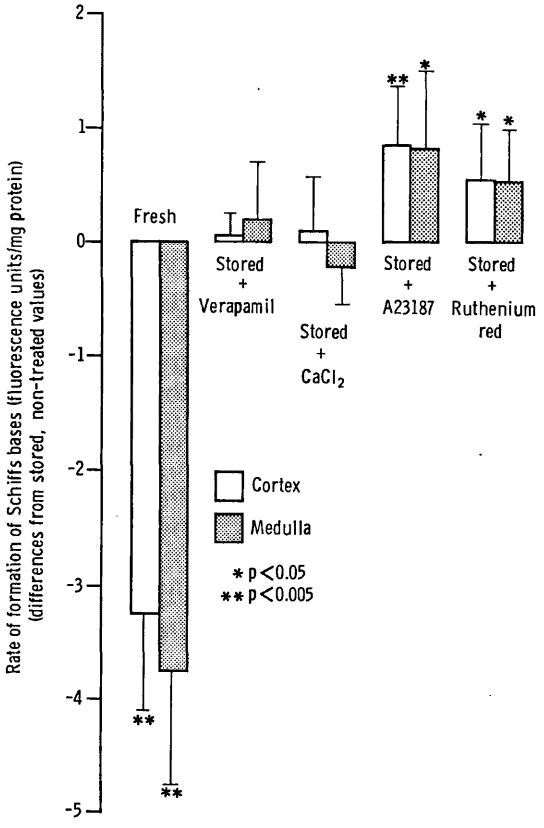

FIGURE 1. The rate of Schiff base formation in homogenates of cortex and medulla from kidneys flushed and stored for 72 hr at 0°C in hypertonic citrate solution containing verapamil (100 μM), $CaCl_2$ (1 mM), A23187 (10 μM), and ruthenium red (5 μM). Values are expressed as the mean difference from stored, nontreated kidneys ±95% confidence interval (n=6). Control values include cortex, 2.06±0.78 fluorescence U/hr/mg protein; and medulla, 2.80±0.73 fluorescence U/hr/mg protein.

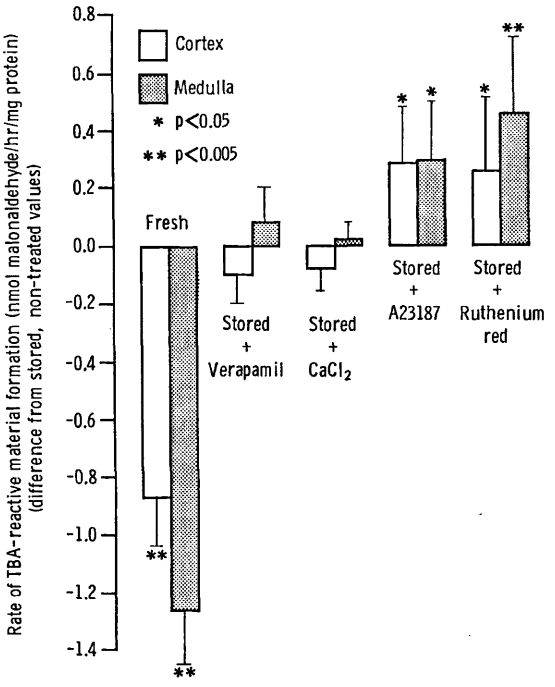

FIGURE 2. The rate of formation of TBA-reactive material in homogenates of cortex and medulla from kidneys flushed and stored for 72 hr at 0°C in hypertonic citrate solution containing verapamil (100 μM), $CaCl_2$ (1 mM), A23187 (10 μM), and ruthenium red (5 μM). Values are expressed as the mean difference from stored, nontreated kidneys ±95% confidence interval (n=6). Control values include cortex, 1.04±0.28 nmol malonaldehyde/hr/mg protein; and medulla, 5.40±0.33 nmol malonaldehyde/hr/mg protein.

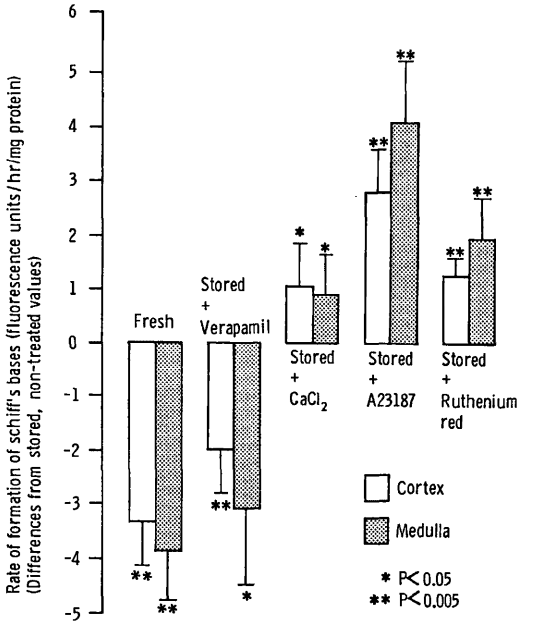

FIGURE 3. The rate of Schiff base formation in homogenates of cortex and medulla from kidneys flushed and stored for 24 hr at 0°C in isotonic saline containing verapamil (100 μM), $CaCl_2$ (1mM), A23187 (10 μM), and ruthenium red (5 μM). Values are expressed as the mean difference from stored, nontreated kidneys ±95% confidence interval (n=6). Control values include cortex, 4.6±1.21 fluorescence U/hr/mg protein; and medulla, 5.70±1.45 fluorescence U/hr/mg protein.

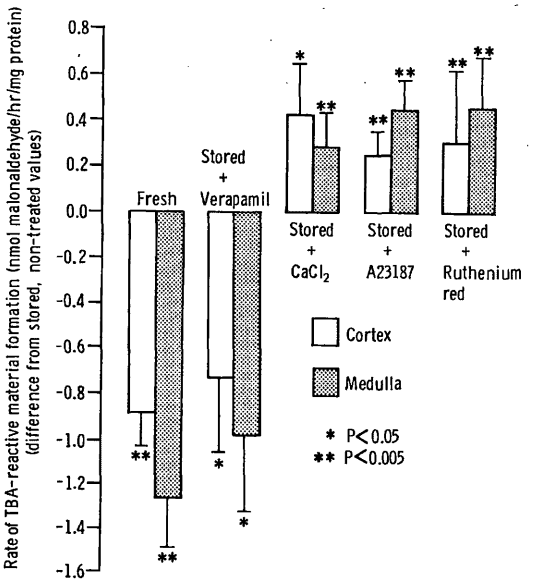

FIGURE 4. The rate of formation of TBA-reactive material in homogenates of cortex and medulla from kidneys flushed and stored for 24 hr at 0°C in isotonic saline containing verapamil (100 μM), $CaCl_2$ (1 mM), A23187 (10 μM), and ruthenium red (5 μM). Values are expressed as the mean difference from stored, nontreated kidneys ± 95% confidence interval (n=6). Control values include cortex 1.25± 0.81 nmol malonaldehyde/hr/mg protein; and medulla, 1.75±1.01 nmol malonaldehyde/hr/mg protein.

voltage-operated channels by the addition of verapamil to the HCA flush and storage solution (group 2) did not decrease the adverse rise in markers of lipid peroxidation following 72-hr storage (Figs. 1 and 2). Furthermore, increasing the extracellular calcium concentration by addition of $CaCl_2$ (1mM) to the HCA solution (group 3) had no significant effect on the extent of lipid peroxidation in either the cortex or medulla of the stored organs (Figs. 1 and 2). These results therefore suggested that extracellular calcium did not play a role in mediating oxidative damage to kidneys when stored in HCA.

In complete contrast to the above results, the addition of verapamil to the saline storage solution (group 6) very effectively inhibited the rise in SB and TBARs in both the cortex and medulla observed after 24 hr storage in this medium (Figs. 3 and 4), and the level of lipid peroxidation markers were very similar to those found in fresh kidneys. The addition of verapamil directly to kidney homogenates (Table 1) had no effect on lipid peroxidation whether from kidneys stored in saline (group 10) or in HCA (group 11). Thus it seems likely that this agent was exerting its effect on the intact cells of the organ during the storage period. Addition of $CaCl_2$ (1 mM) to the saline flush and storage solution (group 7) resulted in levels of lipid peroxidation that were significantly greater than in the kidneys stored for 24 hr in saline alone (Figs. 3 and 4). Thus it appears that extracellular calcium was mediating, to a significant extent, oxidative damage to kidneys when stored in saline solution.

We hypothesized that this important difference between these 2 storage solutions may be due to the ability of the large excess of citrate (55 mM) in HCA to chelate Ca^{++} during the storage period thus preventing, or at least slowing, the entry of extracellular calcium into the renal cells. We therefore measured free Ca^{++} ion concentrations by means of an ion-specific electrode in HCA to which various amounts of Ca^{++} were added to the solution. As can be seen in Figure 5, the Ca^{++} buffering capacity of HCA proved highly effective; for example, the free Ca^{++} concentration was only 1 μM in the presence of 100 μM total Ca^{++} and only 10 μM when 1 mM was added (Fig. 5).

TABLE 1. Measurements of biochemical indices of lipid peroxidation (Schiff bases and TBA-reactive material) in homogenates of cortex and medulla from kidneys stored at 0°C in isotonic saline for 24 hr or hypertonic citrate solution for 72 hr after addition of verapamil (100 μM) direct to homogenates after storage

	Experimental group (n = 6)	Section of kidney	Formation of lipid peroxidation products (change in fluorescence intensity/hr/mg protein) mean ± SD			
			Schiff bases		TBA-reactive material	
			Control	Treated	Control	Treated
10	100 μM verapamil added to the homogenate after storage in saline	Cortex	2.22±0.50	1.82±0.43	3.52±0.64	3.18±0.88
		Medulla	2.28±0.77	2.65±1.93	3.06±0.67	3.50±0.26
11	100 μM verapamil added to the homogenate after storage in HCA	Cortex	2.81±1.08	3.04±1.29	3.46±0.88	3.65±1.45
		Medulla	2.23±0.39	1.92±0.25	4.27±1.35	4.15±1.33

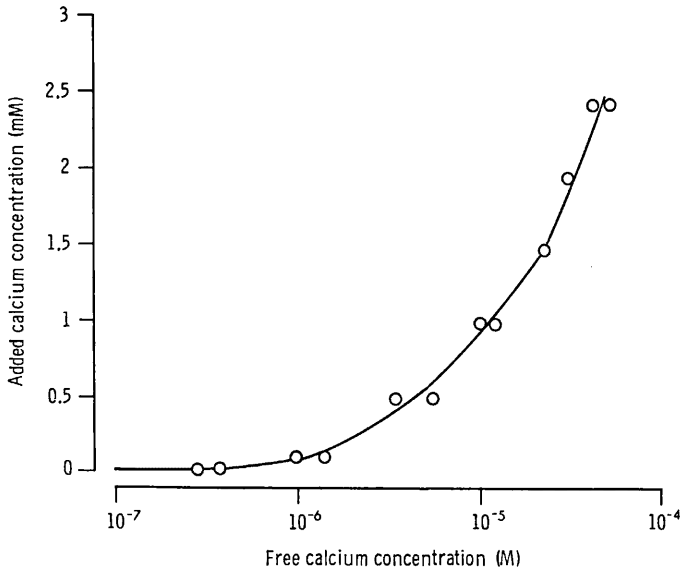

FIGURE 5. Relationship between the added calcium concentration and the free calcium concentration in hypertonic citrate solution (83 mM Na^+, 80 mM K^+, 40 mM Mg^{2+}, 55 mM citrate, 186 mM mannitol).

Further experiments were then carried out to investigate the effect of the calcium ionophore A23187 that renders both plasma membranes and intracellular organelles permeable to Ca^{++}. Addition of this agent to the flush and storage solutions resulted in increased lipid peroxidation markers in both cortex and medulla to levels significantly greater than those observed in kidneys stored for either 72 hr in HCA (Figs. 1 and 2) or 24 hr in saline (Figs. 3 and 4). This demonstrated that redistribution of intracellular Ca^{++} by A23187 was able to elevate oxidative damage to membranes of kidneys stored in HCA in the absence of any effect involving extracellular Ca^{++} entry. One important organelle that sequesters Ca^{++} and keeps cytosolic calcium levels low is the mitochondrion, and this uptake mechanism is inhibited by ruthenium red. As addition of this agent to the flush and storage solutions significantly increased markers of lipid peroxidation in the medulla and cortex of organs stored in either HCA for 72 hr (Figs. 1 and 2), or saline for 24 hr (Figs. 3 and 4), it could be concluded that removal of excess cytosolic Ca^{++} by mitochondria constituted an important protective mechanism during hypothermic storage.

DISCUSSION

Physiologic deterioration of organs as a result of hypothermic storage is likely to be the result of a number of adverse biochemical processes associated with ischemia. We have previously demonstrated that the extent of lipid peroxidation initiated by free radicals is high when organs are stored in a poor preservation medium (saline) and accumulates much more slowly when organs are stored in a good medium such as HCA (*16*). This investigation extends those earlier studies by demonstrating that altered Ca^{++} homeostasis plays a significant role in mediating free radical-induced damage.

Extracellular fluid is rich in Ca^{++} ions (10^{-3} M), and there is a highly regulated gradient across the cell membrane, which is poorly permeable to Ca^{++}, such that cytosolic Ca^{++} levels are maintained at low levels (10^{-7} M). Normally, Ca^{++} may only enter cells via specific voltage-operated channels or receptor-operated channels and is extruded from the cell by a Na^+/Ca^{++} exchange mechanism and ATP-driven pumps. If this permeability barrier is breached, for example by a Ca^{++} ionophore such as A23187, then any extracellular free Ca^{++} present will flood into the cell. Increased cytosolic Ca^{++} levels are associated with many biochemical changes as well as altered cellular morphology such as blebbing, and many studies have demonstrated a close temporal association between elevated cell Ca^{++} levels, severe histologic damage, and cell death, strongly suggesting a causal connection between these events (*19*).

One important consequence of raised cytosolic Ca^{++} levels is the activation of phospholipases (*20, 21*) that remove fatty acids, particularly arachidonic acid, from membranes. These enzymes do not require O_2 or energy so they are likely to be active during the storage period. However, fatty acid catabolism as well as the reincorporation of fatty acids back into phospholipids do require ATP, and both processes will therefore be diminished during hypoxia. Hence, free fatty acids are likely to accumulate leaving increased residual lysophosphatides in the membrane, leading in turn to a disturbed bilayer configuration. It is possible that this altered structure renders the membrane more susceptible to free radical attack; furthermore, free polyunsaturated fatty acids, unprotected by membrane-bound antioxidants such as vitamin E, will also be more prone to peroxidation. In addition, Ca^{++} regulates a number of proteases of which the proteolytic conversion of xanthine dehydrogenase to xanthine oxidase may be important during storage (*22*). Accumulation of xanthine oxidase can later increase the risk of generating free-radical species during reperfusion because of the interaction of breakdown products of adenine nucleotide metabolism with incoming molecular oxygen. Thus altered Ca^{++} homeostasis and the production of reactive free radicals could act synergistically to bring about cell death.

In this study, addition of the voltage-operated channel-block-

ing agent verapamil to the saline flush solution resulted in significant inhibition of the lipid peroxidation experienced in controls without the drug. Since addition of verapamil directly to kidney homogenates had no effect on lipid peroxidation, we conclude that the protective effect of this agent was due to its ability to prevent influx of extracellular Ca^{++} into the cytosol of intact kidney cells during the storage period. The effect of verapamil on saline-stored organs suggests that a significant amount of extracellular Ca^{++} remains in the tissue after flushing with a storage solution; this is not surprising considering that a single-passage vascular flush is unlikely to gain access to all the extracellular spaces. Deliberately raising the concentration of extracellular Ca^{++} by addition of $CaCl_2$ (1 mM) to the saline flush solution resulted in further elevations in the extent of lipid peroxidation, demonstrating that the extent of oxidative damage was dependent on the concentration of extracellular Ca^{++} when kidneys were stored in saline.

Verapamil had no effect on kidneys stored in HCA that demonstrates a highly specific difference between the way kidneys stored in HCA and saline respond to extracellular Ca^{++}. We conclude that protection against extracellular Ca^{++} entry by HCA is due to the ability of the large excess of citrate (55 mM) in this medium to chelate free Ca^{++} ions. Measurements by an ion-specific electrode, of free calcium concentrations in HCA to which various amounts of calcium had been added confirmed the very effective buffering capacity of this medium. Thus entry of free calcium ions through voltage-operated channels will be prevented or at least slowed during the storage period, and it is likely that the addition of $CaCl_2$ (1 mM) had no effect on peroxidative damage as it was sequestered by the citrate leaving only very low amounts of free metal ions in the extracellular medium.

It is interesting that Shapiro and co-workers have demonstrated that addition of verapamil (2.5 μM) to Collins' C_2 flush solution, which does not contain a Ca^{++}-buffering system such as citrate, significantly improved inulin clearance, urine flow rate, and sodium reabsorption in kidneys after 8 hr of simple storage at 0°C followed by isolated perfusion (*23*); since addition of verapamil to the perfusate *after* the ischemic period had no effect, it was concluded that verapamil protected the organ during the ischemic period itself. Other experiments in which verapamil reduced warm ischemic damage did not attempt to determine whether it worked primarily by preventing Ca^{++} influx into cells or through its potent diuretic, vasodilator, or antiplatelet activities (*24–26*).

Intracellular organelles are also important in maintaining Ca^{++} homeostasis. They contain over 90% of intracellular Ca^{++} and provide "sinks" that sequester excess Ca^{++} from the cytosol. Lipid peroxidation of mitochondria (*27*) and the endoplasmic recticulum (*28*) impairs the Ca^{++}-buffering capacity of these structures. As organelles can also be rendered permeable to Ca^{++} by the ionophore A23187, we added this agent to the flush and storage solutions, and assessed peroxidative damage after storage. Both in the HCA- and saline-flushed kidneys this resulted in significantly increased levels of lipid peroxidation when compared to organs stored in HCA or saline alone. This therefore demonstrated that detrimental redistribution of intracellular Ca^{++} can occur during cold storage even in the absence of extracellular free Ca^{++} ions, as in the case of organs stored in HCA, and this results in an increase in oxidative damage to the tissues.

There are several ways in which periods of warm or cold ischemia may bring about an altered Ca^{++} homeostasis. Depletion of O_2 accompanied by falling ATP levels will reduce the cellular ability to extrude Ca^{++} by ATP-dependent pumps, as well as the ability of organelles to sequester Ca^{++} by energy-dependent shuttles, especially at low temperature (*29*). In addition, a falling pH will decrease the affinity of the 10 μM of Ca^{++} normally complexed to soluble substances such as ATP, citrate, and glutamate and will hence release Ca^{++} as free ions (*29*). The movement of other ions is also seriously altered during ischemia and may induce pathologic fluxes in Ca^{++}. In particular, during storage in saline, cells would exchange much of their intracellular K^+ for the Na^+ present in high concentrations outside the cell. Having entered the cell, the Na^+ may then exchange with Ca^{++} present in mitochondria by a passive mechanism that would result in increased free Ca^{++} in the cytosol. In addition, during cold ischemia as the temperature of cells falls, membrane fluidity is diminished, and molecular packing faults developing in the phospholipid bilayer could allow abnormal Ca^{++} movements to occur. Swelling of organs that can occur during storage in saline may also contribute to this effect.

It has previously been demonstrated that cytosolic Ca^{++} levels increase in cultured kidney cells as a result of anoxia and that these levels correlate well with falling ATP concentrations (*15*); in that study, it was concluded that rising cytosolic Ca^{++} was mostly due to release of Ca^{++} from intracellular depots, particularly mitochondria (*15*). In other studies, cellular Ca^{++} in kidney cortex slices incubated at 4°C increased by 50% after only 1 hr of hypoxia, suggesting that Ca^{++} overload commences soon after depletion of energy supplies in tubular cells (*30*). Several studies have demonstrated that anoxic cells take up considerable amounts of Ca^{++} from the extracellular space during reoxygenation, and much of this is accumulated by respiring mitochondria (*31*, *32*).

In our study, whole kidneys that were exposed to ruthenium red for the duration of the storage period exhibited significantly elevated levels of lipid peroxidation products in both the cortex and medulla when stored in HCA or in saline solutions. This may be the consequence of inhibiting mitochondrial Ca^{++} uptake, a process that utilizes the electrochemical gradient across the mitochondrial membrane and that takes precedence over oxidative phosphorylation (*32*). Thus with the onset of ischemia, mitochondria would divert declining energy resources to buffer initial rises in cytosolic Ca^{++} levels. In the presence of ruthenium red, cytosolic Ca^{++} levels would rise, unchecked by mitochondria, and could trigger increased oxidative damage outside the mitochondria. Alternatively, one mechanism of mitochondrial Ca^{++} efflux can be blocked by ruthenium red that may therefore result in the accumulation of abnormally high levels of Ca^{++} ions in these organelles. Oxygen-derived free radical damage to mitochondria in vitro is markedly enhanced by addition of Ca^{++} to the system that leads to increased membrane permeability and decreased ability to synthesize ATP (*7*). Thus following organ storage, Ca^{++}-rich mitochondria would be particularly prone to oxidative damage by free radicals produced on reperfusion. This could be critical to the survival of the cell since restoration of ATP synthesis would be diminished, a situation aggravated still further by Ca^{++} overload activating Ca^{++}-ATPases and consuming valuable ATP resources (*33*).

Another possible mechanism of damage is the release of abnormal amounts of free fatty acids, particularly arachidonic acid, from membranes during ischemia by Ca^{++}-activated phospholipases. As arachidonic acid release is the rate-limiting step

in prostaglandin synthesis, increases in cytosolic Ca^{++} levels during ischemia are likely to have important consequences in the endothelial lining of the vascular bed. In addition, as cyclooxygenation to prostaglandin precursors is stimulated by hydroperoxides formed as a result of lipid peroxidation, but further metabolism to the vasodilator prostacyclin is inhibited, then the formation of the vasoconstrictor thromboxane may be favored following ischemia (*34–36*). Elevated levels of free arachidonic acid produced during ischemia could also lead to formation of leukotrienes that, too, are vasoconstrictors and enhance capillary permeability (*37*).

