

The Regulation of Hormone-Sensitive Lipase in Macrophages

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By

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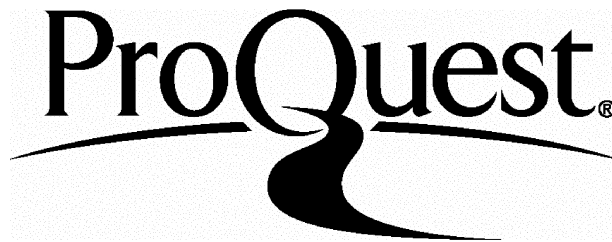
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Abstract

The mechanism by which type 2 diabetes dramatically increases atherosclerotic risk remains poorly understood. Type 2 diabetes is preceded by long periods of insulin resistance and associated abnormalities such as, high serum free fatty acid (FFA) levels, hyperinsulinemia, hyperglycaemia and obesity, collectively described as 'syndrome X'. Due to the fact that atherosclerosis is generally observed at the time of diagnosis of diabetes it is thought that syndrome X could be the cause of increased atherogenesis. Accumulation of cholesterol esters (CE) in macrophage foam cells is an important early event in atherosclerosis. The neutral cholesterol esterase responsible for the hydrolysis of CEs in macrophages is identical to hormone-sensitive lipase (HSL) in adipocytes.

In adipocytes insulin exerts an anti-lipolytic function by decreasing HSL activity, suggesting that insulin may also regulate HSL in macrophages. Therefore the regulation of HSL in macrophages has been studied and compared to that in adipocytes. HSL activity is acutely downregulated by insulin, as it is in adipocytes. However this acute effect of insulin on HSL in macrophages does not appear to be dependent on PI 3-kinase activity, as it is in adipocytes.

Leptin is a hormone, produced in adipocytes, that is raised in the circulation of obese individuals and this hormone was found to acutely increase HSL activity in macrophages. The regulation of HSL activity by leptin is blocked by wortmannin, suggesting that PI 3-kinase is involved.

HSL expression and activity were increased in adipocytes upon chronic stimulation with insulin under high glucose conditions but activity and expression were decreased in macrophages. Chronic incubation with leptin and high glucose also caused a reduction in HSL expression and

activity in macrophages. Insulin and leptin in combination with high glucose also caused an increase in CE accumulation.

Therefore a mechanism linking hyperinsulinemia, hyperglycaemia and obesity to foam cell formation and increased atherogenesis has been identified.

**In memory of my uncle, William Friend
(April 1930 – October 1999)**

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1. INTRODUCTION

1.1 Pathogenesis of Atherosclerosis

The lesions of atherosclerosis represent the principal cause of death in the USA, Europe and part of Asia. The lesions cause changes in blood vessels that can lead to myocardial infarction in the heart, cerebral infarction and stroke in the brain, and loss of function in the peripheral vasculature (Ross, 1995). In the past two decades atherosclerosis has been the subject of intense study. At one time, atherosclerosis was thought to be a degenerative disease that was an inevitable consequence of ageing. Research in the last two decades however, suggests that the disease is not degenerative, but seems to be a chronic inflammatory condition that is converted to an acute clinical event by the induction of plaque rupture, which in turn leads to thrombosis (Wissler and Vesselinovitch, 1983; Berliner et al., 1995; Macke Consigny, 1995).

The mortality rate due to coronary artery disease is at least doubled in the diabetic population and the reason for increased atherosclerosis in diabetics is poorly understood. There is no evidence to suggest that the pathomorphology of the disease, that is the plaque types and composition, is different in diabetics, only that the disease is grossly accelerated (Gerrity and Antonov, 1997). Due to the increased incidence and greater severity, it is crucial to prevent atherosclerosis in diabetic populations. Understanding the mechanisms of accelerated atherogenesis in diabetes will lead to better methods of preventative therapy.

1.2 The Lesions of Atherosclerosis

Three cellular components in the circulation, monocytes, T-lymphocytes and platelets together with two cells of the artery wall, endothelium and smooth muscle interact in multiple ways to generate the lesions of atherosclerosis. The lesions can be divided into three categories; the fatty streak, the intermediate or fibrofatty lesion and the fibrous plaque or advanced complicated lesion of atherosclerosis (Stary et al., 1994). The events involved in the progression of atherosclerosis from fatty streak to fibrous plaque are difficult to elucidate. However, analysis of this sequence in animal models of atherosclerosis, such as the cholesterol-fed rabbit (Finking and Hanke, 1997), selectin deficient mice (Johnson et al., 1997; Dong et al., 1998), and C57BL/6 mice (Schreyer et al., 1998) as a model for diabetes accelerated atherosclerosis, has proven to be particularly useful.

1.2.1 Fatty Streak Formation

The age at which fatty streaks appear differs in different regions of the arterial tree, but they are present in the aorta of virtually every child, regardless of race, sex or environment, by the age of 10 years. From then to age 25 the extent of aortic intimal surface covered by fatty streaks increases from about 10 per cent to 30 to 50 per cent (Ross and Glomset, 1976).

The fatty streak consists of an intimal collection lipid-filled monocyte-derived macrophages, with a varying number of T-lymphocytes. The macrophages, engorged with lipid in the form of cholesterol esters (CE) are called *foam cells*, so named because the CE droplets in the cytoplasm of these cells give them a

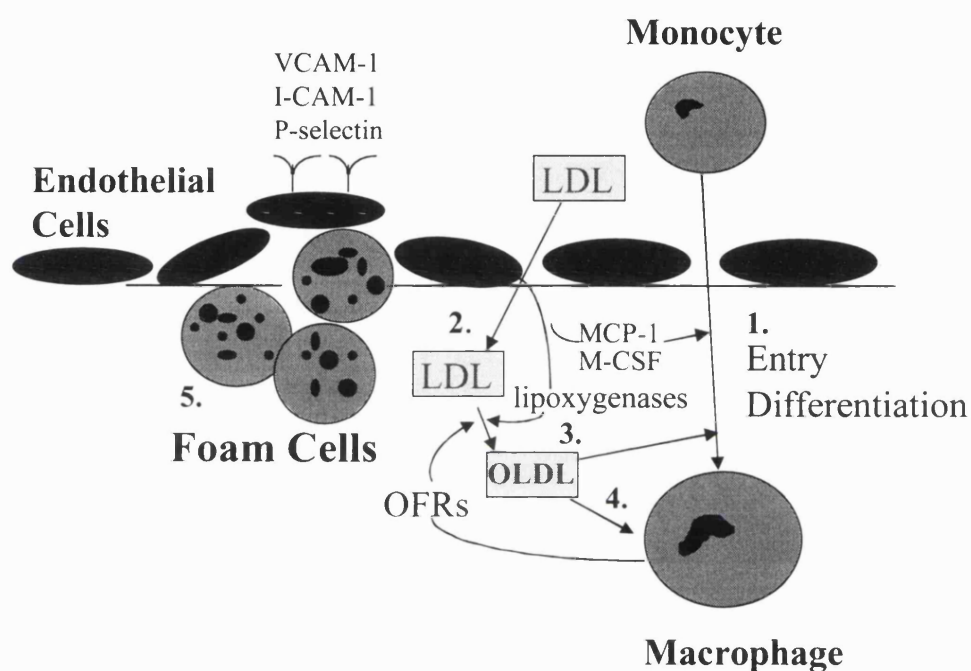
foamy appearance. The foam cells accumulate to form the bulk of the lesion and give the lesion a yellow colouration. The fatty streak is commonly found at sites where changes in blood flow, such as a decrease of flow or back currents, occur at branches and curves in the system (Wissler and Vesselinovitch, 1983).

Atherosclerosis is an example of a human disease which occurs as a result of inappropriate recruitment and activation of leukocytes (Prescott et al., 1997), due to endothelial injury. The original 'response-to-injury' hypothesis, as proposed by Ross in 1973 (Ross and Glomset, 1973), suggested that lesions developed in response to factors released from platelets that had adhered to sites of endothelial damage. Since then the hypothesis has undergone several revisions to take into account accumulating observations (Ross, 1993; Ross and Glomset, 1976; Ross and Harker, 1976). Although the ability to attract leukocytes to the appropriate location in the vasculature is an essential response in defending against bacterial infection and in wound repair, the same processes, if they occur at the wrong location or under inappropriate circumstances can support pathological events. The way in which endothelial damage and macrophage activation contribute to the early stages of atherosclerosis are summarised in figure 1.1.

The luminal surface of a normal artery is covered with a monolayer of endothelial cells attached to a sub-endothelial matrix (Schwartz et al., 1995). The earliest cellular event in atherogenesis is the adhesion of monocytes to the endothelium and their migration into the sub-endothelium through endothelial junctions. Endothelial cells act, not only as a liner for the blood vessels, but play

numerous functional roles. Alteration in one or more of these functions of the endothelium are important in the early stages of atherogenesis (Macke Consigny, 1995)(Ross, 1995), (see table 1).

Figure 1.1 *Fatty streak formation at site of endothelial injury*



1. Endothelial damage results in increased monocyte adherence and entry into the subendothelium where they are activated to form macrophages.
2. Fewer endothelial cell intercellular attachments results in the trans-endothelial migration of low-density lipoprotein (LDL).
3. LDL becomes modified (OLDL)
4. OLDL is taken up by scavenger receptors of macrophages
- (1) OLDL uptake results in accumulation of lipid, mostly in the form of cholesterol esters, forming foam cells.