In conclusion, the data presented strongly suggests that Ca^{++} redistribution takes place during cold storage of kidneys, and this results in increased oxidative damage to the organs. Since addition of free radical scavengers and of iron chelators to the flush solution is also effective at reducing levels of lipid peroxidation in stored rabbit kidneys (*38*), and administration of superoxide dismutase (an enzyme that scavenges $O_2^{\cdot}$ radicals) or allopurinol (a xanthine oxidase inhibitor) to the recipient has been reported to improve poststorage function of porcine kidneys (*39*), it may be that altered Ca^{++} homeostasis during the ischemic period acts synergistically with oxygen-derived free radicals during reperfusion to produce cellular and vascular damage. We have demonstrated that prevention of extracellular Ca^{++} entry either by specific blocking agents such as verapamil or buffering systems such as citrate is an important consideration in the design of a good storage solution. This is in accordance with another report that showed that while 0.5 mM Ca^{++} in the University of Wisconsin solution is optimal for maintaining the structural integrity of perfused dog kidneys, increased organ viability requires control of intracellular Ca^{++} by the use of the calmodulin inhibitor chlorpromazine (*40*). In contrast Ca^{++} levels of up to 0.8 mM have been reported to increase the viability of hepatocytes stored in Euro-Collins' solution without any precautions being taken against a rise in intracellular Ca^{++} levels (*41*). We further demonstrate that intracellular Ca^{++} redistribution during storage of organs in HCA leads to adverse increases in oxidative damage, and we are currently investigating pharmacologic strategies to protect against this eventuality during the harvesting, storage, and engraftment of organs. Administration to the donor (if considered ethical) and incorporation in the flush solutions of agents that inhibit phospholipase activity, inhibit free radical production or scavenge reactive free radical intermediates, would be logical candidates for such a strategy. Further studies are planned to investigate the possible changes in kidney function that may result from such pharmacologic manipulation.

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REPERFUSION INJURY AND RENAL METABOLISM : THE TEMPORAL RELATIONSHIP BETWEEN OXIDATIVE STRESS AND FUNCTIONAL CHANGE

B J Fuller*, J Gower, L Cotterill, G Healing, S Simpkin and C J Green
*Academic Department of Surgery, Royal Free Hospital & School of Medicine, London NW3 2QG and Section of Surgical Research, MRC Clinical Research Centre, Northwick Park Hospital, Harrow, UK.

INTRODUCTION

A large number of studies have implicated oxygen-derived free radical (OFR) stress in the pathology of the so-called 'reperfusion injury' in a number of organs and tissues including brain (1), liver (2), intestine (3), skin (4), heart (5) and kidney (6). This is the type of injury sustained when an organ has been deprived of blood supply for a period of time, and is then subjected to a sudden restoration of blood supply bringing in high concentrations of oxygen; such events occur in many surgical interventions e.g. during repair of tissues after trauma, and particularly in organ transplantation (7). There is growing evidence that OFR scavengers, when introduced under such circumstances, can beneficially influence the outcome of reperfusion (8). However, there is still much debate about the time relationship between OFR events in a tissue and subsequent functional changes, which in turn will influence the timing of administration of any anti-OFR therapy. We have been particularly concerned with reperfusion injury in kidneys (9,10), and the present studies were undertaken to assess renal tissue metabolism (by gluconeogenesis) in rabbit kidneys after ischaemia / reperfusion. Renal cortical tissue gluconeogenesis was chosen as functional test since this is an active process of cortical tubular cells and these cells show characteristic early signs of ischaemia / reperfusion damage as expressed by tubular necrosis.

MATERIALS AND METHODS

Ischaemia / reperfusion in the rabbit kidney

Details of the surgical model have been published previously (9). In brief, adult male New Zealand White rabbits (2-3 Kg) were anaesthetised by injection of fentanyl-fluanisone and diazepam. The abdomen was opened by a midline incision and the left kidney was completely skeletalised. The left renal artery, vein and ureter were clamped for the desired ischaemic period and the abdomen was temporarily closed. Kidneys were removed for assay immediately after 1 or 2 hours of ischaemia, or in other groups after additional blood reperfusion for 1 or 24 hours. Untreated kidneys were used for control assays.

Assays for indices of lipd peroxidation

which has been shown to correlate with degree of ischaemic damage in kidney tissue (9). In this assay kidneys were sliced and the majority of renal tissue (about 8g) was homogenised in buffer to give a 10% w/v concentration, and incubated in open vessels with shaking at 37 C. Production of fluorescent Schiff bases and thiobarbituric acid reactive substances were measured over the time course of incubation (90 minutes) in extracted lipid fractions as described previously (9).

Assay for gluconeogenesis

From the remaining (approximately 2g) tissue, thecortex was dissected from the medulla, and the cortical fragments used to produce tissue slices (0.3 mm thick) on a McIlwain tissue slicer. The slices were weighed and incubated in Krebs Ringer bicarbonate at 37 C, with or without added pyruvate as gluconeogenic substrate as described previously (11). The incubated supernatants were assayed for glucose production, and from this the pyruvate-stimulated glucose synthesis was assessed.

RESULTS AND DISCUSSION

In Table 1 are shown the results for assays of OFR-induced lipid peroxidation products and the tissue metabolism as assessed by gluconeogenesis. It can be seen that ischaemia for 1 and 2 hours showed a rising trend in Schiff base production (groups II and V). Reperfusion tended to increase still further the marker of lipid peroxidation, and this became a statistically-significant increase on reperfusion for 2 hours ischaemia (group VI). A similar trend was seen for TBA-reactive compounds measured at 2 hours ischaemia and with reperfusion.

When gluconeogenesis was examined, it can be seen that ischaemia for 1 hour (group II) caused a significant drop in activity, whilst activity was almost eradicated by 2 hours ischaemia (group V). After reperfusion for 1 hour (groups III and IV) there was an improvement in gluconeogenic activity. This is in spite of the enhanced markers of oxidative stress seen on reperfusion. Only when reperfusion was continued for 24 hours did gluconeogenesis decrease again compared to that seen in the short (1 hour) reperfusion group (group III versus group IV).

These data show that there is no simple relationship between OFR stress in reperfusion injury and changes in a particular metabolic process within the ischaemic organ. Particularly for the kidneys subjected to 2 hours ischaemia, gluconeogenesis was virtually completely absent, yet a short period of reperfusion allowed a significant recovery of this active process. This occurred in spite of the increased evidence of oxidative stress resulting from reperfusion. Only when reperfusion was carried out for prolonged periods (24 hours) did gluconeogenic activity again decrease. Since in all cases the kidney slices were supplied with substrate and oxygen sufficient to allow gluconeogenesis to proceed, the changes could not be attributed to depletion of any of these factors. The recovery of metabolism on reperfusion in the 2 hour group (V) must have resulted from recovery of some organelle function, required co-factor or alleviation of inhibition by waste products which were brought about by the restored blood flow. The secondary decline in gluconeogenic activity during 24 hour reperfusion would then have resulted from some expression of damage which may have been completely unrelated to the primary loss of function at the end of ischaemia.

A similar relationship between recovery of metabolic activity and time of reperfusion has been demonstrated in a model of myocardial ischaemia (12). Here, the cardiac muscle ATP content recovered immediately on reperfusion after ischaemia, but then declined again as reperfusion continued. Taken

Group n=6 in each	Schiff bases*	TBA-react.* substances	Glucose** production
I	1.05 ± 1.04	0.85 ± 0.96	36.8 ± 6.2
II	2.53 ± 0.77	ND	14.4 ± 2.8
III	2.93 ± 0.68	ND	21.6 ± 2.9 [a]
IV	4.93 ± 1.27 [a]	ND	11.2 ± 4.3
V	4.00 ± 0.83	4.32 ± 1.88	2.8 ± 1.4
VI	5.67 ± 0.75	6.28 ± 0.98 [a]	16.1 ± 4.8 [a]

Experimental groups : I - fresh kidney; II - 1 hour ischaemia; III - 1 hour ischaemia + 1 hour reperfusion ; IV - 1 hour ischaemia + 24 hour reperfusion; V - 2 hour ischaemia; VI - 2 hour ischaemia + 1 hour reflow.
* - fluorescence intensity units / hr / mg protein; ** umol glucose / mg / inncubation.
[a] denotes statistically significant difference from ischaemia alone (Student's T test)

together with our data the results suggest a complex relationship between ischaemia / reperfusion and tissue damage with possible primary, secondary and even later events all playing a role. Such interactions need to be considered when undertaking studies of reperfusion damage and any preventive therapy.

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EVIDENCE THAT CALCIUM MEDIATES FREE RADICAL DAMAGE THROUGH ACTIVATION OF PHOSPHOLIPASE A_2 DURING COLD STORAGE OF THE RABBIT KIDNEY

Lisa A Cotterill, Jon D Gower, Barry J Fuller* and Colin J Green

Section of Surgical Research, Clinical Research Centre, Harrow, Middlesex, HA1 3UJ, U.K. and *Academic Department of Surgery, Royal Free Hospital School of Medicine, London, NW3 1QG, U.K.

INTRODUCTION

Renal transplantation involves periods of warm and cold ischaemia which often result in poor renal function and death of tubular cells soon after engraftment. There is growing evidence that oxygen derived free radicals are responsible, at least, in part, for reperfusion injury and may be involved in ischaemic damage[1]. We have shown in previous studies that markers of lipid peroxidation, resulting from oxidative damage, increase in rabbit kidneys following storage[2]. In addition, we have recently reported evidence for a relationship between altered calcium homeostasis and oxidative damage to the rabbit kidney following cold storage[3].

One possible mechanism by which calcium ions may potentiate oxidative damage is through the activation of phospholipases. Free fatty acids (FFA), the products of phospholipase action, may accumulate during periods of cold ischaemia and such accumulation would be detrimental to the cell due to the detergent-like properties of these molecules[4]. In addition, the disruption of the membrane by lysophosphatides, the residual products of phospholipid hydrolysis[5], may render the membrane more susceptible to attack by free radicals.

We have now investigated the free fatty acid status of rabbit kidneys following cold storage in hypertonic citrate (HCA) using gas liquid chromatography. In addition, we have examined the effects of adding the calcium ionophore A23187, and the phospholipase inhibitor dibucaine on rabbit kidneys during the storage period.

MATERIALS AND METHODS

Groups of New Zealand White rabbits (average weight 3kg) were anaesthetised, the abdomen opened by a mid-line incision, and the kidneys completely skeletalised and removed[2]. The renal artery of each kidney was cannulated and the organs flushed with either 30ml sterile isotonic saline solution or 30ml HCA already cooled to 4°C. 10μM A23187 and 250μM dibucaine were added to storage solutions where appropriate. The kidneys were placed in sterile beakers containing 60ml of the identical flush solution and

Samples were ground to a powder using a mortar and pestle while still frozen. Free fatty acids were extracted and methylated with methyl iodide according to the method of Allen[6]. 10μg of the internal standard pentadecanoic acid was added to the samples prior to extraction. Fatty acid methyl esters were analysed by gas-liquid chromatography using a glass column packed with 10% DEGS on chromosorb WAW 80-100 mesh. Fatty acid methyl esters were identified on the basis of their retention times compared to known standards. The rate of malonaldehyde formation was taken as a marker of lipid peroxidation[3]. Statistical analysis was performed using the students paired t-test.

RESULTS AND DISCUSSION

Following storage of rabbit kidneys for 72 hours in HCA, there were significant rises in all observed unsaturated fatty acids oleic acid (18:1), linoleic acid (18:2) and arachidonic acid (20:4) in both cortex and medulla of kidneys (Table 1). No such increases were observed in the saturated fatty acids, stearic acid (16:0) and palmitic acid (18:0) (Table 1). The most conspicuous increase observed in cortex and medulla was the rise in arachidonic acid.

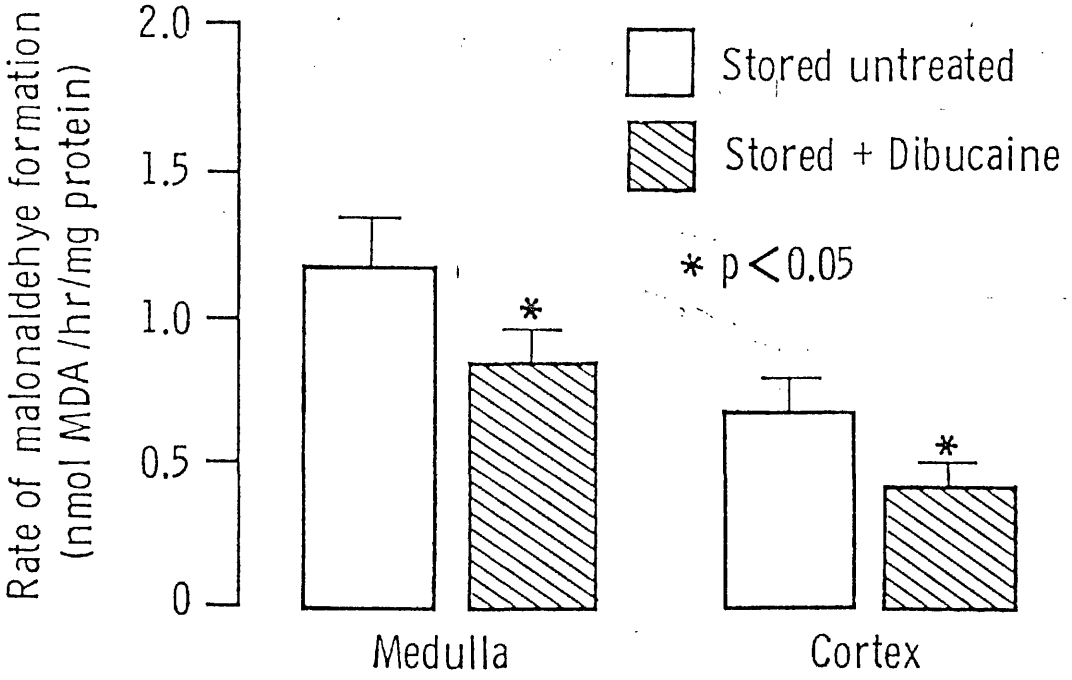
Addition of the phospholipase A_2 inhibitor dibucaine led to a reduction in the accumulation of free arachidonic acid in both cortex and medulla of rabbit kidneys following 72 hours storage in HCA (Table 1). It is likely, therefore, that calcium activation of phospholipase A2 is responsible at least in part for augmenting the release of arachidonic acid during storage. However dibucaine did not completely abolish arachidonic acid accumulation

Table 1. Free Fatty Acid (FFA) analysis in cortex and medulla of rabbit kidneys ± 250μM dibucaine before and after storage in HCA for 72 hours.

	Ratio of 15:0 Standard: FFA			
	CORTEX		MEDULLA	
FFA	Non-stored	Stored	Non-stored	Stored
16:0	4.76 ± 1.99	7.42 ± 3.06	6.22 ± 2.73	6.71 ± 4.02
18:0	6.35 ± 2.69	8.16 ± 3.77	9.32 ± 3.58	7.55 ± 4.85
18:1	0.21 ± 0.32	1.69 ± 0.87*	0.00 ± 0.00	1.06 ± 0.49*
18:2	0.38 ± 0.47	1.85 ± 1.66*	0.28 ± 0.15	0.78 ± 0.39*
20:4	0.13 ± 0.22	1.66 ± 0.59*	0.10 ± 0.06	0.41 ± 0.16*
20:4 250μM Dibucaine		0.44 ± 0.20*		0.21 ± 0.08*

Free fatty acids (FFA) were measured by GLC. Values represent mean ± S.D. of 8 determinations. *Significantly ($P < 0.05$) different from control.

containing 250μM dibucaine.



Values represent mean ± S.D. of 8 determinations. *Significantly ($P<0.05$) different from control.

during storage. Thus it may be postulated that blocked β-oxidation, due to shortage of ATP during ischaemia is a contributory factor in producing FFA accumulation.

In similar studies dibucaine reduced the level of markers of lipid peroxidation (Figure 1), indicating the involvement of phospholipase A_2 in potentiating lipid peroxidation. Disruption of the lipid bilayer through increased phospholipase activity may render the membrane more susceptible to lipid peroxidation. In addition, free fatty acids, without the protective environment of the membrane, form excellent targets for free radical attack.

Increases in the accumulation of free arachidonic acid through phospholipase A_2 activation may lead to a burst in prostaglandin synthesis via cyclooxygenase. Upsetting the delicate eicosanoid balance may have important consequences in the vascular bed of the organ due to production of potent vasoconstrictors[7].

It would appear therefore that an increase in arachidonic acid accumulation may be potentiated by calcium-dependent activation of phospholipase A_2. Such effects are likely to mediate oxygen free radical damage. Exact mechanisms are yet to be elucidated, but there may be a role for phospholipase inhibitors in the improvement of organ preservation.

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THE IMPORTANCE OF OXYGEN FREE RADICALS, IRON AND CALCIUM IN RENAL ISCHAEMIA

Jon D. Gower*, Lisa A. Cotterill and Colin J. Green

Section of Surgical Research,

Clinical Research Centre,

Watford Road, Harrow,

Middlesex, HA1 3UJ

*To whom correspondence should be addressed

INTRODUCTION

Ischaemia encompasses a wide range of clinical conditions and is also an integral part of many surgical techniques, in particular transplantation. Organ retrieval usually involves a short period of warm ischaemia (WI) between cessation of the blood supply and harvesting the organ from the donor. This is followed by a much longer period of cold ischaemia (CI) in which the organ is flushed with and suspended in a cold asanguinous solution for transport to the recipient. The organs are then rapidly reperfused with fully oxygenated blood as soon as the vascular pedicle is reconstructed. Cooling depresses metabolism and very much slows the deterioration of ischaemic organs. However, some organs are particularly susceptible to ischaemic damage and it is currently considered inadvisable to store liver, heart or lungs for longer than 4hr. Kidneys are usually stored for about 24hr but storage periods up to 72hr are not uncommon. There is no definitive safe storage time but rather the longer the period of ischaemia, the less chance there is of an organ functioning immediately upon transplantation. Acute renal failure may occur in transplanted kidneys which become enlarged with a pale cortex and a dark congested medulla and have a drastically impaired excretory capacity. Vascular injury is another possible complication in ischaemically damaged kidneys which are slow to perfuse when revascularised and develop a microagulopathy which results in an outflow block and venous stasis.

Many biological changes associated with ischaemia and reperfusion have been reported. These include depletion of high-energy adenine nucleotides (Calman, Quin & Bell, 1973), accumulation of metabolites such as H^+ ions leading to a significant fall in intracellular pH (Sehr *et al.*, 1979), release of lysosomal enzymes (Pavlock *et al.*, 1984), loss of membrane phospholipids (Southard *et al.*, 1984) and impaired function of intracellular organelles such as mitochondria (Arnold *et al.*, 1985) and endoplasmic reticulum (Schieppati *et al.*, 1985). However, the mechanisms underlying the pathology of organ deterioration due to ischaemia have yet to be conclusively elucidated.

The observation that many pathological changes only become evident after restoration of the blood supply to an ischaemic organ has led to the term 'reperfusion injury'. There is considerable evidence that an important part of reperfusion injury is oxidative damage initiated by the incoming molecular oxygen. Single electron reduction of O_2 leads to the formation of superoxide radicals ($O_2\cdot^-$); further reduction yields H_2O_2 and the highly reactive hydroxyl radical ($OH\cdot$). Under normal physiological circumstances, production of these reactive species is low because O_2 metabolism is carefully controlled by enzymes such as cytochrome oxidase which catalyzes the $4e^-$ reduction of O_2 directly to H_2O. Cells contain a number of protective enzymes such as superoxide dismutase which converts $O_2\cdot^-$ to H_2O_2 and catalase and peroxidases which reduce H_2O_2 to H_2O. There are

also a number of smaller molecules with important biological antioxidant activity such as the lipid-soluble vitamin E, ascorbic acid and glutathione. Although these systems ensure that oxidative damage is kept to a minimum under normal circumstances, this does not appear to be the case when organs are reperfused following periods of ischaemia.

Oxygen-derived free radicals have been implicated in reperfusion damage to many tissues (e.g. Schoenberg *et al.*, 1983; McCord, 1985; Bolli, 1988) including the kidney (Paller *et al.*, 1984; Laurent & Ardaillou, 1986; Ratych & Bulkley, 1986) and in the 'storage-damage syndrome' (Koyama *et al.*, 1985; Fuller, Gower & Green, 1988). One damaging free radical mediated process is the peroxidation of polyunsaturated fatty acids (Wills, 1969), a chain reaction which can result in extensive membrane damage.

We sought evidence for the involvement of oxygen-derived free radicals in renal injury by assaying for markers of lipid peroxidation in homogenates prepared from kidneys which had been subjected to periods of ischaemia. Periods of both warm (Green *et al.*, 1986c) and cold ischaemia (Green *et al.*, 1986a) were found to significantly increase the rate of formation of lipid peroxidation markers during subsequent incubation at 37°C *in vitro*. In the CI experiments, kidneys were either stored in a poor storage medium (isotonic saline) for 24hr or for periods up to 72hr in the more efficacious and clinically-approved hypertonic citrate solution (HCA) developed by Ross, Marshall & Escott (1976). These results showed that there was a good

correlation between the formation of markers of lipid peroxidation measured *in vitro* and the physiological dysfunction of the stored organs upon transplantation *in vivo*. Reperfusion of the ischaemic organs with oxygenated blood *in vivo* generally led to further rises in the extent of lipid peroxidation in these organs. Addition of free radical scavengers (mannitol and uric acid) or the iron-chelator desferrioxamine to the flush and storage solutions significantly inhibited the adverse rises in lipid peroxidation products following the ischaemic period (Green *et al.*, 1986b).

These initial findings provided good circumstantial evidence for a role of oxygen-derived free radicals in post-ischaemic oxidative damage to the kidney. This raised the question of why a period of ischaemia should compromise an organ in this way. One possibility is that during the ischaemic period there is a reduction in the level of antioxidant defenses. Another possibility is that changes occur during ischaemia which result in a burst of oxidant production upon reoxygenation. One such effect is the conversion of the enzyme xanthine dehydrogenase to xanthine oxidase during ischaemia (Roy & McCord, 1983), possibly as a result of a calcium-dependent proteolysis. Upon reoxygenation this enzyme utilises hypoxanthine, which has accumulated due to catabolism of ATP, and forms $O_2 \cdot^-$ radicals from the incoming oxygen. Mitochondrial injury and a build-up of reduced components of the electron transport chain during ischaemia may also increase $O_2 \cdot^-$ production on reoxygenation due

to increased leakage of single electrons onto O_2. In addition, accumulation of polymorphonucleocytes due to the release of chemotactic factors from ischaemic tissues may cause endothelial injury through the extracellular production of free radicals derived from the respiratory burst (Granger *et al.*, 1989).