(Abbreviations: MCP-1 monocyte chemotactic protein-1, m-CSF macrophage colony stimulating factor, OFRs oxygen free radicals)

Table 1: Functions of Vascular Endothelium in Normal and Injured State (taken from P.Macke Consigny, 1995)

Function	Normal Endothelium	Injured endothelium
Permeability	Tight endothelial junctions prevent passage of large molecules to subendothelium	Loss of tight junction Large molecules e.g LDL penetrate into sub-endothelial space
Thrombogenicity	Platelet aggregation is inhibited by PGI ₂ and EDRF-NO secretion	Converted from anti to prothrombic. PGI ₂ and NO secretions are diminished. PAI and tissue factor secretions are increased.
Vasomotor tone	Vasodilation is promoted by secretion of PGI ₂ and EDRF-NO	Vasoconstriction is promoted by less EDRF-NO and PGI ₂ and more endothelin-1 secretion.
Vascular smooth muscle migration and proliferation	Smooth muscle cell migration and proliferation are inhibited by secretion of heparin sulphate and EDRF-NO	Smooth muscle cell proliferation is promoted by less EDRF secretion and increased PDGF and endothelin-1 secretion.
Inflammation	Inflammatory molecules fail to adhere to normal endothelium	Leukocytes are recruited to sites of injury by expression on cell surface of proteins, endothelium-leukocyte and intercellular adhesion molecule

Increased expression of a series of cell surface glycoproteins including intercellular adhesion molecule-1 (I-CAM-1) and vascular cell adhesion molecule-1 (VCAM-1), results in the attachment and adherence of leukocytes (Ross, 1995). It has been reported that P-selectin is an effective tether for monocytes and macrophages and those that adhere to P-selectin are primed for subsequent synthesis and secretion of cytokines and chemokines (Johnson et al., 1997). This requires a second molecule such as platelet activating factor (PAF). This binds to the receptor on the surface of the monocyte initiating a process that includes upregulation of the monocytes' own adhesion proteins (β 2 integrins) and other cellular responses such as change in morphology, chemotaxis, emigration from the vasculature and secretion of proteolytic

enzymes and oxygen radicals (Prescott et al., 1997). After recruitment of circulating monocytes to the sub-endothelium, they are activated to become macrophages, (see step 1, figure 1.1)

Low density lipoprotein or LDL is the major cholesterol carrying lipoprotein. Fatty streaks typically develop at sites where endothelial injury has increased endothelial cell permeability, due to fewer intercellular attachments, enough to allow LDL and other large molecules to penetrate the subendothelium (Goldstein and Brown, 1977)(see step 2, figure 1.1). LDL, once beneath the endothelium, becomes trapped and undergoes a series of chemical changes that result in the formation of modified LDL (OLDL, see step 3, figure 1.1) (Witztum and Steinberg, 1991). This modification involves oxygen free radicals or lipoxygenases of endothelial cell or macrophage origin. The OLDL is a chemoattractant which, in conjunction with other chemoattractant molecules, such as monocyte chemotactic protein (MCP) secreted by injured endothelial cells, enhances the recruitment of monocytes to the sub-endothelial space. The most important effect of OLDL, however, is that it enables cells to take up large amounts of lipid. In contrast to LDL uptake, which is mediated by LDL receptors that are under negative feedback control, modified LDL uptake is mediated by scavenger receptors which are not regulated (Emi et al., 1993; Graham et al., 1993; Loughheed et al., 1997; Witztum and Steinberg, 1991). As a result large amounts of modified LDL can be incorporated into macrophages and smooth muscle cells resulting in CE accumulation and the formation of foam cells.

1.2.2 Intermediate or fibrofatty lesion formation

Fibrotic lesions are characterised by a fibrotic cap composed of smooth muscle cells recruited from the subendothelium and media. The layers of smooth muscle cells alternate with lipid-filled macrophages and T-cells surrounded by a connective tissue matrix of collagen fibrils, elastic fibers and proteoglycans. One of the initial events responsible for the conversion to the fibrotic lesion is loss of endothelial cells covering the fatty streak. At the site of cell loss platelets adhere and release factors, such as heparinase and platelet derived growth factor (PDGF), which promote vascular smooth muscle cell migration and lesion development. Endothelial cells, macrophages and smooth muscle cells all produce mitogens, such as fibroblast growth factor (FGF), EGF (epidermal growth factor), and IGF-1 (insulin-like growth factor), which stimulate proliferation of these smooth muscle cells (Dilley et al., 1987; Ross, 1993)

In adults the main function of vascular smooth muscle cells is to regulate wall tension (i.e. they exist in a contractile phenotype). However, upon migration they revert back to a synthetic phenotype and become packed with sub-cellular organelles to synthesise many growth factors and cytokines. These changes in phenotypic state may relate to cell surface adhesive molecules such as specific integrins. Embryonic smooth muscle cells contain $\alpha 2\beta 1$ integrins, which bind to collagen. Mature cells of the media convert from $\alpha 2\beta 1$ integrins to $\alpha 1\beta 1$ integrins, whereas in the lesions of atherosclerosis most cells have converted back to $\alpha 2\beta 1$. This may explain why these cells are responsive to chemotactic and mitogenic agents and their capacity to migrate directionally under appropriate stimulation (Ross, 1995).

1.2.3 *Advanced lesion formation and disruption*

Coronary atherosclerosis starts early in life, but it is the mature plaques which take decades to develop, that result in the clinically significant occlusion responsible for ischemic heart disease and anginal pain.

The trapping of lipid-laden foam cells beneath the fibrous cap leads to their necrosis, contributing to the mass of extracellular lipid in the core of the lesion (Gerrity and Antonov, 1997). As the name 'atherosclerosis' implies mature plaques consist of two main components; soft lipid-rich *atheromatous* material and hard collagen-rich *sclerotic* tissue. The collagen secreted by smooth muscle cells probably stabilises the plaques against disruption. In contrast, the atheromatous component is by far the most dangerous because it destabilises a plaque, making it vulnerable to rupture and thrombosis (Falk et al., 1995). Plaque disruption occurs most frequently where the fibrous cap is thinnest, most heavily foam cell infiltrated, and therefore weakest. Coronary plaques are constantly stressed by a variety of mechanical and hemodynamic forces that may trigger the disruption of vulnerable plaques. Physical exertion and emotional stress could, for example, trigger plaque disruption due to increased sympathetic activity resulting in increased blood pressure, pulse pressure, blood flow, heart rate and coronary tone.

Subsequent to plaque fracture, thrombosis may occur, which involves platelet adhesion and aggregation as well as activation of the coagulation cascade (Macke Consigny, 1995). OLDL has been shown to induce endothelial cells and monocytes to express high levels of tissue factor, a potent coagulation factor. Plaque rupture would therefore expose flowing blood to high levels of tissue

factor and result in clotting. OLDL also contributes to arterial occlusion by increasing plasminogen activator inhibitor levels, inducing expression of endothelin, inhibiting the expression of nitric oxide synthase and inhibiting the resulting vasodilation (Berliner et al., 1995).

Disruption of vulnerable plaques occurs frequently. Autopsy data indicate that 9% of 'normal' healthy persons are walking around with disrupted plaques (without superimposed thrombosis) in their coronary arteries. The numbers increase to 22% in diabetics. The fact that undetected cardiac ischemia is more common in diabetics magnifies the importance of coronary artery disease in diabetes. Asymptomatic ischemia makes diagnosis more difficult, which means that more heart attacks occur without warning, increasing the probability that they will be fatal (Steiner, 1994).

1.3 Foam Cell Formation

Atherosclerosis can be produced in many species of experimental animal simply by raising plasma LDL-cholesterol levels. The bulk of the earliest lesion of atherosclerosis, the fatty streak, is made up of foam cells, which contain lipid derived from LDL. Consequently much attention is now focused on understanding the aetiology of the fatty streak and the mechanisms by which monocyte-derived macrophages accumulate cholesterol from LDL.

1.3.1. Plasma Lipoproteins

Lipids, such as phospholipids, triacylglycerols and cholesterol are not very soluble in aqueous solution, therefore, they are transported in the circulation as

components of lipoproteins. Plasma lipoproteins consist of a nonpolar core of lipid such as triglyceride or cholesteryl ester surrounded by phospholipid and protein. These particles can be divided into 4 broad categories, on the basis of their functional and physical properties (Mack and Hodis, 1996):

- (i) **Chylomicrons**, which transport exogenous (dietary) triglyceride and cholesterol from the intestines to the tissues.
- (ii) **VLDL** (very low-density lipoprotein) which transport mostly triglyceride, which has been synthesised by the liver from excess carbohydrates, to the tissues.
- (iii) **LDL/IDL** (Low/intermediate density lipoprotein) which transport mainly cholesterol from the liver to tissues.
- (iv) **HDL** (high density lipoprotein) which transports endogenous cholesterol from the tissues to the liver

LDL is the major means of transporting cholesterol to peripheral tissues. All cells require a certain level of cholesterol for maintaining cellular membranes and where this cannot be provided by *de novo* synthesis it is supplied by LDL. LDL contains approximately 75% lipid and 25% protein. Protein constituents of lipoproteins are called apolipoproteins (see below). The lipid composition of LDL in weight is 50% cholesterylesters, 30% phospholipids, 10% unesterified cholesterol and 10% triglycerides. Phosphatidylcholine and sphingomyelin account, respectively, for 65% and 25% of the total phospholipids and linoleic acid is the major fatty acid in LDL lipids (Jackson et al., 1976).