Two metal ions, iron and calcium, are important determinants of free radical mediated processes, cell injury and vascular disturbance and their possible role in the pathology of ischaemic/reperfusion damage in the kidney is now discussed in detail. Particular emphasis is placed on the temporal sequence of events, special attention being directed towards early changes which may occur during the ischaemic period itself and which may therefore be important in mediating the extent of subsequent reperfusion injury.

THE ROLE OF IRON

The importance of transition metals in catalyzing damaging free radical-mediated processes has long been recognised. Of particular biological relevance is the iron-catalyzed formation of hydroxyl radicals ($OH\cdot$) from less reactive precursors ($O_2\cdot^-$ and H_2O_2) via the metal-catalyzed Haber-Weiss reaction (Halliwell, 1978). The highly reactive $OH\cdot$ radical can damage all types of biological macromolecule and initiate lipid peroxidation (Gutteridge, 1984). Other studies suggest that some iron-centred species may themselves catalyze hydrogen abstraction from polyunsaturated fatty acids and hence directly

initiate lipid peroxidation (Minotti & Aust, 1987). Furthermore, iron salts are known to decompose lipid hydroperoxides to reactive peroxy and alkoxy radicals which can attack further molecules of polyunsaturated fatty acids and hence propagate the chain reaction of lipid peroxidation (Halliwell & Gutteridge, 1984). Some low molecular weight chelates of iron such as ATP and EDTA increase the reactivity of the metal (Dunford, 1987) whereas some high molecular weight chelators such as desferrioxamine (DFX) bind iron with high affinity (10^{31}) (Keberle, 1964) and prevent the metal ion from catalyzing adverse reactions (Gutteridge, Richmond & Halliwell, 1979).

Administration of DFX (i.v., 15mg/kg) to rabbits 15 min before reperfusion of kidneys which had been subjected to 60 or 120 min of warm ischaemia was found to significantly inhibit the adverse rises in markers of lipid peroxidation (Green *et al.*, 1986c). DFX was also highly effective at reducing levels of oxidative membrane damage in kidneys which had been subjected to cold ischaemia by storage either in isotonic saline solution for 24 hr (Green *et al.*, 1986b) or for periods of up to 72 hr in HCA (Gower *et al.*, 1989). The most effective regime proved to be i.v. administration of DFX both before the removal of kidneys for storage and before reperfusion of the autotransplanted organs. Analysis of markers of lipid peroxidation in different regions of the kidney revealed that DFX was particularly effective at inhibiting lipid peroxidation in the cortex following ischaemia; whereas in the medulla, which contains relatively high levels of

cyclooxygenase (Robak & Sobanska, 1976), a less marked decrease in lipid peroxidation was observed upon DFX treatment.

These results strongly suggested a role for iron in mediating oxidative membrane damage which is observed on reperfusion. Under normal physiological circumstances, the overwhelming majority of iron in the body is stored in 'safe' sites which prevent transition of its redox state and hence catalysis of damaging reactions involving single electrons. These 'safe' sites include haemoglobin and transferrin in the extracellular medium and ferritin, a predominantly intracellular protein which stores up to 4,500 atoms of iron in the form of ferric hydroxides (Aisen & Listowsky, 1980). There is however growing evidence for a small pool of intracellular iron which is chelated to low molecular weight (LMW) species such as ATP, citrate and glycine (Bakkeren *et al.*, 1985; Mulligan *et al.*, 1986). This pool may represent iron which is in transit from the extracellular milieu (transferrin) to intracellular storage sites (ferritin) or iron which is required for synthetic purposes. It is likely that this pool of LMW iron, though small, has the potential for exerting some degree of oxidative stress to the cell.

We hypothesised that ischaemia may result in altered intracellular iron homeostasis leading to an increase in the level of catalytic forms of the metal. Evidence for this was sought by developing a method for quantitating the amount of intracellular iron available for chelation by DFX (Gower, Healing

& Green, 1989a). Low speed supernatants of tissue homogenates were incubated with an excess of DFX for 60 min and the parent drug and its iron-bound form, ferrioxamine (FX), were extracted using solid phase extraction cartridges and analyzed by reversed-phase hplc with dual wavelength detection (FX was detected at 430nm and DFX at 226nm). Standard curves obtained after known amounts of iron were incubated with DFX demonstrated that, with suitable precautions, the ratio of the area of the FX peak/area of DFX peak gave an accurate determination of DFX-available (DFX-A) iron levels in the 1-25μM range. Using this method, DFX-A iron levels were determined in kidneys subjected to periods of warm and cold ischaemia and subsequently reperfused *ex vivo* with an oxygenated medium on an isolated perfusion apparatus at 37°C.

There were measurable levels of DFX-A iron present in both cortex and medulla of fresh control kidneys prior to any ischaemic insult (Figs. 1 & 2). Following 2hr warm ischaemia or 24hr cold storage in HCA, levels of DFX-A iron significantly increased by about two-fold in both the cortex and medulla (Gower, Healing & Green, 1989b). Storage in HCA for longer periods (up to 72hr) resulted in levels of DFX-A iron which were generally higher, but not significantly so, than after the less damaging 24 hr period of cold ischaemia.

Much more dramatic differences were observed in the levels of DFX-A iron between the ischaemic groups when the stored organs were reperfused (Healing *et al.*, In Press). After 24 hr CI, the levels of DFX-A iron immediately decreased upon reperfusion and

returned rapidly to control levels in both the cortex and medulla within 5 min (Figs. 1 & 2). In contrast, following the more physiologically damaging 48hr period of CI, DFX-A iron levels remained elevated in both regions of the kidney during the first 5 min of reperfusion and returned to control levels only after 30 min. A similar response was observed in the medulla of kidneys when the ischaemic time was increased from 48 to 72hr (Fig. 2); however, in the cortex of these organs, DFX-A iron levels actually increased during the first 5min of reperfusion before returning to control levels after 30min. There were no significant differences in the total iron content of cortex and medulla between any of the groups as measured by atomic absorption spectroscopy . Thus, these results clearly showed that both warm and cold ischaemia led to a redistribution of intracellular iron to forms more available for chelation by DFX. In view of our earlier findings that DFX was more effective at inhibiting increased levels of lipid peroxidation in the cortex following ischaemia (Gower *et al.*, 1989), it is interesting to note that increases in DFX-A iron levels during ischaemia were more pronounced in this region compared to the medulla.

It is likely, though not proven, that the source of increased levels of DFX-A iron during ischaemia was ferritin. Although surprisingly little is known about the release of iron from ferritin, it is thought to involve the reduction of Fe^{3+} to Fe^{2+} (Funk *et al.*, 1985). This reduction could be facilitated by the low oxygen tension encountered as a result of ischaemia and

by the accompanying fall in pH. Such an environment would favour increased levels of reducing agents which may release iron from this protein (Fig. 3). Exogenous redox-active quinones such as adriamycin have been shown to catalyse lipid peroxidation in the presence of ferritin especially under hypoxic conditions (Vile & Winterbourn, 1988). However, the identity of possible endogenous agents capable of performing this function and produced as a result of ischaemia has yet to be established. Experiments *in vitro* have demonstrated that lipid peroxidation in microsomes is stimulated in the presence of purified ferritin and flavin mononucleotide during aerobic incubation following a period of hypoxia (Goddard *et al.*, 1986).

Further release of iron into the DFX-A pool on reperfusion of kidneys stored for longer periods may be the result of iron release from ferritin by reducing agents formed from the incoming oxygen (Fig. 3). Superoxide anions, which may be produced in significant amounts upon reperfusion, have been shown to release iron from ferritin *in vitro* (Biemond *et al.*, 1988; Monteiro & Winterbourn, 1988). Alternatively, other reaction products of superoxide or oxygen itself with as yet unidentified cellular components may play an important role. It is also possible that continued high levels of DFX-A iron during the reperfusion period were due to the impairment of uptake mechanisms, as yet poorly understood, of intracellular iron species into 'safe' sites.

These studies demonstrate that redistribution of intracellular iron to forms more accessible to DFX occurs as an

early event during the ischaemic period itself. The fact that increased levels of iron become available for chelation by DFX makes it highly likely that iron redistribution is an important underlying cause of the increased oxidative damage which occurs upon reperfusion. However, extended and hence more damaging periods of cold ischaemia did not lead to correspondingly higher levels of delocalized iron immediately after the ischaemic period (Fig. 3). Rather, extended periods of elevated delocalized iron levels during the immediate reoxygenation period appeared to be more important in determining the degree of reperfusion damage to rabbit kidneys. Chelation of the increased levels of intracellular LMW iron complexes by agents such as DFX inhibit the initiation and propagation of damaging events such as lipid peroxidation (Fig. 3) and may therefore be a worthwhile approach to combating post-ischaemic renal failure.

THE ROLE OF CALCIUM

Calcium ions play a crucial role in the control of a wide variety of biological functions (Evered & Whelan, 1986). These include regulation of cell metabolism through the activation of a considerable number of enzymes and the maintenance of normal blood flow via controlled production of a range of eicosanoids. Careful maintenance of the gradient between low (10^{-7}M) cytosolic calcium levels and the high extracellular concentration (10^{-3}M) is therefore essential. Under normal physiological circumstances, energy-dependent pumps remove calcium from the

cell and also sequester excess calcium into intracellular organelles (Carafoli, 1987). Calcium ions may enter the cell only through specific voltage or receptor-operated channels (Carafori, 1987) and intracellular calcium levels are also controlled by the receptor-mediated turnover of phosphatidylinositides which involves a GTP-binding protein and a specific phospholipase C (Berridge, 1984).

The rapid depletion of ATP levels upon ischaemia will comprimise calcium pumps and a gain in cellular calcium levels is a consistent feature in tissues subjected to ischaemia and reperfusion (Nayler, 1988). This is likely to lead to a derangement in cell function, and continued elevations in intracellular calcium may cause altered cell morphology and irreversible cell injury (Farber, 1981). There is considerable interest in the possible involvement of calcium in ischaemic/reperfusion damage (Cheung *et al.*, 1986; Opie, 1989). However, it is unclear as to whether altered intracellular calcium homeostasis is an important initiator of damage or whether it plays a much later role in the pathology of ischaemic/reperfusion injury.

We rendered rabbit kidneys cold ischaemic in storage solutions containing various agents which either affect calcium movements or interfere with calcium-dependent enzymes (Cotterill *et al.*, 1989a). Oxidative damage was subsequently assessed by measuring formation of markers of lipid peroxidation in tissue homogenates *in vitro*. Blockage of voltage-operated channels by

verapamil reduced the extent of oxidative damage to low levels in both the cortex and medulla of kidneys following 24hr storage in isotonic saline but had no effect on oxidative damage after more prolonged (72 hr) storage of organs in the superior HCA solution. Elevation of extracellular calcium levels by addition of $CaCl_2$ (1mM) to the storage medium increased oxidative damage to significantly greater levels than in organs stored in saline. alone but also had no effect when added to HCA. It was concluded that influx of extracellular calcium through voltage-operated channels (VOCs) was a significant mediator of oxidative damage to organs following storage in saline. In HCA however, the adverse effect of extracellular calcium appeared to be prevented, or at least slowed. This may have been due to chelation of calcium by the excess of citrate (55mM) in this medium or because the better ionic balance of this storage solution may have prevented activation of VOCs during the ischaemic period.

Storage of rabbit kidneys in the presence of A23187, an ionophore which permeabilizes both plasma and intracellular membranes to calcium, resulted in post-ischaemic rates of lipid peroxidation which were significantly greater than the already elevated levels observed after storage in either saline for 24hr or HCA for 72hr (Cotterill *et al.*, 1989a). Ruthenium red, a polysaccharide dye which inhibits mitochondrial calcium transport, also potentiated oxidative damage to organs following CI in either of the two media. These results suggested that, even in the absence of extracellular calcium effects,

redistribution of calcium within the cell could take place during the ischaemic period and contribute to increased peroxidation of cellular lipids upon reoxygenation.

There are several possible ways in which calcium ions may potentiate free radical mediated post-ischaemic injury. Conversion of xanthine dehydrogenase to xanthine oxidase, which may be an important source of $O_2\cdot^-$ radicals following ischaemia (Roy & McCord, 1983), can be catalyzed by a calcium-dependent protease (McCord, 1985). In our cold ischaemic kidney model, inhibition of xanthine oxidase by addition of allopurinol to the saline storage medium partially prevented the increase in lipid peroxidation following storage in the presence of A23187 (Cotterill *et al.*, 1989b). Mitochondrial injury due to calcium overload of this organelle is often seen upon reperfusion (Arnold *et al.*, 1985) and may cause increased leakage of single electrons from the electron transport chain onto O_2, thus increasing $O_2\cdot^-$ production. Another possible link between raised intracellular calcium concentrations and increased oxidative stress is the involvement of calcium-dependent phospholipases which hydrolyse membrane phospholipids, releasing free fatty acids (FFAs) and leaving residual lysophosphatides in the membrane. This possibility was borne out by the observation that specific inhibition of phospholipase A_2 by addition of dibucaine to the storage solution resulted in significant protection against oxidative membrane damage following ischaemia (Cotterill *et al.*, 1989b).

Evidence for phospholipase activation during CI was obtained by analyzing free fatty acids in freeze-clamped renal tissue by gas liquid chromatography (Cotterill *et al.*, 1989c). Levels of unsaturated FFAs ($C_{18:1}$, $C_{18:2}$, $C_{20:4}$) were found to rise considerably in kidneys stored for 72 hr in the clinically approved HCA solution. No significant changes were observed in the levels of saturated ($C_{16:0}$, $C_{18:0}$) FFAs. The high concentration of free arachidonic acid ($C_{20:4}$) observed after 72hr CI is likely to be of particular importance as release of this fatty acid from the membrane is the rate limiting step in the formation of prostaglandins (Isakson *et al.*, 1978). This may therefore lead to a derangement in the production of these vasoactive substances when the organ is reperfused with resulting important consequences in the endothelial lining of the vascular bed (Schlondorff & Ardaillon, 1986). Indeed, it has been demonstrated that following ischaemic insult there is a decrease in the level of the potent vasodilator prostacyclin and an elevation in the formation of thromboxanes which cause vasoconstriction (Lelcuk *et al.*, 1985; Schmitz *et al.*, 1985), a situation which may contribute to the "no-reflow" phenomenon observed when some organs are transplanted. The release of free arachidonic acid during CI is likely to be the main cause of the increased rate of indomethacin-inhibitable peroxidation via the cyclooxygenase pathway which we have observed in the medulla of stored kidneys (Gower *et al.*, 1989). In addition, increased phospholipase activity may result in the formation of

lipoxygenase products such as leukotrienes which are powerful mediators of vascular shock and have been implicated in ischaemic injury (Lefer, 1985).

Storage of kidneys in the presence of dibucaine or A23187 demonstrated a good correlation between the extent of free fatty acid accumulation and the rate of post-ischaemic lipid peroxidation. Possible relationships between calcium, phospholipase activity and lipid peroxidation are shown in Fig. 4. Early redistribution of intracellular calcium during the ischaemic period leads to activation of phospholipases during the hypoxic period. The released unsaturated FFAs, unprotected by the membrane-bound antioxidant vitamin E, would form excellent targets for free radical attack upon reoxygenation. The resulting alkoxy and peroxy radicals may then initiate peroxidation of membranes directly or may breakdown to relatively stable hydroperoxides which could diffuse to other sites in the cell and stimulate lipid peroxidation through interaction with catalytic iron complexes which regenerate reactive lipid radicals (Halliwell & Gutteridge, 1984). In addition, the concomitant build-up of residual lysophosphatides in the membrane, due to phospholipase activation, alter fluidity and permeability (Weltzem, 1979) and may render membranes more susceptible to free radical attack (Ungemach, 1985). Peroxidation of membrane lipids results in a loss of the fatty acid content of membranes, and also increases lysophosphatide levels (Ungemach, 1985) and membrane rigidity (Demopoulos et al, 1980). As phospholipase A2

activity is higher in rigid membranes (Momchilova, Petkova & Koumanov, 1986), elevated rates of lipid peroxidation upon reoxygenation may lead to further increases in phospholipase activation and the damaging cycle of events shown in Fig. 4 may therefore ensue. This would lead to extensive damage to membranes which would become permeabilized to calcium and hence cytosolic calcium levels would rise further. Oxidative damage has also been shown to inhibit the plasma membrane calcium-extruding system (Nicotera *et al.*, 1985) and loss of the ability of intracellular organelles to sequester calcium (Bellomo *et al.*, 1985). Highly elevated levels of cytosolic calcium resulting from the above sequence of events (Fig. 4) could alter cell morphology causing blebbing (Jewell *et al.*, 1982) and, in conjunction with free radical mediated damage, would lead to irreversible injury to the cell.

Recently we have investigated the possibility that ischaemia followed by reoxygenation may affect the cleavage of membrane-bound phosphatidylinositols (PIP_2) in the kidney. This secondary messenger system involves the formation of inositol triphosphate (IP_3) and diacylglycerol (DAG) (Berridge, 1984). IP_3 mobilizes calcium from intracellular stores and DAG stimulates phosphorylation of protein kinase C (PKc), a process which requires phospholipids and calcium for maximum activity (Berridge, 1984). In the kidney, PIP_2 hydrolysis through activation of α_1-adrenoceptors evokes a multiple response: increased sodium reabsorption (Hesse & Johns, 1984), prostanoid

production and vasoconstriction (Cooper & Malik, 1985), gluconeogenesis (Kessar & Saggerson, 1980) and inhibition of renin release (Matsumura *et al.*, 1985).

In this set of experiments, kidney cortical slices were incubated *in vitro* at 37°C either under an atmosphere of 95% O_2:5% CO_2 (control) or gassed with and incubated under N_2 (hypoxia). After 120min all slices were then oxygenated and incubated aerobically for a further 30 min. The formation of lipid peroxidation products increased during the first 60 min of incubation in the presence of O_2 and then levelled off over the remaining period (Fig. 5). Lipid peroxidation was also evident in the hypoxic slices but proceeded at a slower rate than that of the oxygenated samples and also levelled off after 60 min (Fig. 5). Reoxygenation following 120 min of hypoxia resulted in a significant ($P<0.0001$) increase in the rate of lipid peroxidation which was not observed in the slices kept under N_2 or when slices incubated in the presence of oxygen for 120 min were regassed (Fig. 5). This *in vitro* model system therefore closely mimicked the increase in free radical-mediated oxidative membrane damage which occurs when whole kidneys are subjected to ischaemia and reperfusion.

In order to determine the rate of PIP_2 hydrolysis under these conditions, radiolabel was incorporated into the membrane-bound phosphatidylinositol pool by incubating slices in the presence of myo-(2-^{3}H)-inositol for 60 min at 37°C. The slices were then repeatedly washed and incubated under aerobic or

hypoxic conditions for 120 min followed by oxygenation. Aliquots of slices were taken every 30 min and hydrolysis products of PIP_2 (IP_3 and its subsequent metabolites inositol bisphosphate (IP_2) and inositol monophosphate (IP_1)) were analysed by hplc with scintillation counting (Irvine *et al.*, 1985). The extent of PIP_2 hydrolysis was also quantitated by a simple procedure using short Dowex anion-exchange columns (Berridge *et al.*, 1983). Samples were loaded onto the columns and two fractions were collected; fraction 1 containing inositol and glycerophosphoinositol was first eluted with 60mM ammonium formate/5mM sodium borate (12ml) (fraction 1) and inositol phosphates (IP_1, IP_2 and IP_3) were then eluted with 1M ammonium formate/0.1M formic acid (12ml) (fraction 2). The ratio of the radioactivity in fraction 2 : the radioactivity in fraction 1 was taken as a measure of PIP_2 breakdown.

There was no change in the rate of PIP_2 breakdown in the hypoxic slices during the 120 min incubation period compared with control slices incubated under aerobic conditions either in the presence of calcium or in calcium-free medium containing EGTA (Fig. 6). However, immediately upon reoxygenation of the hypoxic slices incubated in the presence of calcium, PIP_2 breakdown increased rapidly (Fig. 6). This increase was highly significant ($P=0.0002$) and was maintained over the remaining 30 min of aerobic incubation. No increase in PIP_2 breakdown was observed when the slices incubated in the presence of calcium and oxygen for 120 min were regassed with 95% O_2:5% CO_2 (Fig. 6).

Furthermore, reoxygenation of hypoxic slices in calcium-free medium (+EGTA) had no effect on the rate of PIP_2 breakdown and no significant changes occurred in slices incubated in the presence of O_2 and EGTA (Fig. 6).

These results clearly demonstrated that hydrolysis of phosphatidylinositols to secondary messenger products is activated very rapidly upon reoxygenation of renal tissue following a period of hypoxia. Inhibition of the effect by EGTA strongly suggested the involvement of calcium. No changes in PIP_2 breakdown were observed during hypoxia itself. One of the products of lipid peroxidation, 4-hydroxynonenal has been shown to stimulate adenylate cyclase, guanylate cyclase and PIP_2 breakdown *in vitro* (Dianzani *et al.*, 1989). It is therefore possible that increased levels of aldehydic products of lipid peroxidation produced on reoxygenation were responsible for increased PIP_2 hydrolysis. In addition, both lipid peroxidation and high calcium-dependent phospholipase A_2 activity alter membrane configuration (Ungemach, 1985) and this may affect the interaction of phospholipase C with membrane-bound regulatory components or make it more accessible to its substrate (PIP_2). It is also possible that highly altered calcium levels during the immediate reperfusion period were sufficient to evoke a response of the phospholipase C system. Rapid hydrolysis of PIP_2 on reoxygenation following ischaemia would lead to deregulation of receptor mediated function through this intracellular secondary messenger system. One of the consequences of this is likely to

be an imbalance in eicosanoid production and vascular disturbances which may contribute to the pathogenesis of renal damage which occurs under these conditions.

CONCLUSION

It is unlikely that a single biochemical event is responsible for the deterioration of organs subjected to prolonged periods of ischaemia. The investigations described in this chapter show that oxidative damage following renal ischaemia can be significantly inhibited by many different agents which suggests that a complex interaction of a number of factors are responsible for post-ischaemic tissue damage. Evidence has been presented for a number of intracellular changes which occur in renal tissue during ischaemia (summarised in Fig. 7). While all these changes may be considered adverse to normal cell function, they are nevertheless reversible within certain limits. Thus upon reperfusion, ATP may be regenerated and provide energy for calcium pumps and the restoration of calcium homeostasis. Similarly, delocalized intracellular iron also appears to be rapidly sequestered upon reoxygenation when the ischaemic period has been short. However, we believe these early events to be of crucial importance in 'priming' the organ for subsequent reperfusion damage and that the longer the period of ischaemia, the more significant these changes become.