LDL was recognised in the 1930's by Gofman and co-workers as being the lipoprotein whose elevation in plasma was most strongly correlated with atherosclerosis (Goldstein and Brown, 1977). This is particularly evident in individuals with familial hypercholesterolemia (FH). Cells from FH homozygotes completely lack functional LDL receptors. As a result their plasma cholesterol levels are extremely high, and rapid formation of atherosclerosis occurs. In homozygotes death from myocardial infarction can occur as early as the age of five (Voet and Voet, 1995).

In contrast to this positive correlation there is a negative correlation between HDL levels and accelerated vascular disease (Mahley and Innerarity, 1983). HDL has essentially the opposite function of LDL by removing cholesterol from tissues. Circulating HDL acts as a cholesterol scavenger, by extracting cholesterol from the surface of cells. Evidence indicates that HDL transfers cholesterol to VLDL, in a poorly understood process mediated by cholesterol ester transfer protein. VLDL can then be degraded to LDL and is taken up by the liver, which is the only organ capable of disposing of significant quantities of cholesterol.

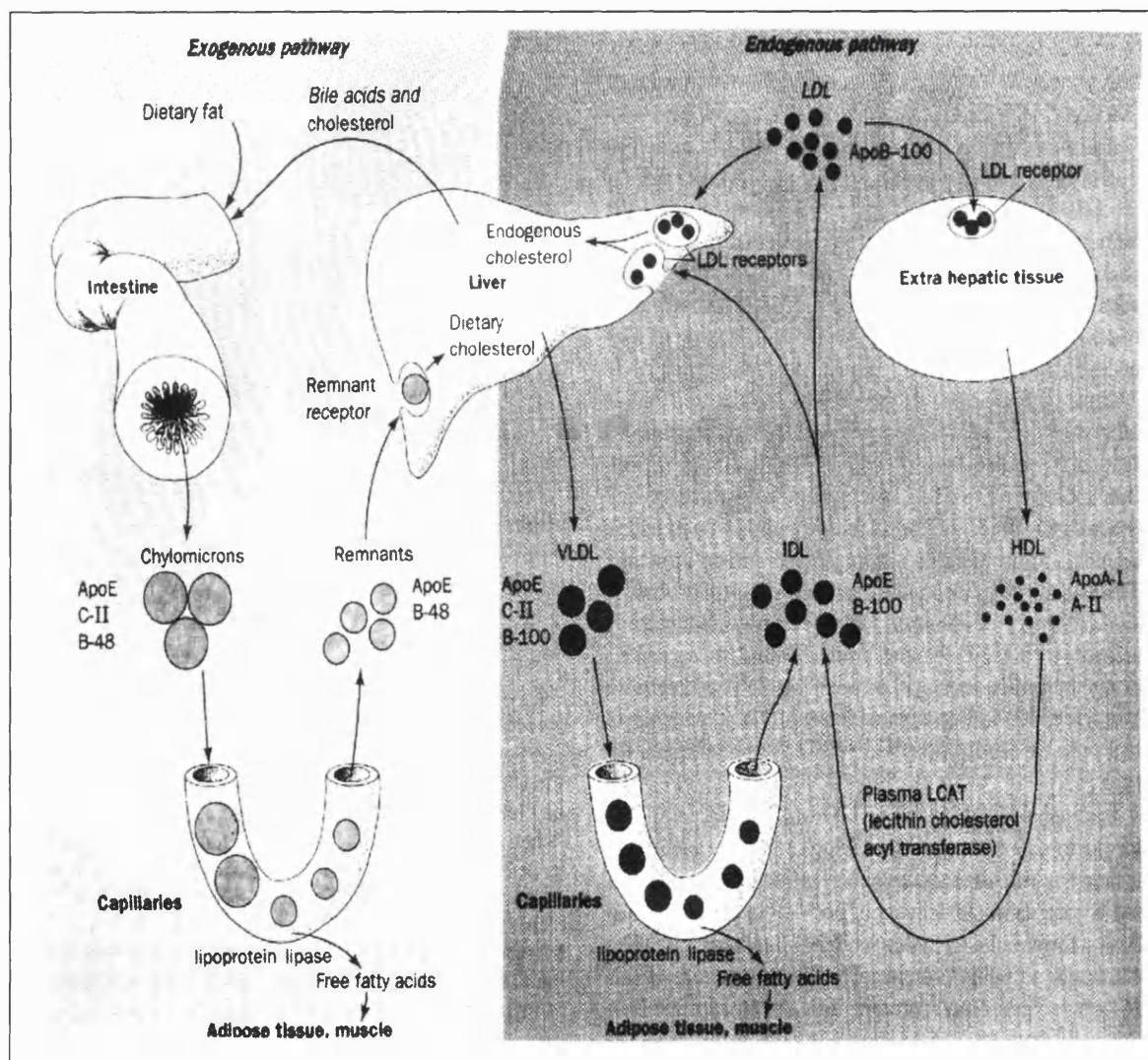
1.3.2. The LDL (apo-B, -E) Receptor

Cells obtain exogenous cholesterol mainly through the endocytosis of LDL particles. Binding of LDL to the LDL (apo-B,-E) receptor leads to endocytosis and utilisation of the cholesterol as well as suppression of endogenous cholesterol synthesis. The number of cell surface receptors for LDL are regulated

so that the cell is supplied with the cholesterol it requires but there is no over-accumulation (Goldstein and Brown, 1977).

Figure 1.2. Triacylglycerol and cholesterol transport

In the plasma triglycerides on chylomicrons are hydrolysed by lipoprotein lipase (LPL). Chylomicron remnants containing CE are transported to the liver. This CE is packaged with triglycerides to form VLDL, and is secreted into the plasma. Triglycerides on VLDL are hydrolysed by LPL, and the lipoproteins are converted to IDL then cholesterol-rich LDL. LDL may be removed from the plasma by the liver and extrahepatic tissues by the LDL receptor.



As mentioned earlier, plasma lipoproteins include one or more protein constituents called apolipoproteins. At least nine apolipoproteins are distributed in significant amounts in different human lipoproteins. It is now well established that the apolipoproteins regulate the metabolism of lipoproteins by mediating the uptake by specific lipoprotein receptors. Apolipoprotein B, the predominant protein constituent of LDL (Alaupovic et al., 1972; Jackson et al., 1976), is therefore involved in LDL receptor recognition. Studies using porcine HDLc, which contains significant quantities of apolipoprotein E (apo-E), showed that this was also capable of binding to the LDL receptor and that the extent of binding was proportional to the content of apoE (Mahley and Innerarity, 1977). The chemical modification of arginine (Mahley et al., 1977) and lysine residues on apolipoproteins (Basu et al., 1976; Weisgraber et al., 1978), prevented binding to the LDL receptor, and therefore demonstrated that these particular amino acids are functionally significant residues in the recognition sites of B and E apolipoproteins for lipoprotein receptors.

1.3.3. The LDL Pathway

Cholesterol biosynthesis and uptake must be tightly regulated. The LDL pathway is responsible for the regulation of uptake, storage and synthesis of cholesterol, and protects the cell from over-accumulation. Details of the pathway were established in the 1970's predominantly by Brown and Goldstein (Brown et al., 1975; Goldstein and Brown, 1977). The human fibroblast in monolayer culture proved to be a useful system in which to investigate this pathway. Binding of LDL to the receptor is the initial event in this pathway. Electron microscopic studies showed that more than 70% of LDL receptor sites

were concentrated in short segments of plasma membrane, where the membrane appeared indented and coated on both sides. These 'coated regions' constitute less than 2% of the total surface membrane of fibroblasts . Invagination of the coated regions leads to internalisation of receptor bound LDL, which is subsequently delivered to the lysosomes. Using LDL that had been radiolabelled, either in its protein component or in its cholesterylester component, it was shown that the LDL is delivered to the lysosome as a unit (Goldstein and Brown, 1974; Goldstein et al., 1975; Goldstein et al., 1975). The protein component is then hydrolysed within the lysosome and excreted from the cell. The cholesterylester component of LDL is also hydrolysed here by an acid lipase, referred to as acidic cholesterol esterase, and labelled cholesterol was found to accumulate within the cell, either in association with membranes or stored in the cytoplasm as cholesterylester (Goldstein and Brown, 1977).

Re-esterification of the cytoplasmic cholesterol, liberated by acidic cholesterol esterase, results in esters with a different fatty acid composition compared to those originally present in plasma lipoprotein. Cholesterol is preferentially re-esterified to oleate and palmitoleate, rather than to linoleate (Goldstein and Brown, 1977).

1.3.4. LDL Pathway as a Protective Factor for Atherosclerosis

As mentioned earlier, the LDL pathway described above functions in man to protect against excess accumulation of cholesterol in cells, such as that which occurs during foam cell formation in atherosclerosis. This is achieved by three

main responses (Mahley and Innerarity, 1983) elicited by the free cholesterol which migrates into the cytoplasm from lysosomes:

- (1) The suppression of cholesterol biosynthesis by regulation of HMG-CoA (β -hydroxy- β -methylglutaryl-CoA) reductase, the enzyme catalysing the rate-limiting step in the *de novo* pathway
- (2) The rate of synthesis of new LDL receptors is decreased, limiting the amount of cholesterol which is taken up by the cell.
- (3) The activation of acyl-CoA cholesterylacyltransferase (ACAT), which esterifies cholesterol allowing it to be stored as cholesterylester lipid droplets

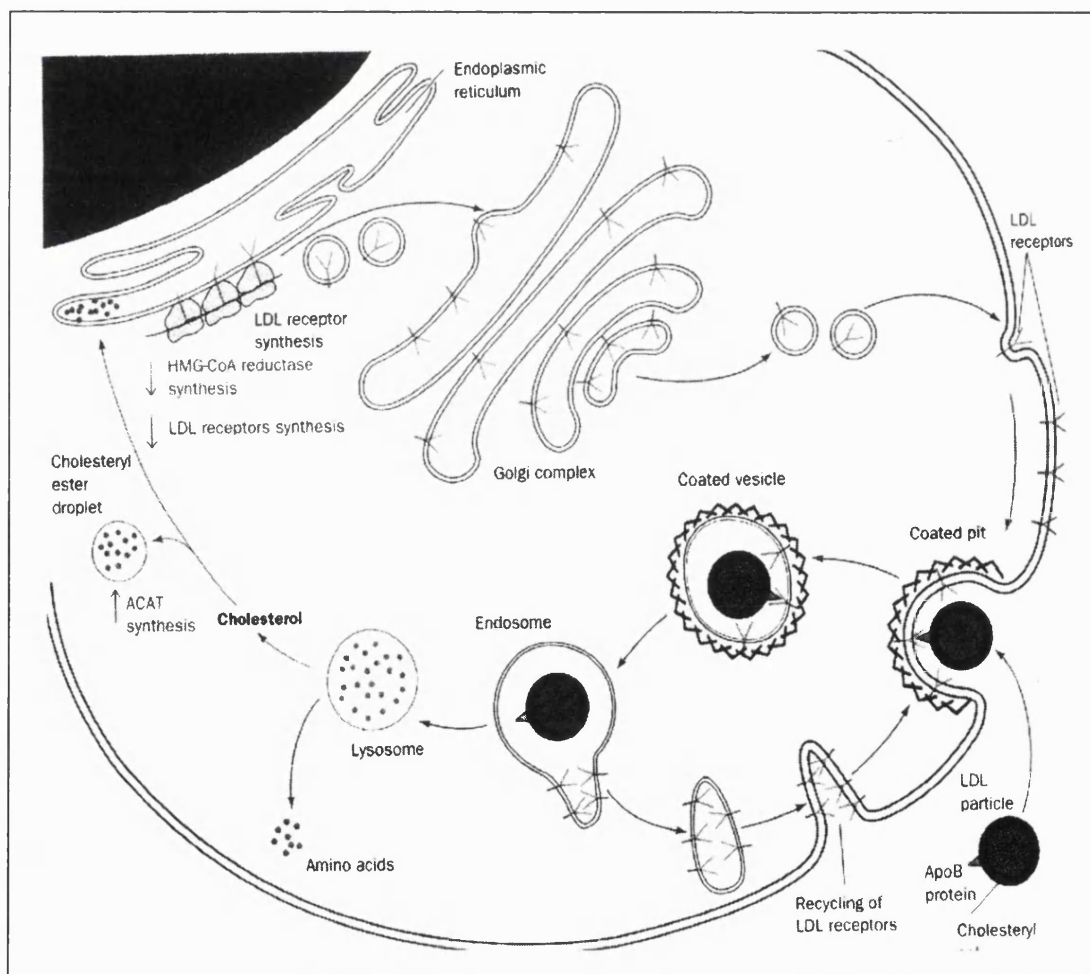
The activity of ACAT remains at a level such that the rate of synthesis of cholesteryl esters equals the rate of their hydrolysis. Therefore, cells in the body are present in this steady state.

The LDL pathway has also been studied in lymphoid cells (Ho et al., 1976; Kayden et al., 1976) and it was found that the same highly regulated process found in fibroblasts, occurs in these cells. The uptake of LDL, giving rise to foam cells and fatty streaks, is therefore likely to be due to pathways independent of the LDL receptor. As mentioned earlier, the observation that lesions rich in foam cells develop in patients deficient in functional LDL receptors (homozygous patients with FH) provides evidence for this (Steinberg et al., 1989). Also

to foam cells by incubation with very high concentrations of LDL (Goldstein et al., 1979). Since foam cells do develop *in vivo* when plasma LDL levels are high it is thought that the LDL must undergo some kind of modification which allows it to be rapidly taken up, fast enough to generate a foam cell.

Figure 1.3. LDL receptor-mediated endocytosis and cholesterol homeostasis

LDL is brought into the cell in endosomes that deliver LDL to the lysosomes, while recycling LDL receptor to the plasma membrane. Lysosomal degradation of LDL releases cholesterol, whose presence decreases the rate of synthesis of HMG-CoA reductase and LDL receptors, while increasing that of acylCoA:cholesterolacyltransferase (ACAT).



1.3.5. *Modification of LDL*

Experiments carried out in fibroblasts showed that acetylation of LDL altered the lipoprotein to the extent that it was no longer recognised by the LDL receptor (Basu et al., 1976). Therefore Brown and Goldstein used ^{125}I labelled acetylated LDL to investigate the system for the uptake of modified LDL in macrophages. They found receptors present on the macrophage surface bind acetyl-LDL resulting in rapid uptake and delivery to the lysosomes and a large accumulation of free and esterified cholesterol within the macrophage (Goldstein et al., 1979). It was later found that LDL modified by malondialdehyde treatment, hypochlorite treatment and oxidation with copper were also taken up rapidly by macrophages (Graham et al., 1993). The receptors which recognise the modified forms of LDL were termed scavenger receptors (Emi et al., 1993)(Lougheed et al., 1997).

Importantly, it was found that LDL could be biologically modified, by incubating overnight with cultured endothelial cells, to an oxidised form (OLDL) recognised by scavenger receptors (Quinn et al., 1985). Evidence suggests that oxidative modification by lipoxygenases and free radicals occurs in atherosclerotic lesions forming OLDL which is subsequently taken up by macrophages in an unregulated manner via scavenger receptors (Witztum and Steinberg, 1991). There are some clues as to how the modification of LDL may alter receptor specificity. As mentioned earlier, arginine and lysine residues of apolipoprotein-B (apo-B) are important for the interaction of LDL with the LDL receptor. Oxidation of LDL causes fatty acid fragmentation creating highly reactive intermediates, such as aldehydes and ketones, which can then complex

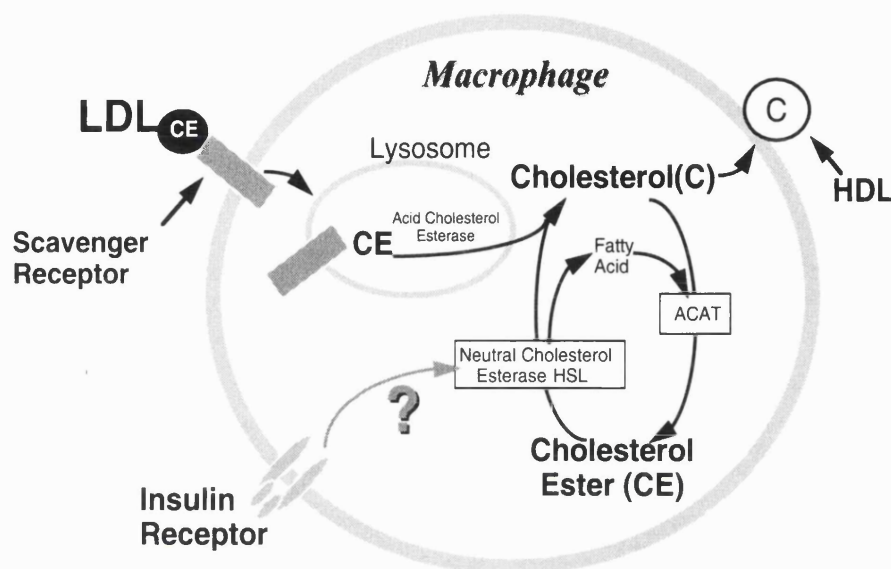
with apo-B increasing the net negative charge of LDL by abolishing the positive charge of the ϵ -amino groups of lysine (Mahley and Innerarity, 1983).

1.3.6. Cholesterol ester cycle in macrophages

The acetyl-LDL system was a useful *in vitro* system for studying the metabolism of cytoplasmic cholesteryl ester droplets in macrophages. Acetyl-LDL is internalised by adsorptive endocytosis and delivered to the lysosomes where the cholesterol esters are hydrolysed, by an acidic cholesterol ester hydrolase, liberating free cholesterol into the cytoplasm (Brown et al., 1979; Goldstein et al., 1979). Much of the cholesterol is re-esterified by ACAT and the resulting cholesterol esters form the lipid droplets. The cholesteryl ester droplets were shown to disappear when acetyl-LDL was removed from the medium (Brown et al., 1979) and only free cholesterol appeared in the medium, suggesting that excretion of stored cholesterol esters requires hydrolysis. Net hydrolysis did not occur in the absence of an acceptor molecule, such as HDL, capable of binding cholesterol and removing it from the cell (Ho et al., 1980).

The turnover of the stored lipids in macrophages was investigated by measuring the synthesis, hydrolysis and excretion of cytoplasmic cholesterol esters separately (Brown et al., 1980). Results demonstrated that these esters undergo a continual cycle of hydrolysis and re-esterification. A cytoplasmic neutral cholesteryl esterase (nCE) liberates free cholesterol which is either excreted from the cell or re-esterified by ACAT. Approximately 50% of the cells stored cholesterol esters are hydrolysed and re-esterified in this cycle each day (Brown et al., 1980).