Upon reperfusion, a burst of $O_2^{\cdot -}$ production from the incoming oxygen, due to a number of possible mechanisms already

discussed, would react with increased levels of catalytic iron to yield much more reactive radical species. Damage to cellular components would ensue, including peroxidation of lipids in membranes already compromised by increased calcium-dependent phospholipase activity (Fig. 7). The resulting loss of integrity of the plasma membrane and intracellular organelles would cause further imbalances in intracellular ion homeostasis. This cycle of self-perpetuating events may perturb the cell sufficiently to cause irreversible cell injury.

In addition to 'gross' biochemical damage, effects on specific systems may contribute to post-ischaemic organ failure through disturbances in the vasculature of the organ (Fig. 7). These include imbalances in eicosanoid production due to a calcium-dependent build-up of free arachidonic acid, production of inflammatory mediators such as leukotrienes, release of chemotactic substances with subsequent adhesion and activation of polymorphonucleocytes, and derangement of receptor-mediated functions such as the phosphatidylinositol secondary messenger system.

The involvement of a wide spectrum of biochemical derangements in ischaemic/reperfusion injury is supported by the fact that many different pharmacological agents have been reported to afford at least some physiological protection to kidneys subjected to ischaemic insult. These include: iron-chelators (Paller *et al.*, 1988); free radical scavengers, including superoxide dismutase (Paller *et al.*, 1984; Koyama *et*

al., 1985; Bosco & Schweizer, 1988) and catalase (Bosco & Schweizer, 1988); allopurinol (Koyama *et al.*, 1985); calcium-antagonists (Gingrich *et al., 1985; Schrier et al.*, 1987); and prostacyclin analogues (Langkopf *et al.*, 1986). However, the wide range of biochemical abnormalities make it unlikely that a single agent will be totally effective. We have investigated the effect of administering (i.v. both before ischaemia and before reperfusion) a combination of the iron-chelator DFX and the cyclooxygenase inhibitor indomethacin on the subsequent viability of kidneys rendered cold ischaemic (Gower *et al.*, 1989). Kidneys were stored for up to 72hr in HCA at 0°C, autotransplanted into the contralateral renal bursa and the animals recovered. After 48hr storage, 3/6 of the kidneys in untreated animals were capable of supporting life whereas all (6/6) of the treated group survived the full 30 days of the experimental period. Following the more damaging 72hr period of CI, only 2/6 of the control group survived compared with 4/6 in the group treated with DFX and indomethacin. Analysis of serum urea and creatinine levels showed that all the failures died of uremia. While the groups were too small for these preliminary results to achieve significance, they did indicate a trend towards improved viability of kidneys subjected to clinically relevant periods of CI in a solution widely used for the storage of human organs.

Now that many of the immunological problems associated with organ transplantation can be successfully controlled, retrieval

of donor organs in optimum condition is becoming an increasingly important aspect of clinical renal transplantation. Hypothermic storage in special solutions with improved ionic composition has already yielded benefits (Belzer & Southard, 1988). It is envisaged that further advances towards increasing both the number and post-storage viability of transplanted organs will come through a better understanding of the underlying causes of ischaemic/reperfusion damage. A combined pharmacological strategy to prevent, or at least slow, several adverse biochemical changes associated with ischaemia including altered intracellular iron and calcium homeostasis, loss of antioxidant protection and increased capacity for free radical generation, would appear to offer great potential.

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FIGURE LEGENDS

Figure 1

The level the desferrioxamine-available iron in the cortex of kidneys subjected to 24, 48 or 72hr cold ischaemia followed by up to 30min *ex vivo* reperfusion at 37°C with an oxygenated asanguinous perfusate. Values represent the mean ± S.E.M. of 6 separate determinations performed in duplicate.

Figure 2

The level the desferrioxamine-available iron in the medulla of kidneys subjected to 24, 48 or 72hr cold ischaemia followed by up to 30min *ex vivo* reperfusion at 37°C with an oxygenated asanguinous perfusate. Values represent the mean ± S.E.M. of 6 separate determinations performed in duplicate.

Figure 3

A schematic diagram depicting the release of iron from ferritin by reducing agents produced during ischaemia and reperfusion, the formation of low molecular weight (LMW) iron chelates and the subsequent catalysis of lipid peroxidation which is prevented by chelation of catalytic iron complexes with desferrioxamine (DFX).

Figure 4

A scheme depicting the possible relationships between increased intracellular calcium (Ca) levels, phospholipase A_2 (PLA_2) activity, lipid peroxidation and membrane damage. FFA - free

fatty acids, LOO· - lipid peroxy radical, LOOH - lipid hydroperoxide, Fe - catalytic iron complex.

Figure 5

Lipid peroxidation in kidney cortical slices incubated at 37°C in Krebs-Ringer bicarbonate solution for 120 min under an atmosphere of N_2 (O) or O_2 (O) followed by regassing with either O_2 (———) or N_2 (- - - -) and incubation for a further 30 min. Values are the means ± 95% confidence limits of 6 separate determinations performed in duplicate. Lipid peroxidation was measured as thiobarbituric acid-reactive material and expressed as malonaldehyde formation.

Figure 6

Phosphatidylinositol breakdown in kidney cortical slices incubated at 37°C in Krebs-Ringer bicarbonate solution containing calcium (2.5mM)(O) or EGTA (10mM) (O) for 120 min under an atmosphere of N_2 (- - - -) or O_2 (————) followed by reoxygenation and aerobic incubation for a further 30 min. Values represent the means of 8 separate determinations performed in duplicate. PIP_2 breakdown was determined by anion-exchange chromatography (Berridge *et al.*, 1983) and expressed as the ratio of fraction 2 (inositol tris-, bis- and mono-phosphates)/fraction 1 (inositol and glycerophosphoinositol).

Figure 7

Summary of some biochemical changes associated with ischaemia and reperfusion.

FIGURE 1

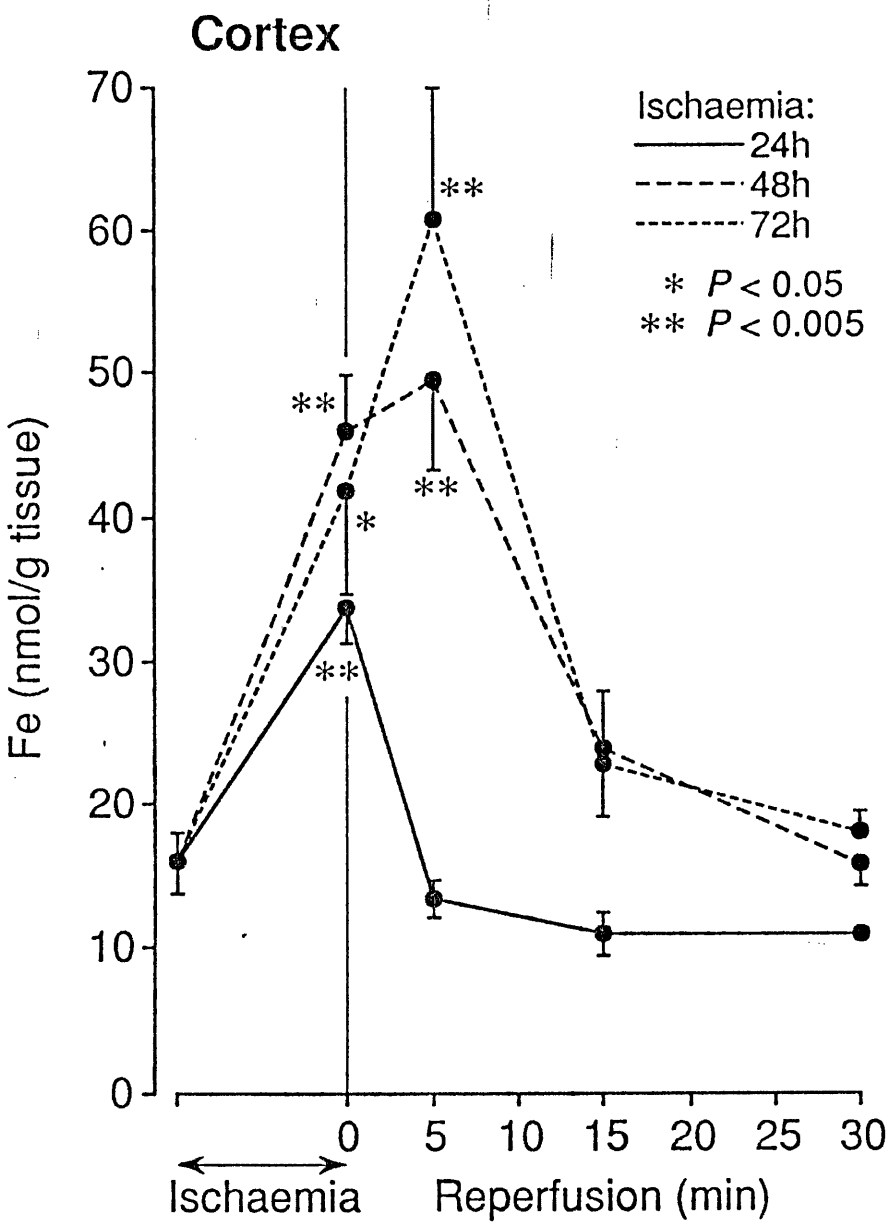

Cortex
Ischaemia:
24h
48h
72h
* P < 0.05
** P < 0.005
Fe (nmol/g tissue)
70
60
50
40
30
20
10
0
0
5
10
15
20
25
30
Ischaemia
Reperfusion (min)

FIGURE 2

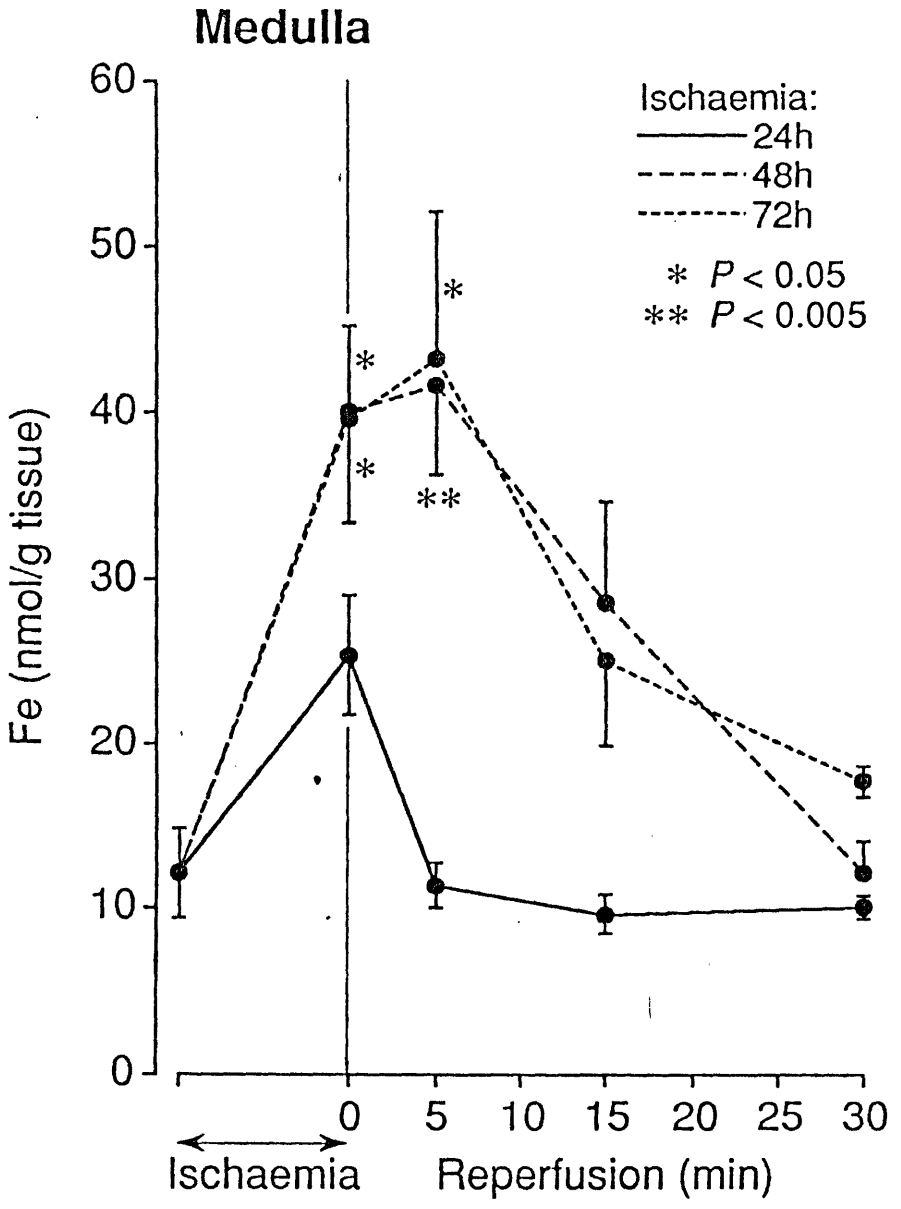

Medulla
Ischaemia:
24h
48h
72h
* P < 0.05
** P < 0.005
Fe (nmol/g tissue)
60
50
40
30
20
10
0
0
5
10
15
20
25
30
Ischaemia
Reperfusion (min)

FIGURE 3

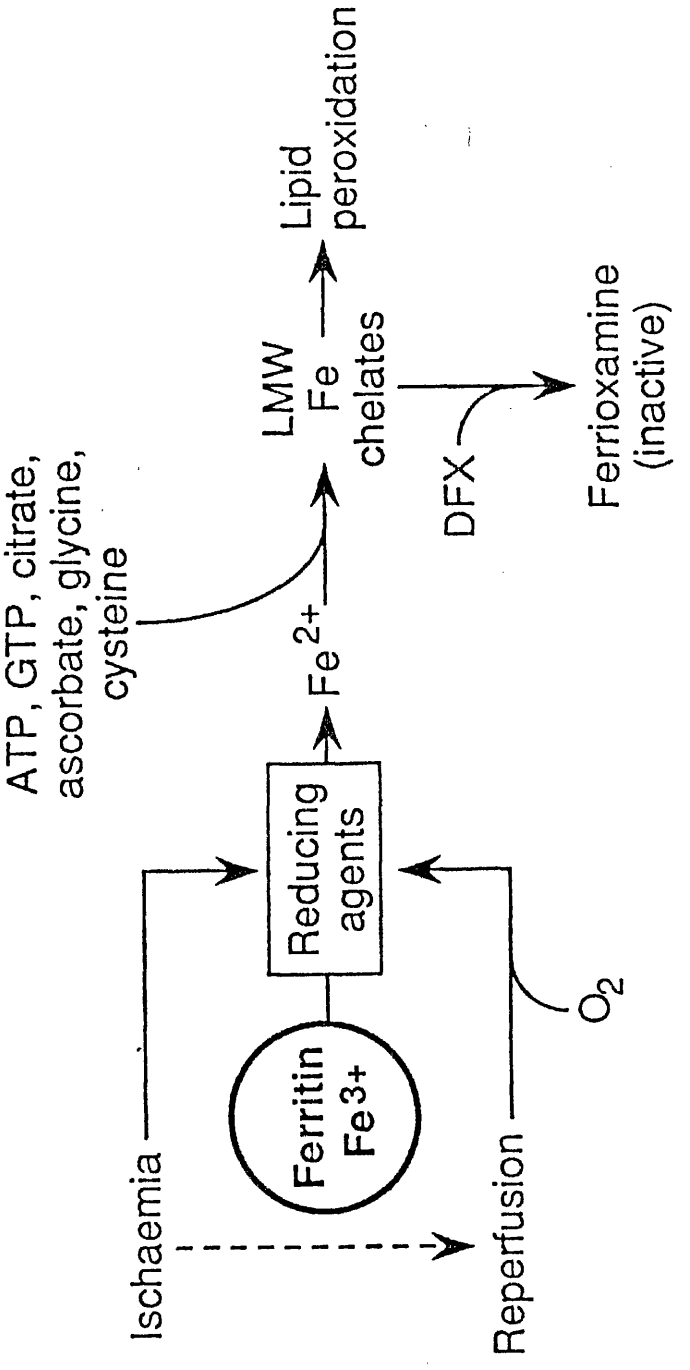
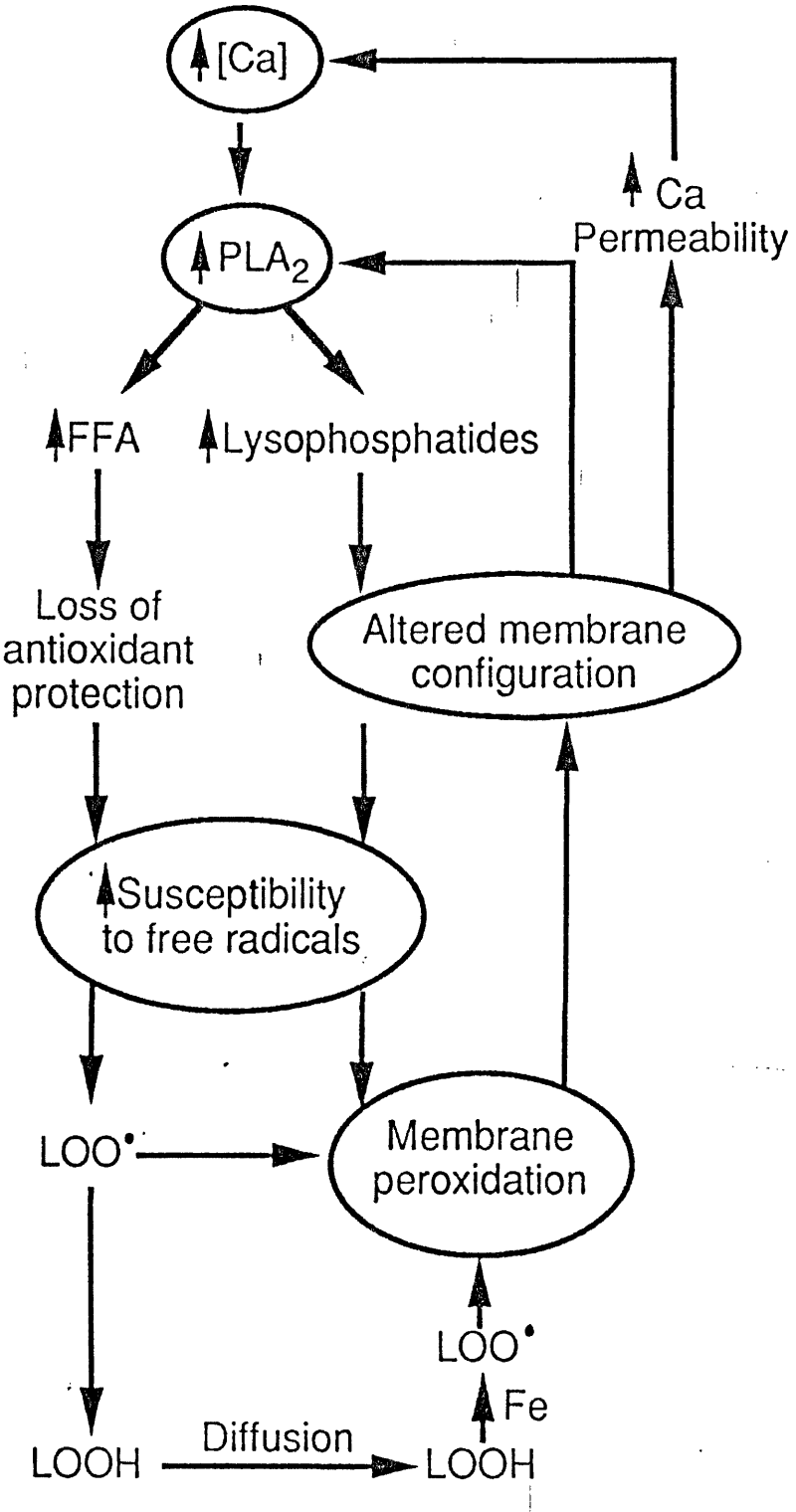

Ischaemia
Ferritin
Fe3+
Reperfusion
O2
Reducing
agents
Fe2+
ATP, GTP, citrate,
ascorbate, glycine,
cysteine
LMW
Fe
chelates
Lipid
peroxidation
DFX
Ferrioxamine
(inactive)

FIGURE 4


[Ca]
Ca
Permeability
PLA2
FFA
Lysophosphatides
Loss of
antioxidant
protection
Altered membrane
configuration
Susceptibility
to free radicals
LOO•
Membrane
peroxidation
LOO•
Fe
LOOH
Diffusion
LOOH