Figure 1.4. *Cycle of cholesterol esterification and hydrolysis in the macrophage*



Cholesterol ester accumulation represents conditions where the rate of esterification exceeds that of hydrolysis. Cholesterol itself can act as an ACAT activator and also downregulates nCE activity, thus promoting cholesterol ester accumulation.

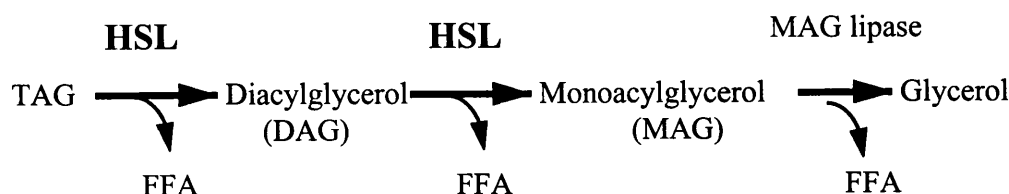
Previous attention has been focussed on the potential therapeutic benefits of reducing ACAT activity and cholesterol esterification in foam cells, but results have been disappointing (Brewer, 2000). In addition, ACAT knockout mice are still found to be subject to atherosclerosis and show extensive free cholesterol

deposition in the skin and brain (Accad and al, 2000), suggesting side effects of ACAT inhibition may be a problem.

Until recently not much attention has been given to the role of nCE in foam cell formation, however studies in atherosclerosis susceptible white Carneau pigeons (Yancey and St. Clair, 1994) showed that the slow rate of CE clearance was due partly to a defect in CE hydrolysis. Also in human THP-1 monocyte-derived macrophage cell line the efflux of stored CE appears to be limited by the activity of nCE (Graham et al., 1996). This highlights the importance of understanding how nCE activity is regulated in macrophages and the potential of this enzyme as a therapeutic target.

1.4 Hormone-Sensitive Lipase

Evidence suggests that the nCE responsible for CE hydrolysis in macrophages is identical to hormone-sensitive lipase (HSL), the rate-limiting enzyme of triglyceride lipolysis. Adipose tissue triacylglycerol (TAG) is the most important energy store in mammals . The release of this energy in the form of oxidisable free fatty acids (FFA) and glycerol is under hormonal and neural control. A key feature of HSL is its ability to be activated by adrenergic stimuli, to release substrates for energy metabolism. Conversely, anabolic hormones, such as insulin, downregulate its activity to promote TAG storage. HSL catalyses the first rate-limiting and second steps in the degradation of adipocyte TAG. The enzyme has a marked, but not absolute, specificity for the primary ester bonds.

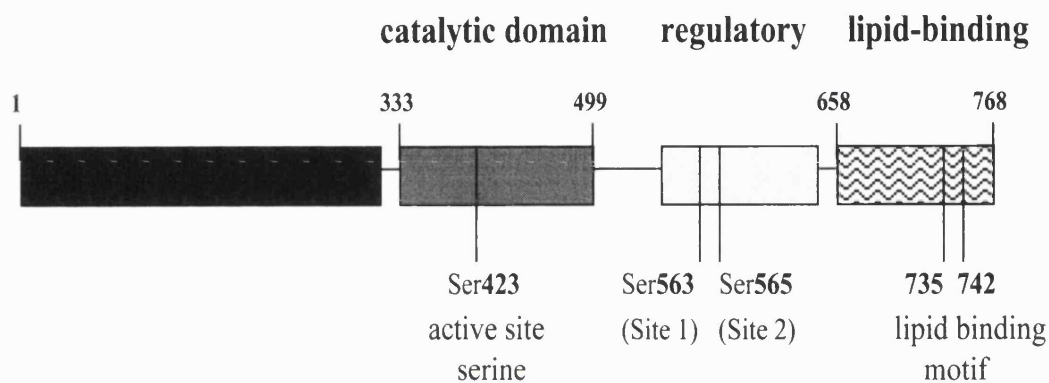


Work with purified HSL demonstrated that it is also active against CEs, the activity against this substrate being approximately equal to that against TAG (Fredrikson et al., 1981). Most other TAG lipases show very little or no cholesterol esterase activity.

Chromosome mapping has localised HSL to the cent-q13.3 region of human chromosome 19. The cDNA encoding the HSL polypeptide has been cloned and sequenced and the primary structure of the 757 amino-acid polypeptide has been deduced (Holm et al., 1988). Common features of lipases and esterases are that they adopt the α/β -hydrolase fold and perform catalysis using a catalytic triad of a serine, aspartic or glutamic acid and histidine. Site-directed mutagenesis has shown that Asp⁷⁰³ and His⁷³³ together with Ser⁴²³ (Holm et al., 1994) are essential for lipase and esterase activities and constitute the catalytic triad of HSL (Osterland et al., 1997). Evidence suggests that HSL has a multi-domain structure (Osterland et al., 1999) and that these domains are encoded by different exons (Langin et al., 1993; Smith et al., 1996). Phosphorylation at two distinct serine residues (site 1 and site 2, see later section 1.5) are important in the regulation of HSL activity. These two sites are present in the regulatory domain (shown below in figure 1.5) encoded by exon 8, the catalytic domain is encoded by exons 5-7, and the lipid binding domain by exon 9 (Osterlund et al., 1996). These three key domains of HSL can be ascribed to the carboxyl terminal region. The role of the 35 kDa amino-terminal region remains unclear, but it

may interact with other regulatory components of the lipolytic and cholesterol metabolism pathways.

Figure 1.5 *Domain structure of rat hormone-sensitive lipase*



1.4.1 Tissue distribution of HSL

Comparisons made between adrenal cholesterol esterase and HSL confirmed that HSL was present in this tissue suggesting that it may have a role in hydrolysing intracellular stores of cholesterol esters supplying free cholesterol for steroidogenesis (Cook and Yeaman, 1982). Immunological and enzymatic evidence suggest that the neutral lipase responsible for TAG hydrolysis in the heart (where endogenous TAG is the major energy store) is also HSL (Small et al., 1989). In addition HSL has been detected in a variety of rat tissues, including testes, ovaries, heart and skeletal muscle, but not in the kidney or liver. More recently HSL has been shown to be expressed, and active in β -cells (Mulder et

al., 1999). A potentially important finding has been the presence of HSL in macrophages (Small et al., 1989). In fact, evidence indicates that HSL is solely responsible for the nCE activity in macrophages, as nCE activity can be completely removed from murine macrophage homogenates by immunoprecipitation with anti-HSL immunoglobulin (Small et al., 1989). Expression of HSL mRNA in human macrophages has been demonstrated using PCR (Khoo et al., 1993; Reue et al., 1997). This finding could have important implications in atherosclerosis, where the regulation of this enzyme may affect CE accumulation and foam cell formation. Indeed very recently it has been demonstrated that there is low level expression of HSL in macrophage-derived foam cells isolated from human and rabbit atherosclerotic plaques and it is suggested that this low level expression may account for the reduced nCE activity observed in arterial foam cells .

Interestingly the size of HSL proteins is variable in the different tissues. In rat adipose tissue HSL is present as an 84kDa protein, whereas in rat heart, ovaries and adrenals there is an 84 and 89kDa protein, and 84 and 55kDa proteins in skeletal muscle . Testes contain two prominent larger bands of approximately 116kDa and 130kDa These higher molecular weight proteins may represent an inactive proform, a differential splicing product or an isoenzyme (Holst et al., 1996). There is also evidence for species-specific expression of HSL. Laurell and coworkers detected a novel HSL mRNA in human tissues (Laurell et al., 1997). In-frame skipping of exon 6 results in mRNA 228bp shorter than the full-length HSL mRNA. As mentioned in the previous section, exon 6 encodes the catalytic site serine, whereas the phosphorylation sites on HSL are encoded by exon 8.

The corresponding 80kDa protein therefore, shows no lipase or esterase activity but contains the phosphorylation sites thought to be important in regulation (see section 1.5).

It is likely that there may be tissue specific control of HSL, due to the difference in receptors and levels of kinases/phosphatases found in the various cell types in which HSL is found.

Table 2: *Tissue distribution and functions of HSL* (taken from S.Yeaman, 1990)

Substrate	Tissue	Function
triacylglycerol	adipose	fatty acid supply
	heart	fatty acid supply
cholesterol esters	adrenals	cholesterol supply
	ovaries	cholesterol supply
	macrophages	cholesterol removal
steroid esters	placenta	steroid action

1.5 The Regulation of HSL in adipocytes

Regulation of HSL activity is best understood in adipocytes where counteracting effects of lipolytic and antilipolytic stimuli fine tune HSL activity to tightly regulate triglyceride (TAG) stores. HSL is regulated by phosphorylation at, at least two sites . Site 1 (the regulatory site) is responsible for the activation of HSL which occurs in response to lipolytic stimuli, such as noradrenaline (Stralfors et al., 1984). This site is phosphorylated by cAMP dependent protein kinase (A-kinase) (Garton et al., 1988; Stralfors and Belfrage, 1983). Cloning

studies (Holm et al., 1988) together with phosphopeptide sequencing (Stralfors and Belfrage, 1983) have identified this residue as a serine at position 563 in the rat HSL sequence.

Tryptic phosphopeptides containing site 1 on bovine HSL phosphorylated by cAMP-dependent protein kinase (SVSEAALTQPEGPLGTDSLK) also contain a second residue, serine 565, (SVSEAAALTQPEGPLGTDSLK) which is not modified by cAMP-dependent protein kinase but which is already partially phosphorylated. This second phosphorylation site, site 2 (basal site), is phosphorylated under basal conditions of lipolysis (Stralfors et al., 1984) and has been shown to be phosphorylated by AMP activated protein kinase and Ca^{2+} /calmodulin-dependent protein kinase II (Garton et al., 1989). Phosphorylation at the two sites are mutually exclusive, that is phosphorylation at site 2 blocks activation of HSL via phosphorylation at site 1 (Garton et al., 1989).

Recently two novel phosphorylation sites on HSL have been identified (Anthonsen et al., 1998). Site-directed mutagenesis and two-dimensional phosphopeptide mapping showed that Ser-659 and Ser-660, as well as Ser-563, are phosphorylated in response to isoproterenol. Furthermore, it is claimed that these are the major activity controlling sites.

1.5.1. Dephosphorylation of HSL

The data indicating that phosphorylation at site 1 and site 2 are mutually exclusive implies that activation of HSL by A-kinase has to be preceded by

dephosphorylation of site 2. Whether this dephosphorylation is an important regulatory step or merely a result of high phosphatase activity in the cell is unclear. Insulin reduces catecholamine-induced lipolysis in adipocytes, by causing a net dephosphorylation of HSL. When cAMP levels are high, insulin-induced dephosphorylation can be explained by a reduction in A-kinase catalysed phosphorylation. However insulin also causes a net dephosphorylation of HSL under conditions where A-kinase is not activated and only the basal site is phosphorylated (Stralfors and Honnor, 1989). Therefore it has been assumed that insulin can cause a reduction in phosphorylation through an A-kinase-dependent pathway involving reduction of cAMP, and through a cAMP-independent pathway. It seems likely that the mechanism employed by insulin for the cAMP-independent effect is activation of a protein phosphatase (Chan et al., 1988; Stralfors and Honnor, 1989). The protein phosphatases responsible for dephosphorylation of HSL in adipocytes have been investigated (Wood et al., 1993). PP2A and PP2C show the same activity against site 1, and together account for 92% of the total phosphatase activity at this site. PP2A is also the predominate phosphatase active at site 2 (50%), at which PP1 (20%) and PP2C (30%) are also active. Total phosphatase activity in adipocyte extracts was 2-3 fold higher against site 2 than site 1. Insulin is known to activate PP1 (Oliver et al., 1988; Ragolia and Begum, 1998; Villa-Moruzzi, 1989), which selectively dephosphorylates the basal site (Holm et al., 1997). This may therefore represent a mechanism by which HSL activity is influenced.

1.6 Signalling pathway regulating site 1 phosphorylation

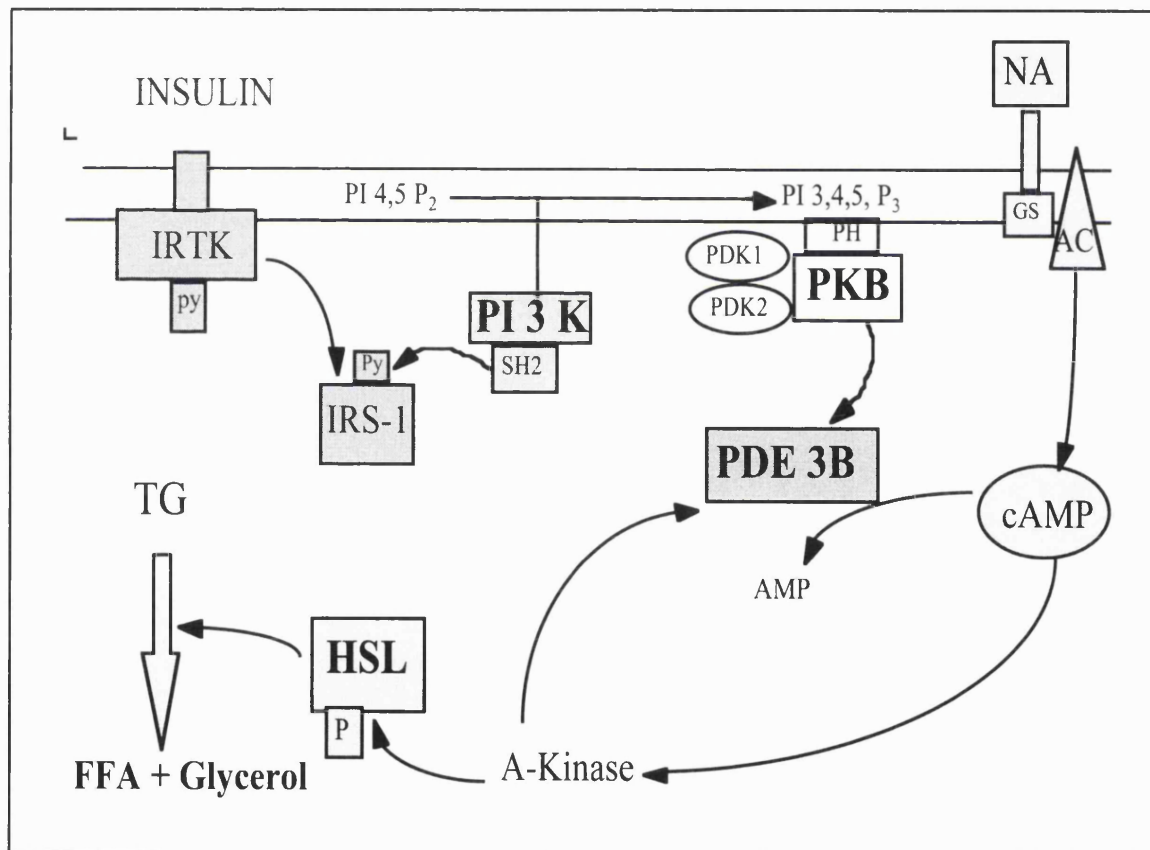
Insulin opposes the effects of hormones that raise cAMP and insulin-induced reduction in lipolysis is associated with a decrease in cAMP. The strategy for dissecting the signalling pathway by which insulin lowers cAMP levels was to start with the target enzyme and analyse each step moving upstream to the insulin receptor.

1.6.1. *cGMP-inhibited cAMP phosphodiesterase (PDE3)*

It was shown in adipocytes that isoprenaline and insulin rapidly increased phosphorylation of a 135kDa protein which has been identified as a cGMP-inhibited 'low Km' cAMP phosphodiesterase (cGI-PDE or PDE3B)(Smith et al., 1991). PDEs are a large group of structurally related enzymes that belong to seven related gene families (PDEs 1-7). They catalyse the hydrolysis of the 3'-5' phosphodiester bond of cyclic nucleotides to regulate the intracellular concentrations and effects of these second messengers (Degerman et al., 1997). PDE3 and PDE4 exhibit a high affinity for cAMP. PDE3s can be distinguished from PDE4s by their high affinities for both cAMP and cGMP, and PDE3 but not PDE4, is inhibited by cGMP. PDE3B is the isoform in adipocytes phosphorylated in response to insulin (Degerman et al., 1996). Both isoprenaline and insulin stimulation results in phosphorylation and activation of PDE3B followed by a reduction in A-kinase activity and inhibition of lipolysis. Therefore PDE3B can be phosphorylated and activated by cAMP-dependent and insulin-dependent kinases (Degerman et al., 1990). Thus, agents that raise cAMP increase both cAMP synthesis and 'feed-back' regulation of cAMP hydrolysis

Figure 1.6 *Regulation of HSL in adipocytes*

Signalling mechanism by which insulin downregulates HSL activity and reduces lipolysis. PI 3-kinase activation results in PIP_3 production, which activates PKB. PKB then phosphorylates and activates PDE3B, which hydrolyses cAMP and reduces cAMP-dependent protein kinase (A-kinase) activity. This results in reduced phosphorylation of HSL and subsequently reduces lipolysis. (IRTK, insulin receptor tyrosine kinase, AC, adenylate cyclase, NA, noradrenaline, FFA, free fatty acid)