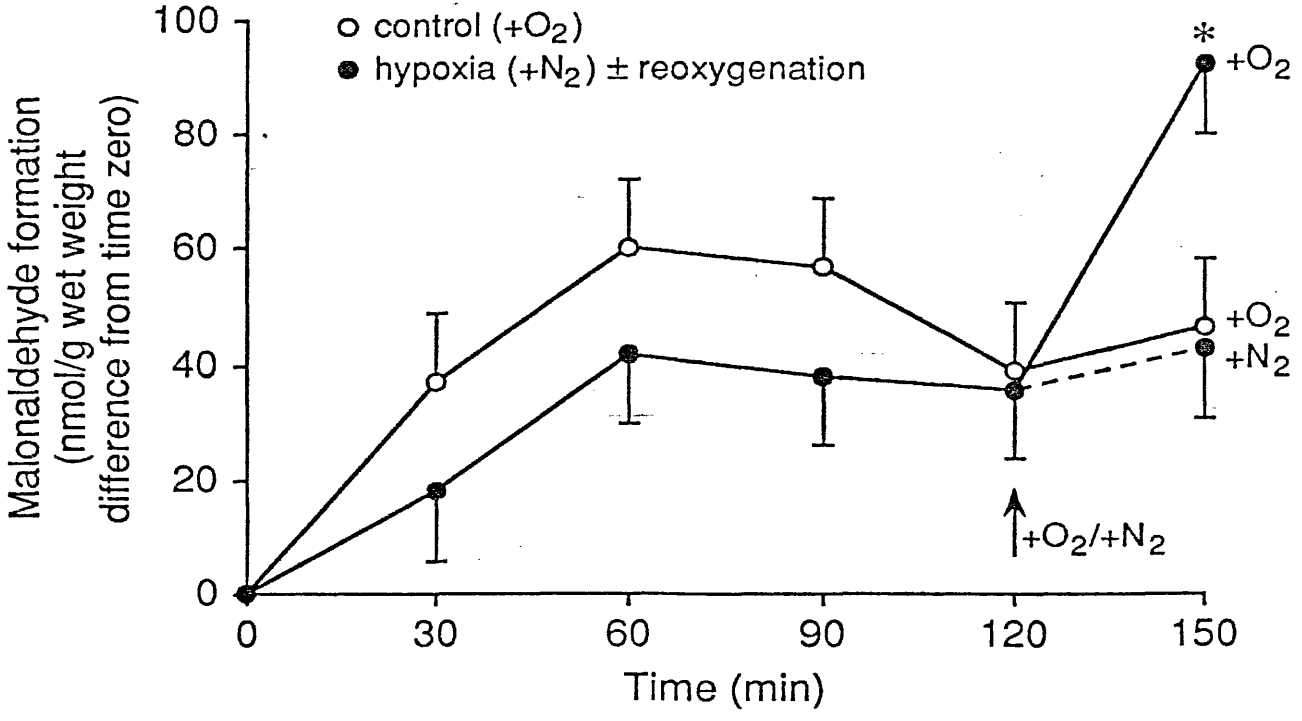
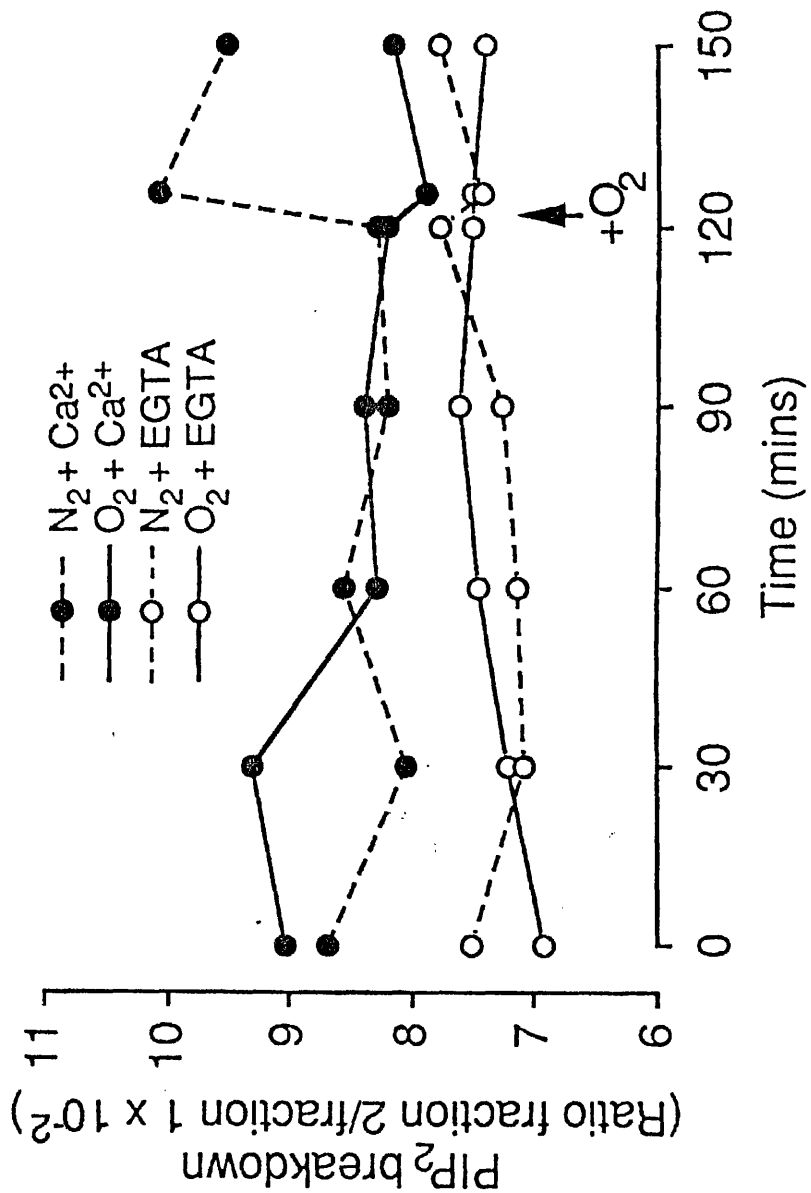

control (+O2)
hypoxia (+N2) ± reoxygenation
Malonaldehyde formation
(nmol/g wet weight
difference from time zero)
100
80
60
40
20
0
0
30
60
90
120
150
Time (min)
*
+O2
+O2
+N2
+O2/+N2

FIGURE 3

FIGURE 6


PIP2 breakdown
(Ratio fraction 2/fraction 1 x 10-2)
11
10
9
8
7
6
0
30
60
90
120
150
Time (mins)
+O2
N2 + Ca2+
O2 + Ca2+
N2 + EGTA
O2 + EGTA

Ischaemia	Reperfusion
↓ ATP Accumulation of Metabolites eg. Hypoxanthine ↓ pH ↑ LMW Iron Ca redistribution	↑ Oxygen Free Radicals ↑ Lipid Peroxidation Imbalance in Prostaglandin/ Thromboxane production Production of Inflammatory Mediators eg. Leukotrienes
Reversible Cell Injury →	**Irreversible Cell Injury**
Organ 'primed' for 'reperfusion injury' →	**Vascular Injury** - Poor Reflow - Odema

FIGURE 7

REOXYGENATION FOLLOWING HYPOXIA STIMULATES PHOSPHATIDYLINOSITOL BREAKDOWN AND LIPID PEROXIDATION IN KIDNEY CORTICAL SLICES

Lisa A Cotterill[1], Jon D Gower[1], Peter K Clark[2],
Barry J Fuller[3] and Colin J Green[1]

[1]Section of Surgical Research and [2]Division of Medical Statistics, Clinical Research Centre, Watford Road, Harrow, Middlesex, HA1 3UJ, England
and
[3]Academic Department of Surgery, Royal Free Hospital and School of Medicine, Pond Street, Hampstead, London, NW3 2QC, England

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Reoxygenation of rabbit kidney cortical slices exposed to hypoxia for 120 min at 37°C in vitro resulted in a rapid increase in phosphatidylinositol hydrolysis to inositol phosphates and this high rate was maintained for at least 30 min. No changes in phosphatidylinositol breakdown occurred during the hypoxic period itself or upon reoxygenation in the absense of calcium (+EGTA). Reoxygenation following hypoxia also resulted in a significant increase in lipid peroxidation. It is possible that phospholipase C activation was the result of increased free radical activity and/or altered intracellular calcium homeostasis.

It is now well established that certain agonists (e.g. hormones and neurotransmitters) evoke a secondary messenger response through the hydrolysis of membrane phosphatidylinositides [1]. Cleavage of phosphatidylinositol-4,5,-bisphosphate (PIP_2) by a specific phospholipase C, controlled by a GTP-binding protein (G-protein) [2] generates two second messenger products, inositol trisphosphate and diacylglycerol. Inositol trisphosphate mobilizes calcium from intracellular stores and diacylgycerol stimulates phosphorylation of protein kinase C, a process which requires phospholipids and calcium for maximum activity [1]. In the kidney, PIP_2 hydrolysis through activation of α1-adrenoceptors evokes a multiple response: increased sodium reabsorption [3], prostanoid production and vasoconstriction [4], gluconeogenesis [5] and inhibition of renin release [6].

We have previously found evidence that intracellular calcium homeostasis becomes altered when rabbit kidneys are subjected to ischaemia [7] and that there is a highly elevated rate of lipid peroxidation in homogenates of kidneys following cold ischaemia [8]. Our present study demonstrates for the first time that reoxygenation following hypoxia stimulates phosphatidylinositol hydrolysis in rabbit kidney cortical slices in vitro

which may be linked to one or both of these metabolic disturbances.

MATERIALS AND METHODS

Cortical slices were prepared from New Zealand White rabbit kidneys as previously described [9], suspended (25% v/v) in Krebs-Ringer bicarbonate solution (KRB) containing pyruvate (10mM) and gassed with 95% O_2:5% CO_2 to restore ATP levels. Myo-(2-^{3}H)-inositol (0.32μM; 19.9 Ci/mmol; Amersham Int.) was incorporated into phospholipids over a 60 minute incubation period at 37°C, the slices were then washed four times and resuspended in fresh KRB (25% v/v).

Aliquots of [^{3}H]-inositol-loaded slices were suspended (10% v/v) in either KRB buffer (2.5mM $CaCl_2$), or calcium-free KRB buffer containing 10mM EGTA, gassed with either 95% O_2:5% CO_2 for 2 minutes (control) or N_2 for 5 minutes to create hypoxic conditions and incubated in tightly stoppered flasks for 120 min at 37°C. All slices were then gassed with 95% O_2:5% CO_2 and incubated for a further 30 minutes. Duplicate aliquots (750μl) were removed at 0, 30, 60, 90, and 120 min and following the final incubation period. Samples were immediately added to chloroform:methanol (2:1 v/v; 3ml), vortexed for 2 minutes and the aqueous phase separated by centrifugation.

Water-soluble inositol phosphates were analysed by hplc (Partisil SAX 10 column; 4.6mm i.d.x 25cm; HPLC Technology Ltd., Cheshire) after spiking with adenine nucleotide markers according to the method of Irvine *et al* [10]. The eluent was collected in 1.25ml fractions and the radioactivity of each fraction determined by liquid scintillation counting. PIP_2 breakdown was also determined by separation of inositol phosphates on Dowex columns (2ml of a 50% (w/v) slurry of Dowex-1 resin, 100-200 mesh, X8 in the formate form, BDH Chemicals Ltd., Dorset) [11]. Inositol and glycerophosphoinositol were eluted with 60mM ammonium formate/5mM sodium borate (12ml) (fraction 1) and inositol phosphates in 1M ammonium formate/ 0.1M formic acid (12ml) (fraction 2). The ratio of the radioactivity in fraction 2 : the radioactivity in fraction 1 was taken as a measure of PIP_2 breakdown.

Lipid peroxidation was determined in duplicate aliquots (1ml) of the slices at 30min intervals throughout the incubation period by measuring the formation of thiobarbituric acid-reactive material as previously described [8]. This assay was calibrated using malondialdehyde tetraethyl acetal (Sigma Chemical Company Ltd., Dorset).

Statistical analysis was performed using analysis of variance [12]. The residuals from the analysis of variance were checked for Normal distribution using the Shapiro-Wilks W test [13], and for equal variance in the atmosphere, chemical and time point groups using the Schweder test [14]. When constructing confidence intervals and comparing the means of groups using t tests the appropriate mean square error from the analysis of variance was used as the variance estimate.

RESULTS

Incubation of [^{3}H]-inositol-labelled kidney cortical slices resulted in the formation of radiolabelled inositol phosphate and smaller amounts of inositol bisphosphate and inositol trisphosphate (Fig. 1). There was no change in the rate of PIP_2 breakdown (measured by Dowex chromatography) in

the hypoxic slices during the 120 min incubation period in an atmosphere of N_2 compared with control slices incubated under aerobic conditions either in the presence of calcium or in calcium-free medium containing EGTA (Fig. 2). However, immediately upon reoxygenation of the hypoxic slices incubated in the presence of calcium, PIP_2 breakdown increased rapidly (Fig. 2). Statistical analysis of the results (Table 1) showed that this increase was highly significant ($P=0.0002$) and was maintained over the remaining 30 min of aerobic incubation (i.e. no significant fall in value; $P=0.21$). No increase in PIP_2 breakdown was observed when the slices incubated in the presence of calcium and oxygen for 120 min were regassed with 95% O_2: 5% CO_2 (Fig. 2, Table 1). Furthermore, reoxygenation of hypoxic slices in calcium-free medium (+EGTA) had no effect on the rate of PIP_2 breakdown and no significant changes occurred in slices incubated in the presence of O_2 and EGTA (Fig. 2, Table 1).

The formation of lipid peroxidation products increased during the first 60 min of incubation in the presence of O_2 and then levelled off over the remaining period (Fig. 3). Lipid peroxidation was also evident in the hypoxic slices but proceeded at a slower rate than that of the oxygenated samples ($P=0.002$ comparing overall mean of control vs overall mean of hypoxia up to 120 min) and also levelled off after 60 min (Fig. 3). Reoxygenation following 120 min of hypoxia resulted in a significant ($P<0.0001$) increase in the rate of lipid peroxidation which was not observed in the slices kept under N_2 or when slices incubated in the presence of oxygen for 120 min were regassed (Fig. 3).

DISCUSSION

The results clearly demonstrate that hydrolysis of phosphatidylinositols to secondary messenger products is activated very rapidly on reoxygenation of renal tissue following a period of hypoxia. This is the first report of such an effect and inhibition by EGTA strongly suggested the involvement of calcium. No changes in PIP_2 breakdown were observed during hypoxia itself. Recently it has been reported that phospholipase C activity, measured in synaptosomal fractions *in vitro*, is stimulated as a result of ischaemia of brain tissue *in vivo* [15]. In that report, metabolism of exogenous phosphatidylinositols added after the ischaemic period was measured whereas in our study hydrolysis of endogenous membrane-bound phosphatidylinositols was measured in kidney cortical slices actually during hypoxia and reoxygenation.

In agreement with our previous studies on whole rabbit kidneys rendered ischaemic *in vivo* [8], reoxygenation of cortical slices rendered hypoxic *in*

vitro resulted in an elevated rate of lipid peroxidation. This is likely to be the result of a number of events occurring both during hypoxia and reoxygenation including: redistribution of intracellular iron [16] which is involved in the initiation of lipid peroxidation; altered intracellular calcium homeostasis which activates phospholipase A_2 and renders membranes more susceptible to free radical attack [17]; and increased production of oxygen-derived free radicals due to mitochondrial dysfunction and activation of xanthine oxidase [18].

One of the products of lipid peroxidation, 4-hydroxynonenal has been shown to stimulate adenylate cyclase, guanylate cyclase and PIP_2 breakdown in vitro [19]. It is therefore possible that increased levels of aldehydic products produced on reoxygenation were responsible for increased PIP_2 hydrolysis. In addition, both lipid peroxidation and high calcium-dependent phospholipase A_2 activity alter membrane configuration [20] and this may affect the interaction of phospholipase C with membrane-bound regulatory components or make it more accessible to its substrate PIP_2. Alternatively altered calcium homeostasis may be involved in other ways. During ischaemia, cytosolic calcium levels may increase due to failure of energy-dependent pumps, although this perturbation did not effect PIP_2 hydrolysis during hypoxia. However, on reoxygenation much more dramatic changes to calcium homeostasis can occur due to loss of calcium-sequestering ability of damaged intracellular organelles and increased permeability of the plasma membrane allowing entry of extracellular calcium. It is possible that highly altered calcium levels on reoxygenation were sufficient to evoke a response of the phospholipase C system.

Rapid hydrolysis of PIP_2 on reoxygenation following ischaemia would lead to deregulation of receptor mediated function through this intracellular secondary messenger system. One of the consequences of this is likely to be an imbalance in eicosanoid production and vascular disturbances which may contribute to the pathogenesis of renal damage which occurs under these conditions.

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Table 1

The effect of reoxygenation on phosphatidylinositol breakdown in kidney cortical slices following incubation under hypoxic or aerobic conditions in the presence of calcium or EGTA

	O_2 + Ca^{++}	N_2 + Ca^{++}	O_2 + EGTA	N_2 + EGTA
	Phosphatidylinositol breakdown (ratio fraction 2/fraction 1 X 10^{-2})*			
pre-O_2 mean ratio	8.21	8.29	7.51	7.80
post-O_2 mean ratio	7.97	10.07	7.53	7.45
mean change	-0.24	+1.78	+0.02	-0.35
95% CI for change	-1.11,0.63	0.91,2.65	-0.85,0.89	-1.22,0.52
P-value+	0.58	0.0002	0.97	0.43

Analysis of variance: Mean square error = 0.746; Degrees of freedom = 42
Means are based on 8 separate determinations performed in duplicate ± 95% confidence interval (CI). Kidney cortical slices were incubated at 37°C in KRB containing calcium (2.5mM) or EGTA (10mM) under an atmosphere of N_2 or O_2 for 120 min (pre-O_2) and then regassed with O_2 (post-O_2). *PIP_2 breakdown was determined by anion-exchange chromatography as described in Methods.

+ P-value for t-test comparing pre-O_2 and post-O_2 mean rates.

FIGURE LEGENDS

Fig. 1. Analysis by hplc of inositol phosphate formation in rabbit kidney cortical slices labelled with ^{3}H-inositol and incubated at 37°C for 60 min in KRB. GPI - glycerophosphoinositol, IP_3 - inositol trisphosphate, IP_2 - inositol bisphosphate, IP_1 - inositol monophosphate.

Fig. 2. Phosphatidylinositol breakdown in kidney cortical slices incubated at 37°C in KRB containing calcium (2.5mM) (o) or EGTA (10mM) (0) for 120 min under an atmosphere of N_2 (- - - -) or O_2 (————) followed by reoxygenation and aerobic incubation for a further 30 min. Values represent the means of 8 separate determinations performed in duplicate. PIP_2 breakdown was determined by anion-exchange chromatography as described in Methods.

Fig. 3. Lipid peroxidation in kidney cortical slices incubated at 37°C in KRB for 120 min under an atmosphere of N_2 (o) or O_2 (0) followed by regassing with either O_2 (————) or N_2 (- - - -) and incubation for a further 30 min. Means are based on 6 separate determinations performed in duplicate ± 95% confidence limits. Analysis of variance: Mean square error = 195.1; Degrees of freedom = 17. Lipid peroxidation was measured as TBA-reactive material and expressed as malonaldehyde formation.

*$P<0.0001$ comparing pre-O_2 values with post-O_2 values (mean change = 56.6, 95% confidence interval = 39.6, 73.6).

Abbreviations:
KRB = Krebs-Ringer bicarbonate solution containing pyruvate (10mM)
PIP_2 = phosphatidylinositol-4,5,-bisphosphate.

FIGURE 1

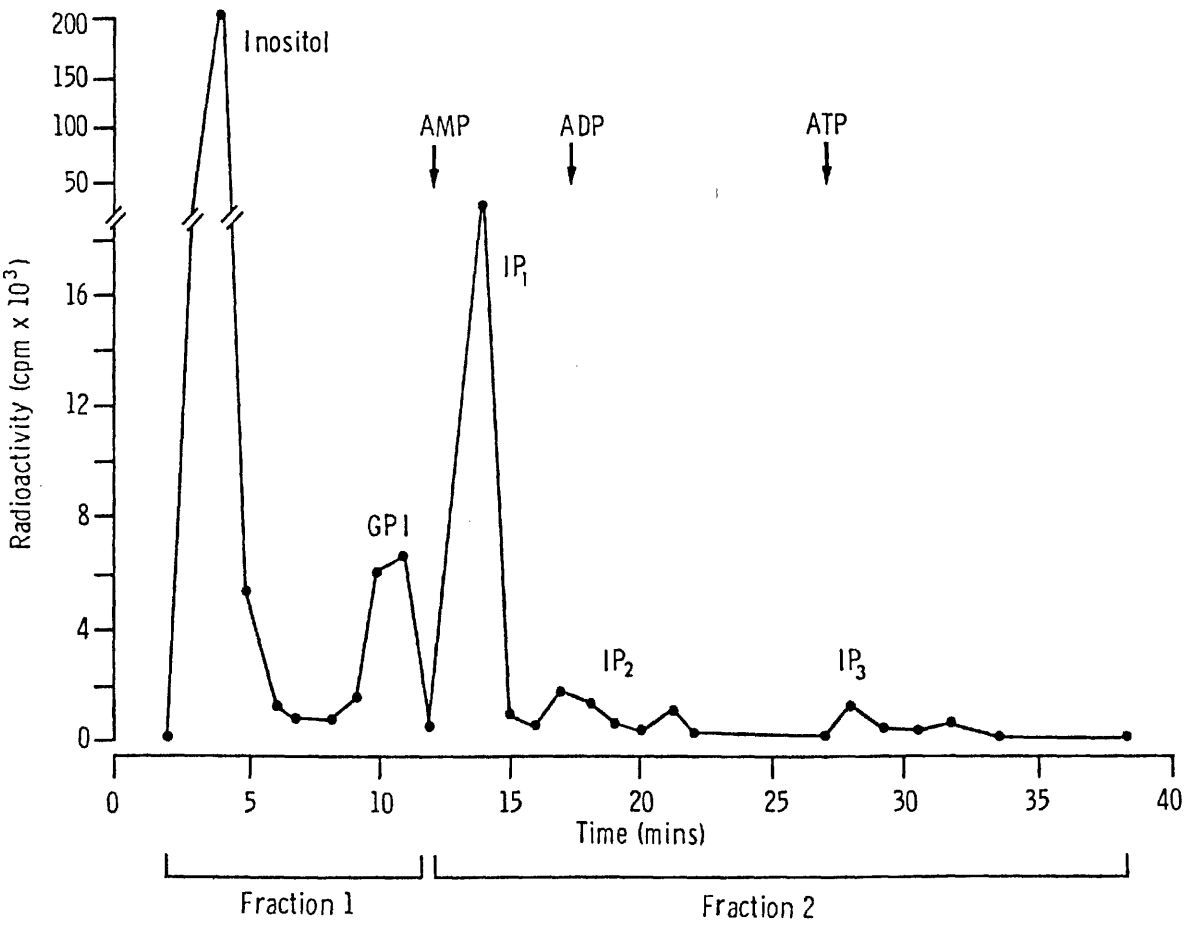

Inositol
AMP
ADP
ATP
GPI
IP1
IP2
IP3
Radioactivity (cpm x 10³)
200
150
100
50
16
12
8
4
0
0
5
10
15
20
25
30
35
40
Time (mins)
Fraction 1
Fraction 2