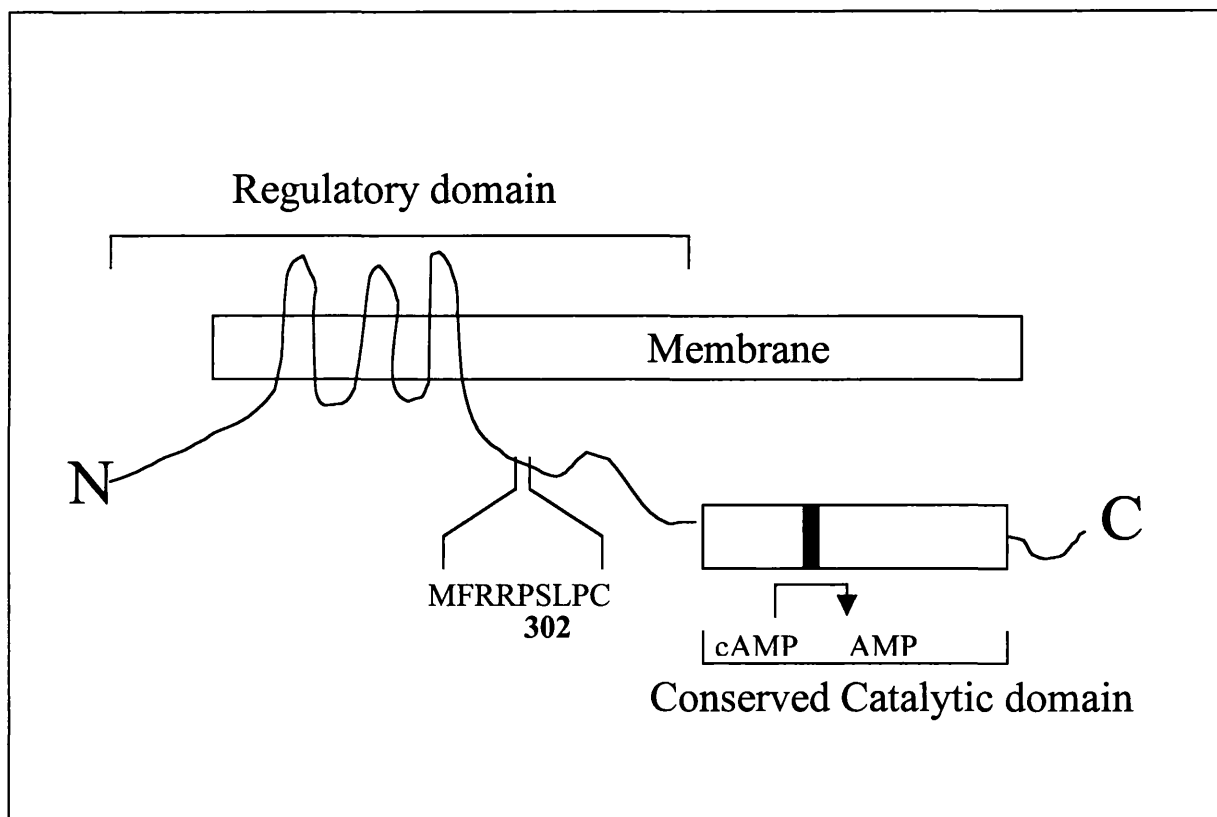


A single serine residue located in an A-kinase consensus sequence [-MFRRPS(302)LPCISREQ-] is phosphorylated in response to insulin and isoprenaline (Rahn et al., 1996). The effects of the two hormones on phosphorylation of Ser302 are more than additive suggesting the anti-lipolytic action of insulin involves cross talk between insulin and cAMP-signalling pathways and A-kinase is involved in sensitising the insulin signalling pathway (Degerman et al., 1996). More recently another serine residue has been found to be phosphorylated by an insulin-stimulated kinase, which is discussed later (section 1.6.3).

Also, insulin has been shown to inhibit lipolysis in human skeletal muscle (Enoksson et al., 1998), where HSL has been identified and is activated in a similar way to that in adipocytes (Small et al., 1989). However, it was found that the mechanism employed by insulin did not involve PDE3B. The main PDE activity in this tissue is PDE4, but specific inhibitors of PDE3, 4 and 5 were ineffective in blocking effects of insulin on lipolysis but they could be abolished by a non-selective inhibitor (Enoksson et al., 1998). This suggests that insulin inhibits lipolysis in different tissues by activation of different PDEs.

Figure 1.7 Model of domain structure of PDE3B

The deduced sequence of rat PDE3B predicts five or six helical, potentially transmembrane, segments in the N-terminal hydrophobic region. The serine 302 within an A-kinase consensus sequence, is phosphorylated in response to insulin and isoproterenol. Also the C-terminal catalytic domain is shown.

**1.6.2. Phosphatidylinositol 3-kinase (PI 3-kinase)**

Incubation of rat adipocytes with wortmannin, a potent and specific PI 3-kinase inhibitor (Arcaro and Wymann, 1993), completely blocked insulin-induced phosphorylation and activation of PDE3B and therefore the antilipolytic action of insulin (Okada et al., 1994; Rahn et al., 1994). These studies suggested that

activation of PI 3-kinase activity was required upstream of PDE3B in the antilipolytic signalling chain.

PI 3-kinases phosphorylate the 3'-OH position of the inositol ring of phosphatidylinositol (PI), generating PI(3)P, PI(3,4)P₂ and PI(3,4,5)P₃. Class I_A PI 3-kinases are heterodimers made up of a 110 kDa catalytic subunit (p110) and an adaptor/regulatory subunit (p85) (Carpenter et al., 1990). They have preference for PI(4,5)P₂ producing PI(3,4,5)P₃ (Hunter, 1995). Class I_A PI 3-kinases signal downstream of tyrosine kinases and play a central role in insulin signalling to metabolic pathways (Shepherd et al., 1998). Insulin treatment increases PI 3-kinase recruitment to, and activity in anti-phosphotyrosine immunoprecipitates (Hayashi et al., 1992). The insulin receptor has intrinsic tyrosine kinase activity and during insulin stimulation a protein known as, insulin receptor substrate (IRS) is tyrosine phosphorylated. This protein contains many potential tyrosine phosphorylation sites and acts as a docking protein for signal-transducing molecules (Sun et al., 1991). The p85 subunit of PI 3-kinase contains two SH2 regions (src homology regions) which bind phosphorylated tyrosine residues in membrane bound receptors and other cytoplasmic proteins (Escobedo et al., 1991; Otsu et al., 1991). PI 3-kinase is recruited to the signalling pathway by binding to phosphorylated YXXM motifs present on IRS (Shepherd et al., 1996). This allows translocation of the cytosolic PI 3-kinase to the membranes where their lipid substrates are present.

As well as the lipid kinase activity, a serine/threonine protein kinase activity was found to be associated with PI 3-kinase that phosphorylated the 85 and

110kDa subunits (Carpenter et al., 1993). This Mn^{2+} -dependent protein kinase activity associated with p110 phosphorylates a specific serine, Ser⁶⁰⁸, in the p85 subunit, resulting in inhibition of PI 3-kinase activity (Carpenter et al., 1993; Dhand et al., 1994). It was established that this activity was intrinsic and that phosphotransfer usually occurs within the p110/85 heterodimer (Dhand et al., 1994). The inhibition of activity by phosphorylation is a possible mechanism by which PI 3-kinase activity is kept low in quiescent cells and activity is turned off after stimulation. An isoform of IRS, IRS-1, was found to be another target for this kinase. The protein kinase activity, as well as lipid kinase activity, is inhibited by wortmannin (Lam et al., 1994; Tanti et al., 1994). It is thought this phosphorylation of IRS-1 may play a role in down-regulation of insulin signalling.

Mammals have three p110 isoforms (p110 α , p110 β , p110 δ), encoded by three separate genes, p110 α and β are widely distributed in mammalian tissues, whereas p110 δ is mainly found in leukocytes (Vanhaesebroeck et al., 1997). At least 7 adaptor subunit proteins have been identified generated by alternative splicing of three different genes (p85 α , p85 β and p55 γ) (Fruman et al., 1998).

1.6.3. *Protein kinase B*

In an attempt to identify the kinase that phosphorylates PDE 3B and lies downstream of PI 3-kinase, Wijkander et al investigated several kinases which are activated by insulin through wortmannin-sensitive mechanisms (Wijkander et al., 1998). Studies were performed on MAP kinase and p70 S6 kinase, but inhibition of these kinases did not effect insulin-induced activation of PDE 3B.

Results strongly suggested that Protein Kinase B (PKB) is responsible for the phosphorylation of PDE3B *in vitro*.

The 60kDa serine/threonine kinase, AKT/PKB, was identified relatively recently. The carboxyl terminal of the protein contains a catalytic domain related to both protein kinase A and C and the amino terminal has a pleckstrin homology (PH) domain, which facilitates protein-lipid binding. PKB was found to be activated by a variety of different growth factors including insulin. The PI 3-kinase inhibitor wortmannin (Franke et al., 1995) and a dominant-negative mutant of PI 3-kinase inhibited activation of PKB, indicating that it lies downstream of PI 3-kinase (Burgering and Coffey, 1995). It was found that PI(3,4,5)P₃ but not PI or PI(4,5)P₂ can bind PKB at its' PH domain and activate the enzyme. Other studies showed that PIP₃ binding was not sufficient to activate PKB fully. PKB was shown to be phosphorylated upon stimulation and phosphatase treatment abolishes activity (Kohn et al., 1996). Two different kinases were found to phosphorylate PKB in the presence of PIP₃, 3 phosphoinositide dependent protein kinase I (PDK1), at Thr³⁰⁸ in the activation loop of the kinase domain (Walker et al., 1998), and PDK 2, which phosphorylates Ser⁴⁷³ near the carboxyl terminal. The model for activation is that PIP₃ binds to the PH domain of PKB, forcing its translocation to the membrane and exposing Thr³⁰⁸ for phosphorylation by PDK1, which is constitutively active. Ser⁴⁷³ of PKB is then phosphorylated by PDK2. After phosphorylation at both sites PKB can detach from the membrane and phosphorylate its targets within the cell, (for review, (Downward, 1998)). More recent studies have confirmed that PDE 3B is a physiological substrate of PKB and that PKB-mediated

phosphorylation on Ser²⁷³ of PDE 3B is important for activation (Kitamura et al., 1999). This is therefore distinct from the insulin-induced phosphorylation at Ser³⁰², previously shown to activate PDE3B (see 1.6.1).

1.7 HSL Translocation

Phosphorylation of HSL clearly plays a major role in the regulation of the enzyme and is well described (Stralfors and Honnor, 1989). However, there is a discrepancy between the lipolytic response in adipocytes to rises in cAMP levels (more than 50 fold) and the stimulation of HSL activity *in vitro* upon phosphorylation of the enzyme by A-kinase (less than 2 fold) (Kawamura et al., 1981; Steinberg and Huttunen, 1972).