FIGURE 2

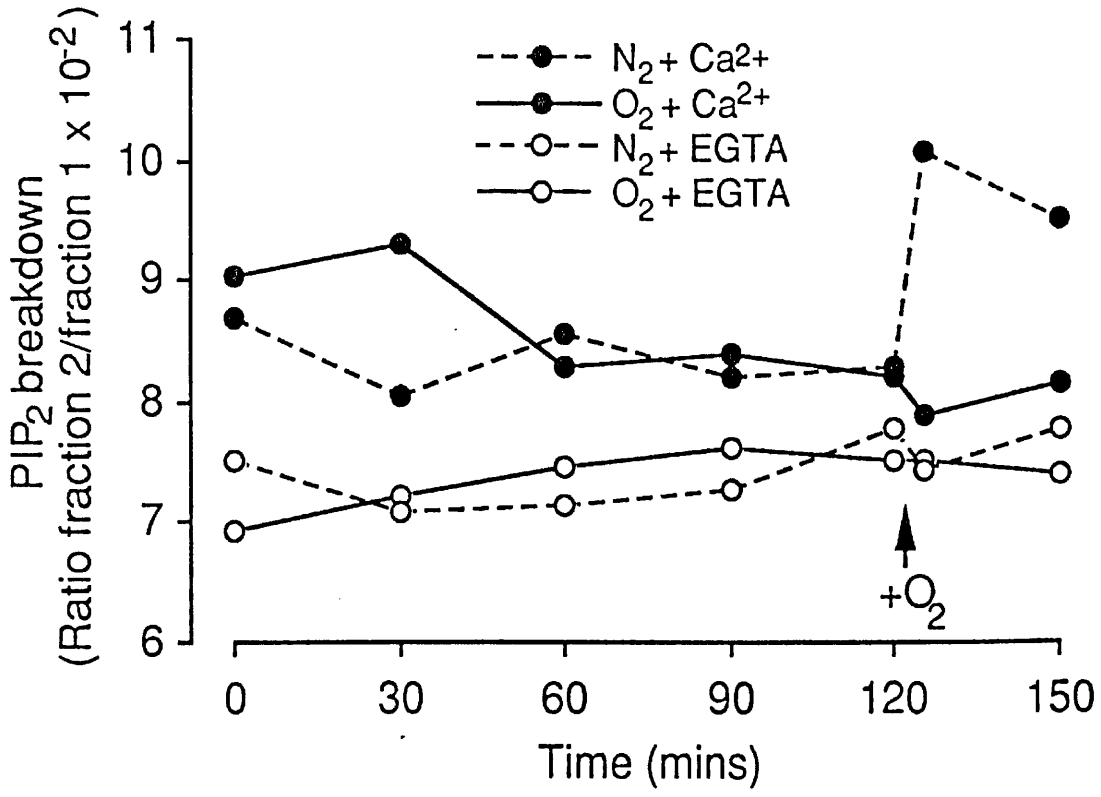
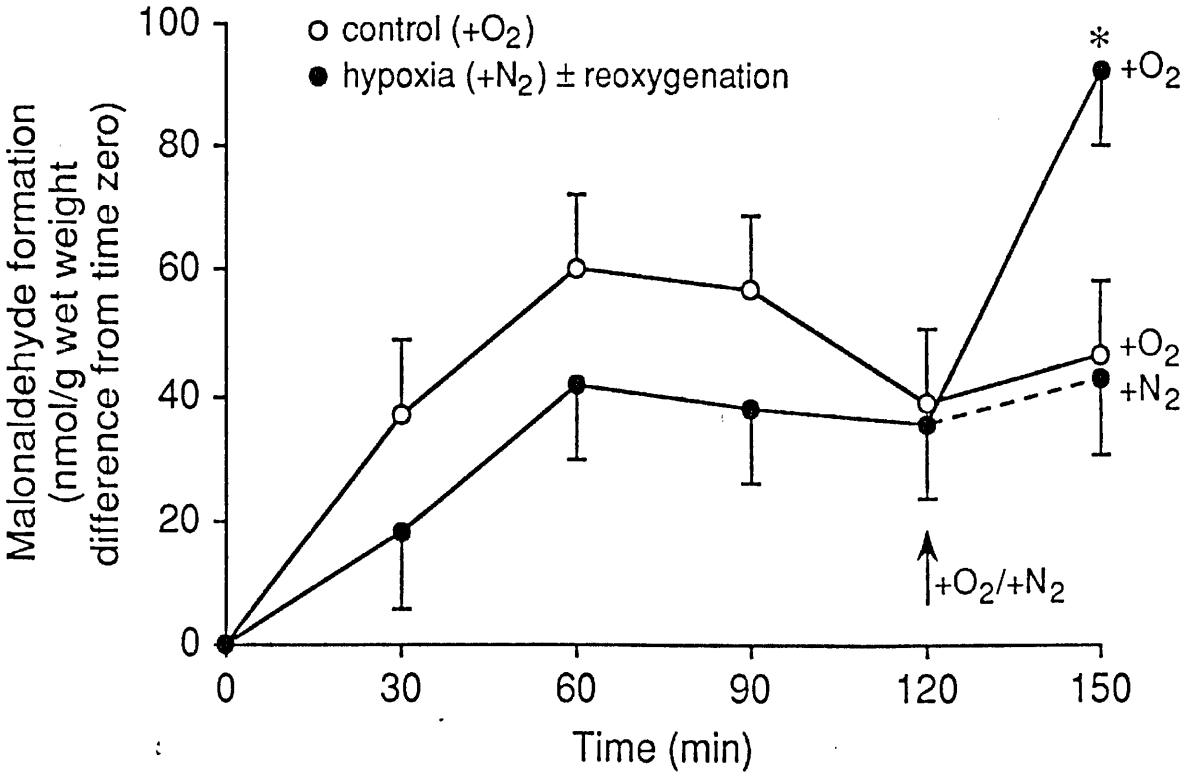

PIP2 breakdown
(Ratio fraction 2/fraction 1 x 10-2)
11
10
9
8
7
6
N2 + Ca2+
O2 + Ca2+
N2 + EGTA
O2 + EGTA
+O2
0
30
60
90
120
150
Time (mins)

FIGURE 3


100
80
60
40
20
0
Malonaldehyde formation
(nmol/g wet weight
difference from time zero)
o control (+O2)
● hypoxia (+N2) ± reoxygenation
*
+O2
+O2
+N2
+O2/+N2
0
30
60
90
120
150
Time (min)

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THE IMPORTANCE OF IRON, CALCIUM AND FREE RADICALS IN REPERFUSION INJURY: AN OVERVIEW OF STUDIES IN ISCHAEMIC RABBIT KIDNEYS

C.J. GREEN, J.D. GOWER, G. HEALING, L.A. COTTERILL, B.J. FULLER and S. SIMPKIN

Section of Surgical Research, MRC Clinical Research Centre, Northwick Park Hospital, Harrow, Middlesex HA1 3UJ

An overview of a series of experiments attempting to link iron and calcium redistribution and release of free fatty acids with falls in pH and adenine nucleotide levels during cold storage of rabbit kidneys is presented. The data reviewed strongly suggest that these events are inextricably linked to subsequent reperfusion injury. Circumstantial evidence incriminating iron was provided by experiments showing that iron chelation decreased reperfusion injury after warm (WI) and cold ischaemia (CI) in rat skin flap and rabbit kidney models. Evidence for a role for calcium was provided when it was found that a calcium channel blocking agent added to the saline flush solution before storage inhibited lipid peroxidation, whereas chemicals which caused release or influx of calcium into the cell exacerbated oxidative damage. Additional involvement of breakdown products of adenine nucleotides was suggested by the protection from lipid peroxidation afforded by allopurinol. Involvement of calcium-activated phospholipase A_2 was strongly suggested by increases in free fatty acids during cold storage and both this increase and lipid peroxidation were inhibited by addition of dibucaine to the storage solution.

KEY WORDS: Iron, calcium, reperfusion injury, ischaemia, kidney.

Retrieval of donor kidneys in optimum condition is one important aspect of clinical renal transplantation. The organs are usually subjected to a short period of warm ischamia (WI) between cessation of the blood supply and harvesting, followed by various periods of cold ischamea (CI) whilst they are stored and transported to the scheduled recipient. They are then rapidly reperfused with fully oxygenated whole blood as soon as the vascular pedicle is reconstructed. Typically, the preservation time in renal transplantation is less than 24 hours but may be as long as 72 hours: other organs are more sensitive to ischaemic damage, and it is considered inadvisable to store liver, heart or lung for longer than 4–12 hours. There is no clear CI time beyond which subsequent function can be predicted to fail, but it is an indictment of our current harvesting and storage techniques that in some centres as many as 50% of transplanted kidneys fail to function immediately, resulting in patients requiring dialysis until the organs recover sufficiently to support life.

The causes of deterioration during prolonged periods of *ex vivo* storage remain unresolved. Several pathological events have been confirmed including depletion of high-energy adenine nucleotides,[1] accumulation of metabolites, particularly H^+ ions,[2] autolysis by release of lysosomal enzymes,[3] damage to cellular membranes with loss of constituent phospholipids[4] and vascular injury that results in oedema, loss of circulating proteins, loss of erythrocyte deformibility, sludging and leakage of blood

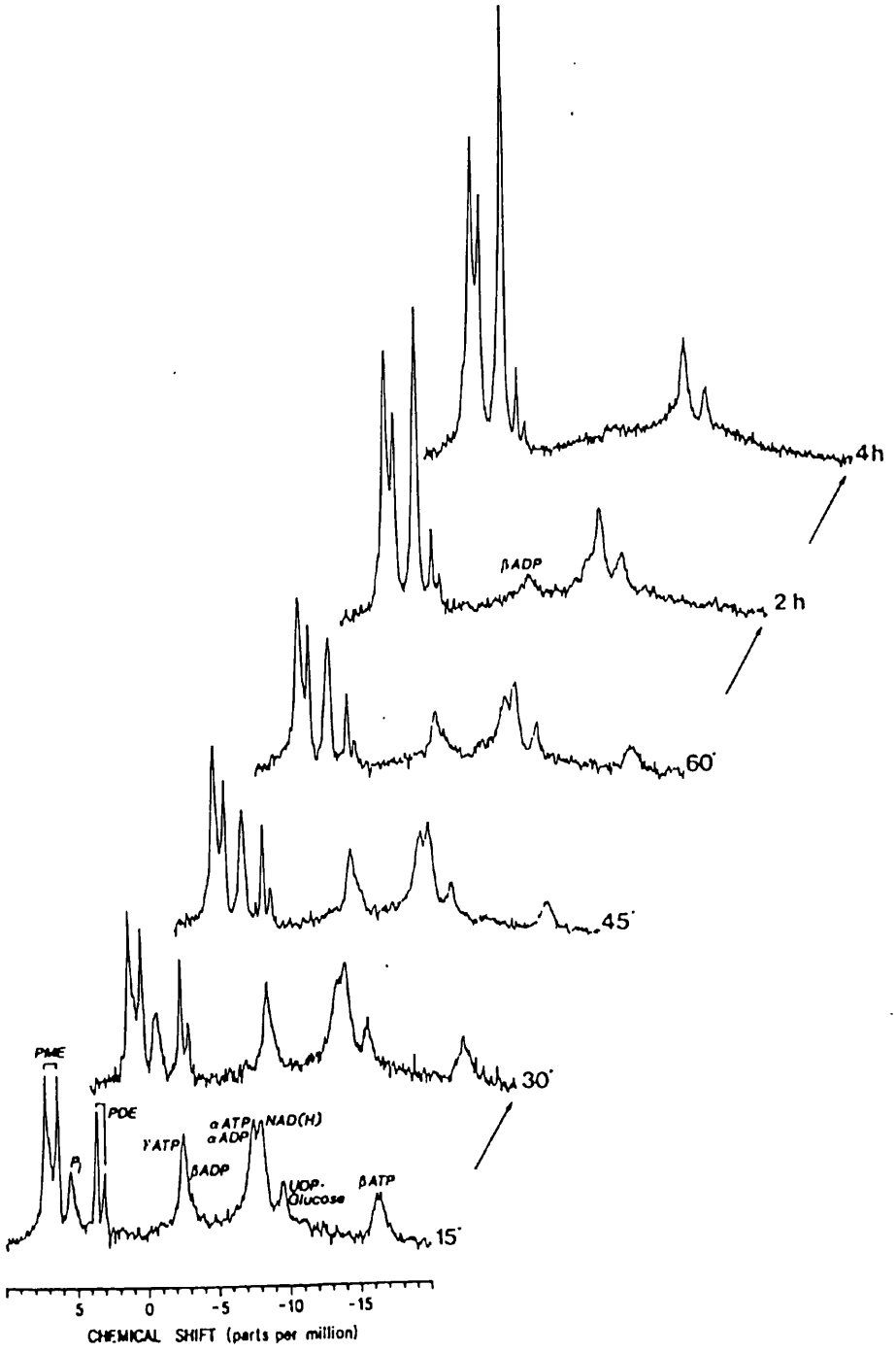

FIGURE 1 ^{31}Phosphorus nuclear magnetic resonance spectra for a rat liver stored at 4–8°C after vascular flush with hypertonic citrate solution. Time of cold storage increases from the first spectrum (bottom) at 15 min up to 4 hr. Signals for α, β, and γ phosphates of ATP, α and β phosphates of ADP, inorganic (P_i), phospho-mono and di-ester — PME, PDE are identified. It can be seen that during cold ischaemia, there is a decrease in ATP and ADP, with an increase in PME (including AMP) and P_i. There is also a fall in pH (not depicted) (after Fuller *et al.*, 1987). Reproduced with permission of the Society for Experimental Biology Symposium XXXX1.

cells into the extravascular compartment. Severely damaged kidneys are slow to perfuse when revascularised, become mottled in appearance and develop a microcoagulopathy which results within minutes in an outflow block and venous stasis. The spectrum of perservation damage is similar if not identical to that seen in simple normothermic renal ischaemia although hypothermia undoubtedly slows the rate of deterioration. For example, from our own studies using ^{31}P NMR spectroscopy with cold preserved rat liver and kidneys, ATP and ADP peaks virtually disappear after 2–4 hr CI and this is accompanied by a rise in inorganic phosphate levels (Figure 1). Measured pH falls to around 6.9 in 6–8 hr of simple refrigeration after flush with either Euro-Collins, hypertonic citrate or the recently formulated lactobionate-hydroxy ethyl starch (UW) solutions.[5] In contrast, in rabbit kidneys, such changes can be anticipated within 10–30 mins of WI and these organs are irreversibly damaged within 2 hours.[6]

It has become fashionable to talk about 'reperfusion injury' almost as if this occurs independently of the ischaemic period itself or as if the damage incurred during ischaemia was incidental rather than essential in predisposing the organ to failure. Mere commonsense dictates that this cannot be so. Nevertheless, it is true that at a visual level (both microscopic and macroscopic) many pathological changes only become evident after restoration of blood flow through the organ. For example, several workers have shown that most myocardial damage occurs during the early phases of recirculation and reoxygenation after normothermic ischaemia.[7,8] In some of our earlier experiments with rabbit kidneys attempting to correlate increases in lipid peroxidation either with warm or cold ischaemia *per se* or with additional effects attributable to reperfusion after transplantation, we noted significant increases in oxidative stress associated with reperfusion; in some cases, reperfusion injury was superimposed on increased lipid peroxidation attributable to ischaemia alone, whilst in others it was the only significant damage demonstrable.[6,9]

From that evidence and the work of others which provided circumstantial evidence incriminating oxygen-derived free radicals in the 'storage-damage' syndrome[10–12], we made two base line assumptions around which to design a series of experiments. The first was that lowered adenine nucleotide levels were central to a number of events *during* ischaemia; the second was that damage to the endothelium and its subsequent interraction with incoming blood would inevitably be linked to reperfusion injury. Accumulation of reducing equivalents and fall in pH could be expected to facilitate release of iron from ferritin stores and could therefore increase the susceptibility of the organ to lipid peroxidation on reperfusion. Decreased ATP levels upset Ca^{++} homeostasis as the differential between high extracellular (10^{-3}M) and low cytosolic (10^{-7}M) concentration is maintained by membrane-bound ATP-dependent pumps. Increased cytosolic Ca^{++} in turn would stimulate several Ca^{++}-dependent processes. For example, concentrations of free fatty acids (particularly polyunsaturated acids) would increase under the influence of Ca^{++}-dependent phospholipase A_2; Ca^{++}-dependent protein kinases would be activated; leukotrienes are produced as a consequence of enhanced lipoxygenase activity; and increased hypoxanthine from breakdown of adenine nucleotides would provide a substrate for xanthine oxidase (converted by Ca^{++}-dependent proteolysis from xanthine dehydrogenase) to produce superoxide radical anions and thence hydroxy radicals. Some of these events might be most important during ischaemia in predisposing the organ to reperfusion injury but then have indirect consequences during reperfusion. Increased free Ca^{++} and arachidonic acid released from membranes could, for example, upset the delicate

balance in prostaglandin turnover such that vasoconstriction and platelet aggregation was favoured in the vascular bed during reperfusion with blood. Likewise, the vasoconstrictor effects of leukotrienes would be important during reperfusion. This scenario is supported by evidence in which initial infusion with a prostacyclin analogue has been shown to protect rat livers after cold storage,[13] and continuous infusion of PGI_2 prevented microcoagulopathy during xenograft hyperacute rejection.[14] Furthermore in rats, subsequent development of acute tubular necrosis can be prevented if ischaemic kidneys are initially reperfused with diluted blood instead of whole blood.[15] After considering these possibilities, we decided to direct our studies toward direct and circumstantial evidence for the interrelationship between iron, Ca^{++} and free fatty acids in cold ischaemic damage using rabbit kidneys as one model and to seek pharmacological means of alleviating this damage in clinical renal transplantation.

THE ROLE OF IRON

We investigated whether iron is important in ischaemia and reperfusion injury by assessing the value of deferrioxamine (DFX) in a model of warm ischaemia (WI) in which we had previously demonstrated that significant rises in markers of lipid peroxidation Schiff bases (SB) and TBA-reactive material (TBARs) were proportional to 60 or 120 min WI and still further significantly increased after 60 min of reperfusion.[6] DFX, administered to rabbits at 15 or 50 mg/kg i.v. 15 min before reperfusion of kidneys which had been subjected to 60 or 120 min of WI, was found to inhibit detrimental deviations in each marker of lipid peroxidation including glutathione redox activity.[16] This was considered likely to reflect an effect of DFX *in vivo* rather than an *in vitro* effect during incubation of homogenates since relatively high concentrations of DFX had to be added directly to homogenates to achieve similar alterations in lipid peroxidation *in vitro*.[17] DFX also inhibited peroxidation when added to the saline solution used to flush rabbit kidneys prior to 24 hr storage at O°C but, in these experiments, the kidneys were not transplanted and reperfused.[18]

In another model of warm ischaemia and reperfusion injury[19], intravenous injection of DFX to rats 10 min before engraftment of vascularised skin flaps which had been totally ischaemic at 37°C for 14 hours, not only stopped the rise in markers of lipid peroxidation observed in untreated animals, but also prevented the flaps from becoming necrotic (100% necrosis in untreated rats, 8% in DFX treated rats).

In further experiments in which DFX was compared with other agents added to homogenates of cold-stored rabbit kidneys, it was found that DFX effectively inhibited lipid peroxidation in the renal cortex but less so in the medulla whereas the cyclooxygenase inhibitor indomethacin inhibited oxidative stress in the medulla rather than the cortex.[20] Based on this information, experiments were conducted to assess these two agents *in vivo* administered either separately or concurrently.[21] To mimic the clinical situation more closely, rabbit kidneys were flushed with hypertonic citrate solution (HCA) and stored at 0°C for 48 or 72 hours when they were either immediately homogenised and assayed for lipid peroxidation or were autotransplanted and then examined after 60 min reperfusion. There was a significant rise in SB and TBAR levels in both cortex and medulla after 48 and 72 hr storage compared with fresh unstored kidneys. These levels were further elevated when the kidneys were

replanted and reperfused for 60 min. Administration of DFX (50 mg/kg) 15 min before harvesting and before reperfusion reduced the production of SB and TBARs in the cortex after storage to significantly ($p < 0.001$) lower levels, indeed near to control levels. A significant ($p < 0.02$) reductiom was also observed in medulla. Indomethacin (3 mg/kg) alone had no effect on cortical levels of lipid peroxidation but significantly reduced SB and TBARs in the medulla ($p < 0.01$) following storage for 72 hrs. Administration of DFX (50 mg/kg) and indomethacin (3 mg/kg) together prior to storage effectively inhibited lipid peroxidation in both cortex and medulla when compared with untreated stored controls ($p < 0.001$) The combination of the two agents also effectively inhibited the observed rises in SB after 48 hr and 72 hr of CI with 60 min reperfusion.[21] Taken together with the data in which the agents were added to homogenates, these results provided some evidence for the existence of two separate pathways of lipid peroxidation in medulla of ischaemic kidneys — one iron-catalysed and the other cyclo-oxygenase catalysed.

Although these studies provided some circumstantial evidence of a role for iron in storage damage, we now wanted more direct evidence that iron was involved in its initiation. We therefore set out to develop a method for measuring levels of loosely bound iron available for chelation by DFX in tissue homogenates by reversed-phase HPLC and then used this to measure chelatable iron in the stored organs.[22] Rabbit kidneys were divided into cortex and medulla, homogenised in 0.1 M *Tris*-HCl buffer, pH 7.4, and centrifuged at 10,000 gav for 15 mins. Triplicate aliquots of supernatants were incubated with DFX (2 mM) for 1 hour, and the parent drug and its iron-bound form ferrioxamine (FX) were extracted using Bond-Elut c_{18} cartridges. Quantitation of the two forms of drug was achieved using reversed-phase HPLC with u.v. detection and the ratio of FX:DFX was calculated. The amount of chelatable iron in each sample was determined from a standard curve obtained from triplicate standards containing 0, 10 or 25 nmoles iron/ml subjected to the same procedure. The total iron content of the samples was determined by atomic absorption spectroscopy.[22]

Measurement of the FX:DFX ratio in kidney homogenates revealed significant increases in the DFX-chelatable iron pool in both the medulla and cortex after warm and cold ischaemia. In fact, it was virtually doubled after 2 hr of WI and after 24 hr of cold storage at 0°C, whereas the total iron pool remained essentially unchanged.[22] Hence, it appears that iron had been redistributed to more available forms as a result of ischaemia. Since the release of ferritin-bound iron involves reduction of ferric iron to the ferrous state, and we know that pH falls and reducing species accumulate as a result of ischaemia, it is likely that the extra available iron measured was derived from ferritin. It cannot, of course, be stated with certainty that the iron measured in this study is in a form which is available to catalyse the initiation of lipid peroxidation. However, the availability of iron for chelation with DFX strongly suggests that it is available for catalysis of free radical reactions. Redistribution of iron to more accessible pools as a result of ischaemia may be an important factor underlying the increased levels of lipid peroxidation under these circumstances, and provides a biochemical explanation for the effectiveness of DFX.

These studies were extended still further[23] by taking rabbit kidneys which had been subjected to 2 hr WI or 24 hr CI and reperfusing them *ex vivo* at 37°C on a suitable circuit using an oxygenated ($95\%O_2$:$5\%CO_2$) asanguinous crystalloid solution to which had been added haemaccel to provide colloid osmotic pressure. After perfusion for 5, 10, 15, 30 or 60 min the kidneys were removed from the circuit and assayed for

DFX-chelatable iron as above. It was found that although iron levels were raised initially, they returned to normal within 15 min of reperfusion. This period, although short, would be sufficient for initiation of free radical reactions and reperfusion injury.

THE ROLE OF CALCIUM

Raised concentrations of cytoplasmic Ca^{++} have been linked to irreversible cell injury in many systems[24] but whether this is the common denominator *initiating* cell death or is merely a post-mortem *result* of cell death is difficult to determine. Some studies do suggest that Ca^{++} must be a prime suspect as the trigger for a catastrophic cascade of events. For example, the presence of Ca^{++} has previously been reported to induce damage to isolated renal mitochondria *in vitro*[25] and potentiated oxygen free radical-mediated damage to plasma membranes in isolated hepatocytes.[26] As the differential between high extracellular (10^{-3} M) and low cytosolic (10^{-7} M) concentrations is normally maintained by membrane-bound ATP-dependent pumps which extrude Ca^{++} from the cell, and sequester excess cytosolic Ca^{++} in mitochondria and endoplasmic reticulum, it would not be surprising if this mechanism failed as ATP levels fall during ischaemia. This idea is supported by some studies which demonstrated that Ca^{++} homeostasis is deranged in tissues after WI[27] and that cytosolic Ca^{++} levels rise in isolated cells exposed to anoxia.[28]

We investigated these possibilities by studying rabbit kidneys which had been flushed before cold storage with either a poor preservation solution (isotonic saline) or with hypertonic citrate solution (HCA) which is used routinely in clinical renal transplantation. To these solutions were added various agents which either affect Ca^{++}movements or interfere with enzymes which are thought to require Ca^{++} for their activation. Assessment was again based on alterations in lipid peroxidation following *in vitro* incubation of homogenates of stored organs. The agents used were verapamil (100 μM), a Ca^{++}-channel blocker; A23187 (10 μM), an ionophore which renders membranes permeable to Ca^{++}; calcium chloride (1 mM $CaCl_2$) to increase extracellular concentrations of Ca^{++}; ruthenium red (5 μM) which inhibits mitochondrial Ca^{++} uptake; dibucaine (250 μM) which inhibits Ca^{++}-activated phospholipases by competing with Ca^{++}-binding sites; and allopurinol (5 mM), a xanthine oxidase inhibitor.

Addition of the Ca^{++}-channel blocking agent verapamil to the isotonic saline solution used to flush and store kidneys for 24 hours, resulted in significant inhibition in formation of lipid peroxidative products encountered in untreated stored control kidneys both in cortex (Figure 2) and medulla (figure 3); indeed, the markers for peroxidation were close to levels measured in fresh unstored kidneys. Since the addition of verapamil directly to kidney homogenates had no effect on lipid peroxidation, it appears likely that the protective effect of verapamil was due to its ability to prevent the influx of extracellular Ca^{++} into the cytosol of intact cells *in vivo* during the storage period.[29]

The importance of extracellular Ca^{++} in mediating oxidative damage to cold stored kidneys was also suggested by the observation that addition of $CaCl_2$ (1 mM) to the saline storage medium resulted in a significant increase in peroxidative markers. Furthermore, permeabilisation of the cell membrane to Ca^{++} by addition of the ionophore A23187 also significantly increased these indices (Figures 2 and 3). Inhibi-

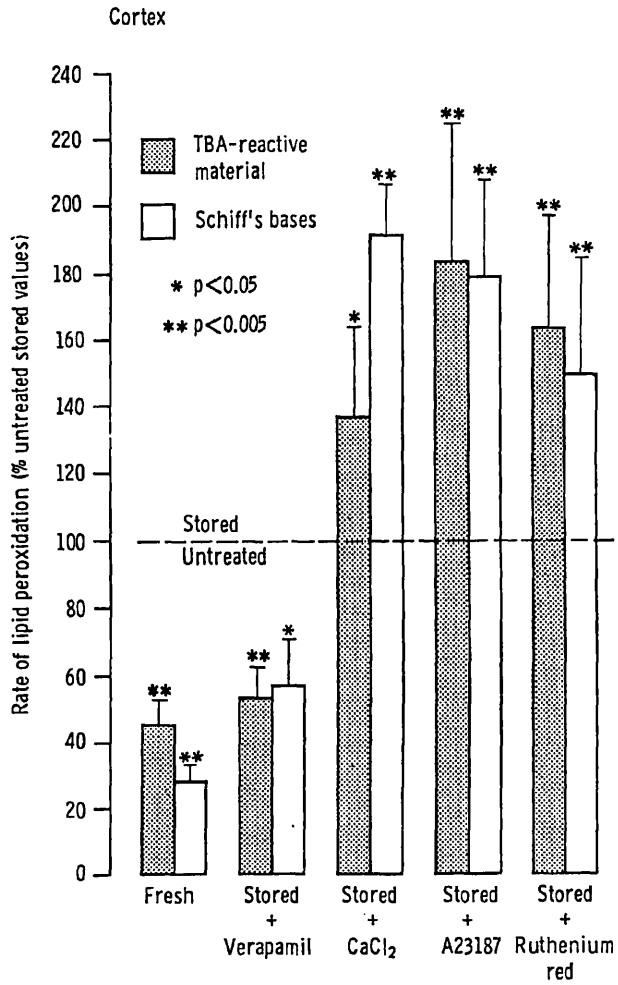

FIGURE 2 The rate of formation of two markers of lipid peroxidation (TBA-reactive material and Schiff's bases) in homogenates of renal CORTEX from kidneys flushed and stored for 24 hours at O°C in isotonic saline containing verapamil (100 μM), $CaCl_2$ (1 mM), A23187 (10 μM) and ruthenium red (5 μM). Values are mean $\pm$ S.E.M (n = 6) expressed as % of control (stored in saline only). 100% values: TBA: 0.81 $\pm$ 0.17 nmol malonaldehyde/hr/mg protein; Schiff's base: 4.67 $\pm$ 1.04 Fluorescence units/hr/mg/protein.

tion of mitochondrial Ca^{++} uptake by ruthenium red during cold storage also resulted in a significant increase in membrane peroxidation, suggesting an important role for mitochondrial sequestration of Ca^{++}.

Addition of verapamil or $CaCl_2$ to HCA, however, did not affect the rise in lipid peroxidation observed after 72 hr cold storage. This was thought likely to be due to the ability of the large excess of citrate (55 mM) in this medium to itself chelate Ca^{++} during the storage period. Measurement of the free Ca^{++} concentration when various amounts of calcium were added to the citrate solution demonstrated the very effective Ca^{++} buffering capacity of this medium and supported this conclusion — for example, the free Ca^{++} concentration was only 1 μM in the presence of 100 μM total calcium and 10 μM when 1 mM calcium was added. This property of citrate may therefore contribute to the well known efficacy of HCA, although the increased rate of lipid peroxidation observed after 72 hr storage suggests that significant amounts of

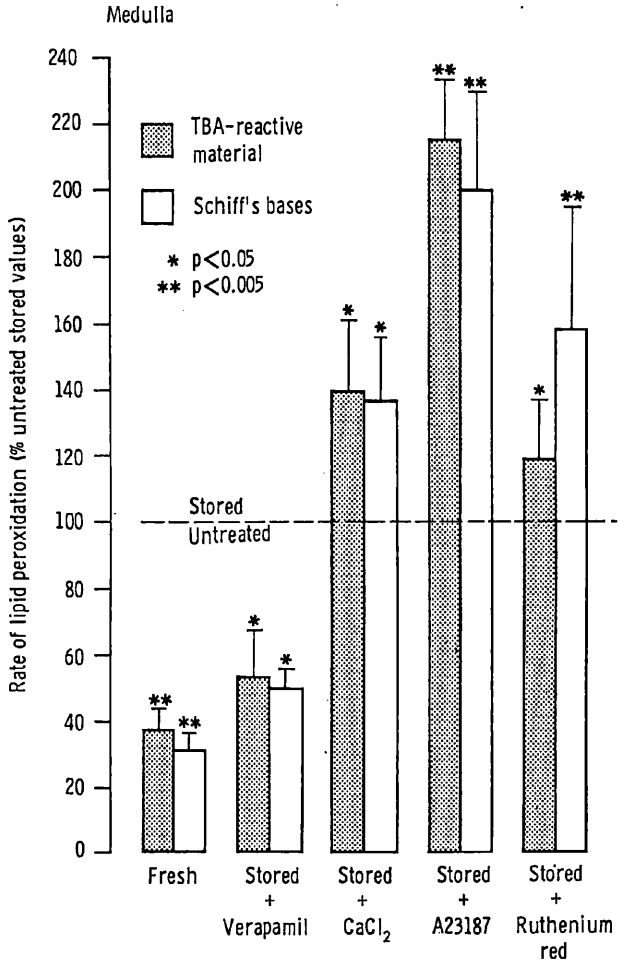

FIGURE 3 The rate of formation of two markers of lipid peroxidation (TBA-reactive material and Schiff's bases) in homogenates of renal MEDULLA from kidneys flushed and stored for 24 hours at 0°C in isotonic saline containing verapamil (100 μM), $CaCl_2$ (1 mM), A23187 (10 μM) and ruthenium red (5 μM). Values are means $\pm$ S.E.M. (n = 6) expressed as % of control (stored in saline only). 100% values: TBA: 1.35 $\pm$ 0.26 nmol malonaldehyde/hr/mg protein; Schiff's base 5.70 $\pm$ 1.48 fluorescence units/hr/mg protein.

Ca^{++} may still enter the cell over an extended period or that other mechanisms may be responsible.

There are several mechanisms by which increased cytosolic Ca^{++} levels could potentiate free radical damage to cell membranes. Phospholipase A_2 is activated by Ca^{++} and removes fatty acids from membranes by a process which does not require oxygen or energy and could function during the storage period. However, both fatty acid catabolism and reincorporation of fatty acids back into phospholipids do require ATP and will therefore be slowed during anoxia. Hence, free fatty acids are likely to accumulate, and configurational disturbances in membranes due to accumulation of lysophosphatides could, as a result, render them more susceptible to free radical attack and peroxidation. Consistent with this hypothesis is the demonstration that dibucaine, an inhibitor of phospholipase A_2, decreased the formation of TBARs when added to the saline flush. Furthermore, addition of dibucaine together with A23187, inhibited the rises described above when A23187 was added alone.

Increased cytosolic Ca^{++} may also be important in enhancing free radical production by activating proteases which convert the enzyme xanthine dehydrogenase to xanthine oxidase.[30] This would be particularly important during reperfusion of compromised cells presented with molecular oxygen; the oxidase utilizes hypoxanthine which has accumulated from adenine nucleotide degradation during ischaemia, to yield xanthine, superoxide anion and, in the presence of transition metal catalysts, hydroxyl and iron-complexed free radicals which are highly reactive. In our experiments, addition of the xanthine oxidase inhibitor allopurinol alone to the saline flush certainly inhibited the rate of peroxidation. When it was added together with A23187 it prevented the increased peroxidation encountered when A23187 was added alone.

The data obtained in this group of experiments strongly suggest that Ca^{++} redistribution takes place during cold storage of kidneys resulting in elevated cytosolic Ca^{++} levels. Several reasons are likely. Influx of extracellular Ca^{++} may result from the inability of anoxic cells to maintain a Ca^{++} gradient across the membrane as this is dependent on ATP-pumps. In addition, low temperatures depress membrane fluidity and this could also influence Ca^{++} movements across cell membranes. Lipid peroxidation of the cell membrane may render it permeable to Ca^{++} which would therefore leak in from the extracellular space. Intracellular Ca^{++} redistribution could also contribute to elevated cytosolic levels during ischaemia as energy-dependent sequestration by mitochondria and endoplasmic reticulum is depressed.

To investigate the possible activation of phospholipase A_2 as a result of raised cytosolic Ca^{++} levels, we then carried out a series of experiments, in which free fatty acids (FFA) were measured directly by gas liquid chromatography. Again using the rabbit kidney model with the organ flushed with saline and stored for 48 hr, or with HCA and stored for 72 hr, we found that increasing concentrations of FFA correlated with increases in TBA reactive markers of lipid peroxidation. Addition of dibucaine (250 μM) to the flush solution inhibited these rises in both FFA and TBARs whereas addition of the Ca^{++} ionophore A23187 alone lead to significant increases in FFA and TBARs over and above the increases detected in the stored untreated kidneys. Perhaps of greatest importance, the increases in polyunsaturated fatty acids were significantly greater than saturated fatty acids and the release of arachidonic acid was most marked when A23187 was added to the solution.

CONCLUSIONS

Though far from resolving the spatial and temporal sequence of these events, we conclude from these data that an early fall in adenine nucleotide levels accompanied by increased H^+ is associated *during* cold ischaemia with increased cytosolic Ca^{++} levels, a redistribution of iron from ferritin stores and activation of phospholipases which release fatty acids from the confines of phospholipid bi-layers. The organ is thus rendered highly susceptible to reperfusion injury in two possible ways. First, exposure to high concentrations of molecular oxygen on reperfusion allows free radical reactions to overwhelm any remaining scavenging capacity and may proceed to irreversible peroxidative damage. Second, raised arachidonic acid metabolism via cyclo-oxygenase pathways may upset the balance in the vascular bed to favour constriction and platelet aggregation. It is unlikely that the two processes are mutually exclusive.

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A Role for Calcium in Cold Ischaemic Damage to the Rabbit Kidney?

Lisa A. Cotterill, Jon D. Gower, Barry J. Fuller*, Colin J. Green

Surgical Research Group, Division of Comparative Medicine,
Clinical Research Centre, Watford Road, Harrow, Middlesex, HA1 3UJ.

*Academic Department of Surgery, Royal Free Hospital,
School of Medicine, Pond Street, Hampstead, London, NW3 2QG.

Abstract

This study describes an investigation into the possible role of calcium ions in the oxidative damage to kidneys which takes place as a result of cold storage.

Rabbit kidneys were stored at $0^{o}C$ for 24 hours in isotonic saline (a poor storage medium) or for 72 hours in hypertonic citrate solution (HCA) (a good storage medium). These periods of storage resulted in significant increases in lipid peroxidation in kidney homogenates which were reduced when the calcium antagonist verapamil was added to the saline solution. Addition of calcium chloride or the calcium ionophore A23187 to the saline solution significantly increased the rate of lipid peroxidation in homogenates of the stored organs.

Addition of these substances to the HCA storage medium had no effect on oxidative damage to the kidney which may be attributable, at least in part, to the ability of citrate present in this solution to chelate calcium.

These results demonstrate a relationship between lipid

peroxidation and calcium fluxes and suggest a role for this cation in the oxidative damage resulting from cold ischaemia.

Introduction

Renal transplantation involves periods of both cold ischaemia (organ storage) and warm ischaemia (removal and replantation of the organ) followed by reperfusion with oxygenated blood. These procedures often result in poor renal function and death of tubular cells immediately after engraftment. There are likely to be several causes for this damage.

Investigations in a number of systems have linked irreversible cell injury with increases in the concentration of calcium in the cytoplasm (1). This suggests the possibility that elevated intracellular calcium levels may be the common denominator which triggers cell death and it is therefore possible that calcium ions play a role in the degenerative processes resulting from combined warm ischaemia and cold storage of kidneys.

Under normal conditions the cytoplasmic calcium concentration is 10^3-10^4 fold lower than that of the extracellular fluid. Low cytoplasmic calcium levels are maintained by a number of processes: membrane-bound ATP-dependent pumps actively remove calcium from the cell and excess calcium is sequestered by mitochondria and the endoplasmic reticulum. Dysfunction of these systems results in an increase in cytoplasmic calcium levels which lead to biochemical

changes, alterations in the morphology of the cell and eventually to irreversible cell death.

There is growing evidence that oxygen-derived free radicals are responsible, at least in part, for reperfusion injury and we have shown that cold storage of rabbit kidneys results in highly elevated levels of lipid peroxidation products formed during the incubation of homogenates in vitro (2,3). Lipid peroxidation affects calcium homeostasis in a number of ways. Peroxidation renders the cell membrane more permeable to calcium ions which readily leak in from the extracellular milieu. Oxidative damage to the mitochondrion and endoplasmic reticulum membranes partially destroys their ability to sequester calcium, so leading to an increase in cytoplasmic free calcium (4,5).

Elevated cytoplasmic calcium levels result in the activation of several biochemical processes which are likely to have important consequences to ischaemic tissues. Activation of calcium dependent phospholipases results in further breakdown of cell membranes (6), the release of free fatty acids and the formation of lysophosphatides which are toxic to the cell (6). Phospholipase activity is the rate limiting step in prostaglandin synthesis and lipoxygenation (7) thus these pathways may be stimulated following ischaemia. An alteration in prostaglandin homeostasis and the formation of lipoxygenase products some of which are chemotactic and cause vasoconstriction, such as

leukotriene B4 (8,9) are likely to result in vascular changes which may contribute to the "no-reflow" phenomenon.

A number of ATPases are also activated as a consequence of calcium overload and these will consume valuable ATP resources already made scarce by the ischaemic conditions (10). In addition, xanthine dehydrogenase is converted to xanthine oxidase by calcium-dependent proteolysis and this enzyme may be responsible for the production of oxygen-derived free-radicals during reperfusion (11).

During the early stages of cell injury, redistribution of intracellular calcium pools may be important. It has been shown that when kidneys are subjected to periods of warm ischaemia, mitochondrial dysfunction coupled with ATP depletion results in failure of the calcium uptake processes of this organelle (12). Renal ischaemia also decreases the ability of endoplasmic reticulum to sequester calcium (13). Thus a redistribution of intracellular calcium takes place and an increase in cytosolic calcium levels has been observed in cultured kidney cells subjected to anoxia (14). During reperfusion there is an influx of extracellular calcium as a consequence of surface membrane changes resulting in massive calcium overload. Some of this calcium accumulates in the mitochondria, probably by passive processes, leading to further mitochondrial and cellular damage (15).

Prevention of ischaemic damage before transplantation continues to pose a major challenge and there is currently considerable

interest in the use of calcium antagonists, particularly slow calcium channel blockers such as verapamil. Certainly, it would appear that verapamil affords some protection to kidneys subjected to periods of cold or warm ischaemia followed by reperfusion (16-20). The beneficial effects of calcium antagonists may be due to changes in renal hemodynamics in the reperfusion period as well as direct effects on calcium fluxes.

The relationships between lipid peroxidation, calcium ions and cell death are complex and as yet not fully understood. The present experiments were designed to investigate the possible relationships between calcium ions and lipid peroxidation in kidneys stored at $0^{\circ}C$. The organs were either flushed and stored at $0^{\circ}C$ in isotonic saline solution (a poor storage medium) for 24 hours or in hypertonic citrate (HCA) solution (a good storage medium currently used in clinical transplantation) for 72 hours. The effects of adding either the calcium-channel blocking agent verapamil, calcium chloride or the calcium ionophore A23187, which evokes an increase in cytoplasmic calcium levels, to these storage solutions on the rate of lipid peroxidation in homogenates of medulla and cortex were studied. These experiments were designed to gain a better understanding of the degenerative processes which take place under these conditions with a view to improving the viability of stored organs awaiting transplantation.

Materials and Methods

Chemicals: Verapamil hydrochloride was purchased from Abbott Laboratories Ltd (Kent) and the calcium ionophore A23187 was purchased from Sigma Chemical Company Ltd (Poole, Dorset). Other reagents were obtained from standard suppliers and were of the highest quality available.

Operative Procedure: Eight groups of adult New Zealand white rabbits (average weight 3kg) were anaesthetised by intramuscular injection of 0.2ml/kg fentanyl-fluanisone (Hypnorm ®), followed by a slow intravenous injection of diazepam (valium 20 ®) at 1.0mg/kg. Frusemide (3mg/kg) and 300 i.u./kg of heparin were also administered by intravenous injection. The abdomen was opened by a mid-line incision and the kidneys were completely skeletalised and removed. The renal artery of each kidney was cannulated and the organs flushed with 30mls of sterile HCA or 30mls of sterile isotonic 0.9% sodium chloride solution (saline) already cooled to $4^{\circ}C$. The kidneys were placed in sterile beakers containing 60mls of the identical flush solution and stored in ice at 0° for 24 hours in saline or 72 hours in HCA.

Kidneys were treated according to the protocol outlined below. In experiments in which kidneys were stored in solutions containing verapamil (100μM), calcium chloride (1mM) or A23187 (10μM), the contralateral kidneys were stored under identical conditions without the addition of these agents thus providing

controls.

Experimental Protocol:

Group (1) : Kidneys were not stored.

Group (2) : Kidneys were flushed with 30mls 0.9% w/v sodium chloride and stored for 24 hours at 0°C. Verapamil (100μM) was added to homogenates of these kidneys after storage.

Group (3) : Kidneys were flushed with 30mls HCA and stored for 72 hours at 0°C. Verapamil (100μM) was added to homogenates of these kidneys after storage.

Group (4) : Kidneys were flushed with 30mls 0.9% w/v sodium chloride solution containing 100μM verapamil and stored for 24 hours at 0°C.

Group (5) : Kidneys were flushed with 30mls HCA containing 100μM verapamil and stored for 72 hours at 0°C.

Group (6) : Kidneys were flushed with 30mls 0.9% w/v sodium chloride solution containing 1mM $CaCl_2$ and stored for 24 hours at 0°C.

Group (7) : Kidneys were flushed with 30mls HCA containing 1mM $CaCl_2$ and stored for 72 hours at 0°C.

Group (8) : Kidneys were flushed with 30mls 0.9% w/v sodium chloride solution containing 10μM A23187 and stored for 24 hours at 0°C.

Preparation of the Kidneys: Kidneys were dissected into cortex and medulla, homogenised in phosphate-saline buffer (pH 7.4)

and incubated at 37°C for 60 mins in a shaking water-bath. The rate of lipid peroxidation was determined by the measurement of lipid soluble Schiff's bases and the formation of TBA-reactive material and related to the protein content of the homogenates as described previously (3).

Statistical analysis was performed using the students paired t-test.

Results

As previously reported (2,3), the cold storage of kidneys in either isotonic saline solution or HCA solution resulted in elevated levels of lipid peroxidation products in homogenates of both the cortex and medulla (Figures 1 and 2).

Addition of verapamil to homogenates of kidneys after storage (Groups 2 and 3) had no significant effect on the formation of Schiff's bases or the production of TBA-reactive material (data not shown).

When kidneys were flushed with isotonic saline solution to which verapamil had been added (Group 4), significant decreases were observed in both measures of lipid peroxidation in the cortex and medulla (Figures 1 and 2). However verapamil had no effect when added to the HCA storage solution (Group 5, data not shown).

Addition of $CaCl_2$ to the isotonic saline solution (Group 6) significantly increased the rates of lipid peroxidation in both the cortex and medulla of the stored kidney (Figure 3) but no effects were observed when $CaCl_2$ was added to the HCA storage

Figure 1

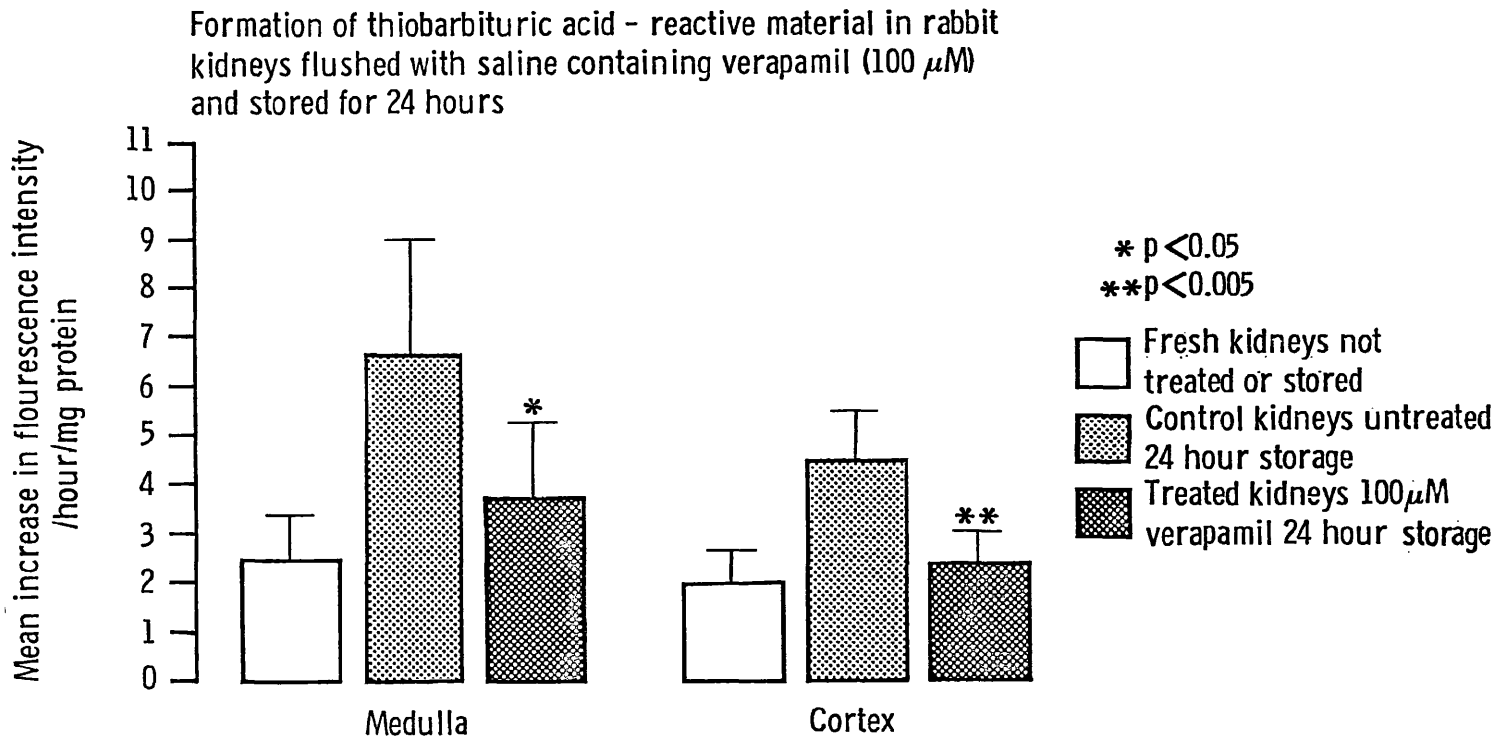

Formation of thiobarbituric acid - reactive material in rabbit kidneys flushed with saline containing verapamil (100 μM) and stored for 24 hours
Mean increase in flourescence intensity /hour/mg protein
11
10
9
8
7
6
5
4
3
2
1
0
*
**
Medulla
Cortex
* p <0.05
**p <0.005
Fresh kidneys not treated or stored
Control kidneys untreated 24 hour storage
Treated kidneys 100μM verapamil 24 hour storage

Figure 2

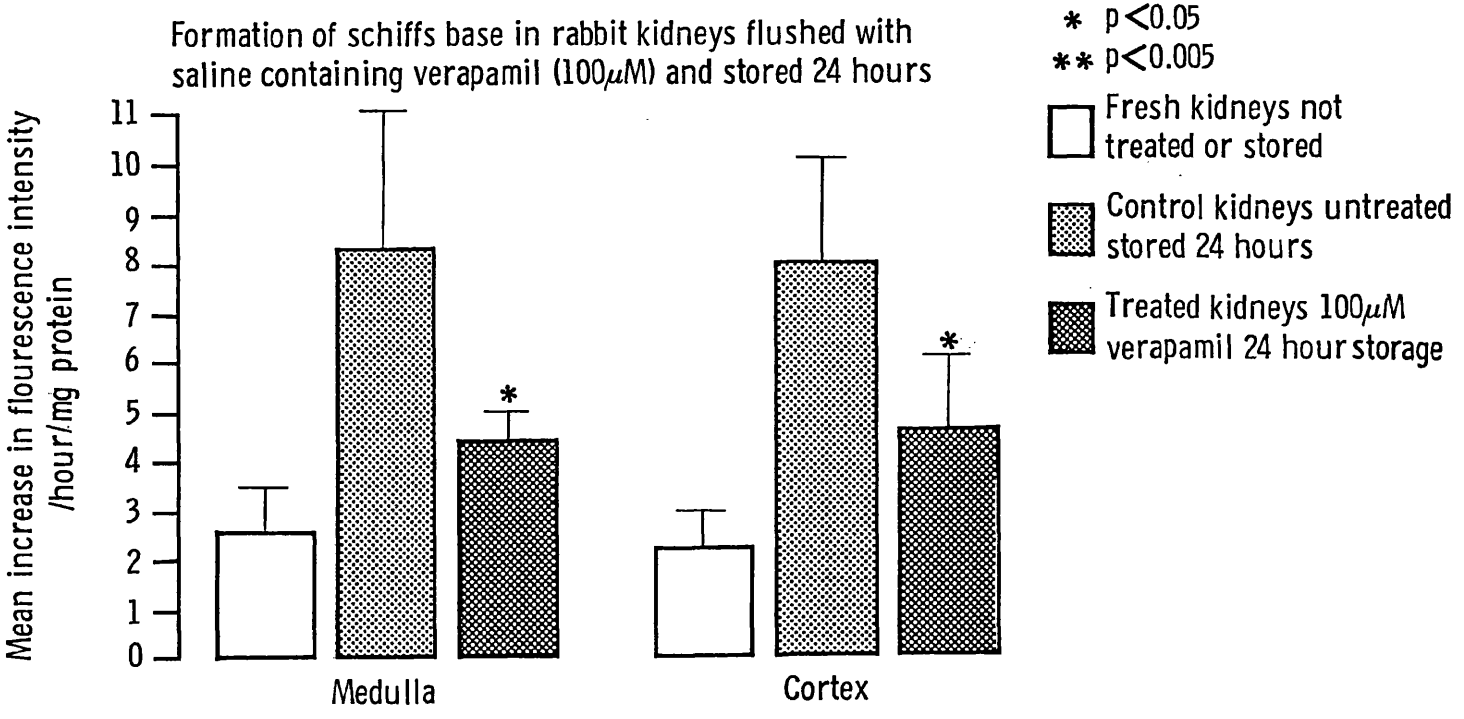
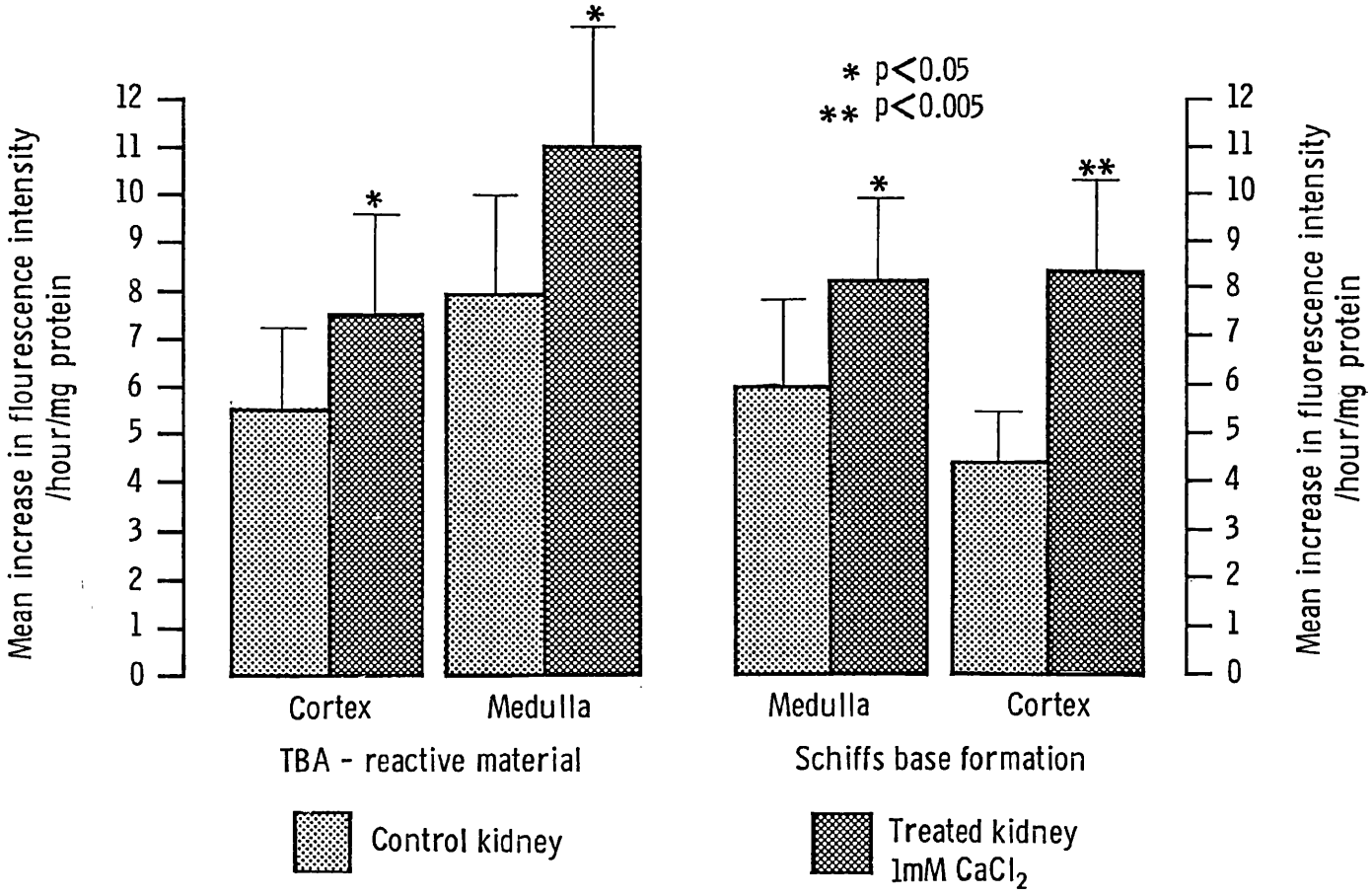

Formation of schiffs base in rabbit kidneys flushed with saline containing verapamil (100μM) and stored 24 hours
Mean increase in flourescence intensity /hour/mg protein
0
1
2
3
4
5
6
7
8
9
10
11
*
*
Medulla
Cortex
* p<0.05
** p<0.005
Fresh kidneys not treated or stored
Control kidneys untreated stored 24 hours
Treated kidneys 100μM verapamil 24 hour storage

Figure 3

Formation of thiobarbituric acid-reactive material and schiffs base in rabbit kidneys flushed with saline containing $CaCl_2$ and stored for 24 hours



storage solution (Group 7, data not shown).

Addition of the calcium ionophore A23187 to the isotonic saline solution (Group 8) significantly increased both Schiff's base formation and the production of TBA-reactive material in the cortex and medulla of the stored kidney (Figure 4).

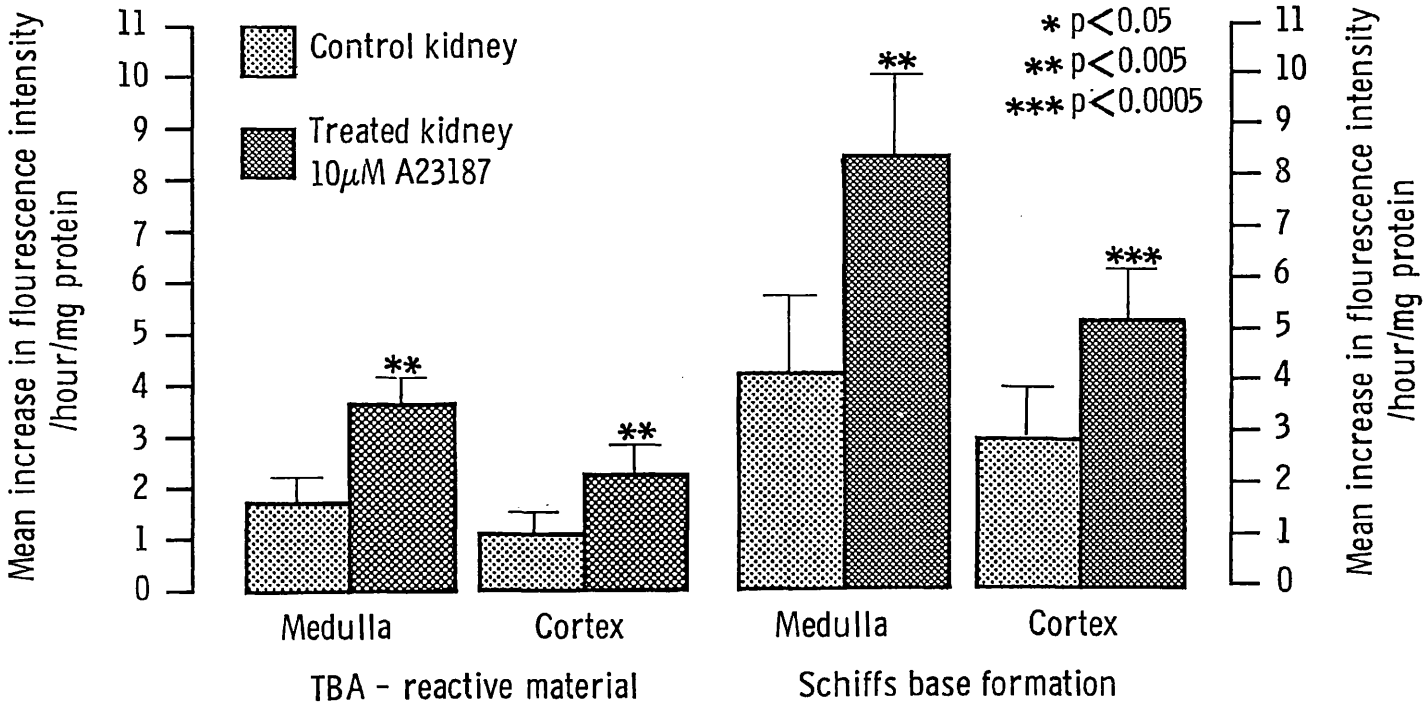
Discussion

These results demonstrate that increases in peroxidative damage during cold ischaemia of kidneys may be attributable in part to influx of calcium into the cytosol. Verapamil decreased lipid peroxidation in kidneys stored 24 hours in saline but had no beneficial effect when added directly to homogenates of kidneys after the storage period. It seems likely that the effect of verapamil is modulated through its ability to decrease calcium influx into the cytosol of intact kidney cells during the storage period rather than an interaction with intracellular components in the homogenised tissue.

Verapamil had no effect on lipid peroxidation in kidneys stored for 72 hours in HCA storage solution. Hence, the known value of HCA as a good storage medium compared to saline may be partly due to the ability of citrate present in the HCA solution to chelate calcium. This idea is further supported by the observation that addition of $CaCl_2$ (1mM) to the storage solutions resulted in a significant increase in peroxidative damage only to kidneys stored in saline and not in the HCA solution which contains a large excess of citrate (55mM).

Figure 4

Formation of thiobarbituric acid-reactive material and schiffs base in rabbit kidneys flushed with saline containing A23187 and stored for 24 hours



The relationship between cytosolic free calcium levels and lipid peroxidation in stored kidneys was further demonstrated by use of the ionophore A23187 which permeabilises the cell membrane to calcium and increased the rate of lipid peroxidation in kidneys stored for 24 hours in isotonic saline solution.

This study strongly suggests that prevention of increased cytosolic calcium levels is an important consideration for maintenance of the viability of organs during storage. Although the associations between calcium levels, oxidative damage and cell viability are not fully understood, it would appear that chelation of extracellular calcium during cold storage and blockage of calcium entry into the cell by agents such as verapamil may be useful approaches to protecting against ischaemic damage.

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kidneys produced copious urine after operation and most of the animals deteriorated through hepatic failure rather than renal failure even after 10 days of storage.

172. *Lipid Peroxidation in Ground Squirrel Kidneys after 7- and 10-Day Cold Storage is Markedly Reduced during Hibernation.* C. J. GREEN, L. WANG, B. J. FULLER, J. GOWER, G. HEALING, AND S. SIMPKIN (Section of Surgical Research, MRC Clinical Research Centre, Northwick Park Hospital, Harrow, Middlesex HA1 3UJ, United Kingdom).

As the kidneys of ground squirrels appear to be more tolerance of *in vitro* cold ischemia if harvested when the animals are hibernating rather than when awake (during the same winter months) we compared their susceptibility to oxidative damage and lipid peroxidation by *in vitro* assays (TBA-reactive material and Schiff-base formation) at these times and then measured the vitamin E status of these homogenates. Both kidneys were removed from Columbian ground squirrels (*Citellus columbiani*), flushed via the renal artery with 3 ml of cold (4°C) hypertonic citrate solution (at pressure 50 cm H_2O) and were then stored at 0°C for 7 or 10 days. They were then homogenized, incubated at 37°C, and assayed by the methods previously described from our laboratory. Schiff-base formation and TBA-reactive material rose significantly ($P < 0.01$) in both 7- and 10-day-stored kidneys compared with unstored fresh kidneys. Of more interest was that the measures of peroxidation were significantly ($P < 0.01$) lower in the hibernating kidneys than in those from awake animals. It was also notable that the ratio of α-tocopherol to retinol was higher in the hibernating than in the awake squirrels at 0 and 7 days of storage but had equalized after 10 days of storage.

173. *Evidence that Damage to Rabbit Kidneys following Cold Storage Is Mediated by a Calcium-Dependent Phospholipase A_2.* L. A. COTTERILL, J. D. GOWER, B. J. FULLER, AND C. J. GREEN (Section of Surgical Research, MRC Clinical Research Centre, Northwick Park Hospital, Harrow, Middlesex HA1 3UJ, United Kingdom).

We have studied the accumulation of free fatty acids following ischemia of rabbit kidneys. Rabbit kidneys were stored at 0°C either for 48 hr in isotonic saline solution (a poor storage medium) or 72 hr in hypertonic citrate solution (a good storage medium). These periods of storage resulted in significant increases in the accumulation of unsaturated free fatty acids. Addition of the phospholipase A_2 inhibitor dibucaine to hypertonic citrate solution significantly reduced the accumulation of arachidonic acid during storage of kidneys. Addition of the calcium ionophore A23187 to hypertonic citrate solution significantly increased the accumulation of arachidonic acid during storage of kidneys. These results provide evidence of a possible role for calcium activation of phospholipase A_2 in releasing arachidonic acid from the membrane during storage. The resulting accumulation may mediate oxidative damage to membranes following ischemic insult.

174. *Oxidative Damage to Kidney Membranes during Cold Ischaemia: Evidence of a Role for Calcium.* J. D. GOWER, L. A. COTTERILL, B. J. FULLER, AND C. J. GREEN (Section of Surgical Research, Clinical Research Centre, Watford Road, Harrow, Middlesex HA1 3UJ, United Kingdom).

Storage of rabbit kidneys at 0°C for periods of 72 hr after flushing with hypertonic citrate (HCA) solution, or 24 hr when flushed with isotonic saline, resulted in significant increases in Schiff base and thiobarbituric acid-reactive markers of lipid peroxidation *in vitro*. The extent of lipid peroxidation was not significantly altered by addition of verapamil (100 μ*M*), a Ca^{2+} channel blocking agent, or calcium (1 m*M* $CaCl_2$) to the HCA storage solution. In contrast, verapamil significantly reduced the extent of lipid peroxidation in kidneys stored in saline solution and a significant increase in oxidative damage occurred when $CaCl_2$ was added to this storage solution. Thus the extent of lipid peroxidation in kidneys stored in saline was significantly mediated by extracellular Ca^{2+}, whereas in HCA this was efficiently chelated by the large excess of citrate (55 m*M*) in this medium which prevented, or at least slowed, its entry into the renal cells. Lipid peroxidation was, however, significantly increased in kidneys stored in both HCA and saline solutions by addition of the calcium ionophore A23187 (10 μ*M*) or the polysaccharide dye ruthenium red (5 μ*M*) which inhibits mitochondrial uptake of Ca^{2+}. This strongly suggested that altered intracellular Ca^{2+} homeostasis during the storage period played an important role in the development of oxidative damage to kidneys stored in both these media.

175. *Preservation of Renal Tubules with Glutathione and Adenosine.* M. AMETANI, P. VEURGDENHIL, F. O. BELZER, AND J. H. SOUTHARD (Department of Surgery, University of Wisconsin, Madison, Wisconsin).

The University of Wisconsin solution (UW solution) effectively preserves livers, kidneys, and pancreases for 2 to 3 days. This solution contains a number of agents, including impermeants to suppress hypothermic-induced cell swelling; a colloid, hydrogen ion buffer; and metabolites. The mechanism of protection