A possible explanation lies in the observation that there is redistribution of triglyceride lipase activity upon lipolytic stimulation in adipocytes (Hirsch and Rosen, 1984). It was noted that there was loss of activity from the so-called infranant fraction and an increase in the pellet fraction. This was the first suggestion that interaction of the lipase with its substrate and associated intracellular membranes may be an important feature of regulation. In unstimulated fat cells HSL is predominantly cytoplasmic and redistributes to the fat droplet upon stimulation by lipolytic hormones (Egan et al., 1992). This association of HSL with the fat droplet may result from the export of a fat-associated protein, as has been proposed for the adipocyte protein perilipin. The phosphoprotein, termed perilipin, was first identified by Mooney and Bordwell and it was shown that it was phosphorylated in response to isoproterenol, and that insulin partially counteracted this effect (Mooney and Bordwell, 1991). The

protein was found to be multiply phosphorylated by A-kinase *in vivo* and was closely associated with the lipid storage droplet (Greenberg et al., 1991). Perilipins were originally reported to be adipose specific (Blanchette-Mackie et al., 1995; Greenberg et al., 1991) however, more recently, they were also found to be associated with cholesterol ester droplets in steroidogenic adrenal cortex and leydig cells (Servetnick et al., 1995). Reduction of perilipin levels at the surface of the lipid droplet is required for stimulation of lipolysis. TNF- α induced lipolysis is associated with decreased expression of perilipin protein. Isoproterenol, on the other hand stimulates the migration of perilipin from the lipid droplet, while not affecting total cellular levels (Souza et al., 1998). These data may explain how TNF- α increases the rate of lipolysis in adipocytes without altering levels of HSL (Green et al., 1994).

Recently Syu and Saltiel have identified a novel HSL-interacting protein called lipotransin, that appears to translocate HSL to the lipid droplet and may play an important role in controlling the compartmentalisation of the lipase in cells (Syu and Saltiel, 1999). The model for lipotransin function is as follows; upon phosphorylation HSL can interact directly with lipotransin, which serves to dock the protein at the outer surface of the lipid droplet. Once bound, lipotransin can then undergo a cycle of ATP hydrolysis, permitting the dissociation of HSL and its direct association with the fat droplet. When cells are exposed to insulin, the complex is frozen, blocking the dissociation of HSL. This is a current model and additional experiments will be needed to validate it.

1.8 Type 2 Diabetes

The metabolic function of insulin includes regulation of processes such as glucose uptake, glycogen synthesis and lipolysis. Insulin is secreted by the pancreatic β cells in response to increased levels of plasma glucose resulting in glucose uptake into fat tissue and muscle and reduced hepatic glucose production. Type 2 diabetes is characterised by; resistance to insulin action on glucose uptake, impaired inhibition of hepatic glucose production and dysregulated insulin secretion (Kahn et al., 1996).

Diabetes Mellitus is the most common metabolic disorder worldwide. Type 2, or non-insulin dependent diabetes mellitus (NIDDM), accounts for more than 90% of cases. Prevalence of type 2 diabetes is affected by environmental factors such as, diet, physical activity, and age as well as genetic predisposition (Kahn, 1998). Many type 2 diabetics have hyperglycaemia despite higher than normal concentrations of plasma insulin, or hyperinsulinemia. Initially there is increased insulin secretion to compensate for the decreased sensitivity of target tissues to insulin. Overt diabetes only occurs when the pancreatic β cells fail to compensate for insulin resistance, therefore, hyperinsulinemia may be present in a substantial proportion of the 'normal' non-diabetic population (Reaven, 1996). The fact that insulin resistant persons are able to compensate by secreting enough insulin to overcome insulin resistance does not mean that this hyperinsulinemia is not having any adverse effects in tissues other than fat, liver and muscle.

1.8.1 Syndrome X, diabetes and coronary heart disease

In hyperinsulinemic individuals who have not yet developed type 2 diabetes there is an association between insulin resistance, hypertension, hypertriglyceridemia/dyslipidemia and obesity. This cluster of abnormalities which predispose to atherosclerosis and cardiovascular diseases are described as 'syndrome X' or 'the insulin resistance syndrome'. Several epidemiological studies have shown that mortality rates due to coronary artery disease (CAD) are at least doubled in diabetic subjects (Steiner, 1994; Stout, 1993). However, the incidence of CAD in NIDDM is not related to duration of diabetes. A possible explanation for this and the observation of atherosclerosis at the time of diagnosis of NIDDM is that there is increased atherogenesis caused by the combination of risk factors that make up syndrome X (Steiner, 1994). Some of these risk factors are discussed below.

1.8.2. Dyslipidemia

In diabetes the most common change in circulating lipids is hypertriglyceridemia which reflects an increase in the number of VLDL particles (Ferrannini, 1997). There is evidence that increased levels of VLDL are a risk factor for cardiovascular disease (Mack and Hodis, 1996). Insulin acts to reduce the synthesis of VLDL by the liver and release of TAGs into the circulation. Therefore, insulin resistance-associated hyperinsulinemia results in enhanced hepatic VLDL synthesis and hypertriglyceridemia. Hypertriglyceridemia has been shown to enhance monocyte binding to endothelial cells, an important early event in the atherosclerotic process (Hoogerbrugge et al., 1996). Lipoprotein lipase is an insulin-regulated enzyme

that regulates the removal of TAG from VLDL which is then degraded to LDL and removed from the peripheral circulation. A decrease in insulin action in Type 2 diabetes would therefore contribute to increased VLDL levels (Orchard, 1990). Increased levels of VLDL are associated with reduced HDL levels, as the breakdown products of VLDL are assembled into HDL. Another explanation for the inverse relationship between HDL and TAG concentration relates to the activity of cholesterol ester transfer protein promoting the movement of CE from HDL to VLDL. Thus high VLDL levels results in loss of CE from HDL and lower plasma HDL-cholesterol concentration.

The most powerful predictor of CAD is increases in LDL and LDL is the major supplier of cholesterol to foam cells. The mortality rate due to CAD at any given serum cholesterol concentration is approximately four times greater in those with diabetes, however, LDL levels are similar in diabetics to those seen in the general population. This suggests that any given amount of LDL is more atherogenic in a diabetic. Changes which may occur to LDL to make it more atherogenic include glycation and oxidation which promote uptake by macrophages (Orchard, 1990) and the presence of smaller and denser LDL (Steiner, 1997). Analysis of LDL particle size has shown that persons with smaller LDL (diameter $\leq 255\text{\AA}$) have higher plasma TAG concentrations and lower HDL concentrations. Because similar changes in plasma TG and HDL are associated with insulin resistance it seems likely that small LDL is also associated with insulin resistance. A study of 100 normal persons has shown those with small dense LDL were more insulin resistant and glucose intolerant, hyperinsulinemic, hypertensive, hypertriglyceridemic and had lower HDL

concentrations (Reaven et al., 1993). This suggests that small dense LDL can be added to the cluster of abnormalities which make up syndrome X (Reaven, 1996).

1.8.3. Hypertension

The prevalence of hypertension is greater in the diabetic population and peripheral vascular disease, aortic calcification and CAD occur more commonly in those diabetic patients that also have hypertension (Steiner, 1994; Stern and Tuck, 1996). One reason for this may be defects in ion channels as several membrane ion transport systems are altered by insulin, such as the Na,K-ATPase pump, the Ca²⁺-ATPase pump and the Na⁺/H⁺ antiporter system (Stern and Tuck, 1996). This results in sodium retention and an increase in cytosolic calcium and growth of smooth muscle cells. Other effects of insulin which may promote vasoconstriction and increase blood pressure are enhanced expression of endothelin-I in endothelial cells and increased sympathetic nervous system activity (Reaven, 1996). Reduction in blood pressure has also been reported with better glucose control in diabetic subjects, implying that abnormalities in both glucose and insulin can modulate blood pressure (Stern and Tuck, 1996).

1.8.4. Hyperinsulinemia and increased atherosclerosis

Several prospective studies have shown that hyperinsulinemia is associated with atherosclerosis in the general population (reviewed in, (Steiner, 1994)) and a more recent study provides support that endogenous hyperinsulinemia increases the risk for CAD in patients with type 2 diabetes (Lehto et al., 2000). Clinical observations that IDDM patients with low insulin requirements showed

delayed vascular damage and a higher survival rate than those requiring larger doses also support the idea that insulin is a risk factor for atherosclerosis (Joron and Webb, 1991).

It has already been mentioned that hyperinsulinemia influences a number of potentially atherogenic factors such as alterations in lipids and development of hypertension. In addition to this, hyperinsulinemia is associated with increased levels of plasminogen activator inhibitor 1 (PAI-1). Fibrin is deposited in the occlusion of coronary arteries and may contribute to plaque growth by stimulation of cell proliferation and by the binding and accumulation of low-density lipoprotein (Juhan-Vague et al., 1991). PAI-1 is a physiological inhibitor of fibrinolysis and increased PAI-1 would therefore result in decreased removal of fibrin (Stout, 1993). Elevated PAI-1 is found in patients with CAD, hypertension, obesity and hypertriglyceridemia suggesting an association with insulin resistance and hyperinsulinemia. To further support this, treatment with anti-diabetic drugs have shown parallel reductions in insulin and PAI-1 levels (Juhan-Vague et al., 1991). At physiological concentrations insulin stimulates the proliferation and migration of cultured arterial smooth muscle cells, and cholesterol synthesis and LDL binding in both smooth muscle cells and monocyte macrophages (Stout, 1990). These multiple effects of insulin (summarised in table 3.) would be expected to contribute to lesion formation and suggest a potential direct role of insulin in the development of atherosclerosis.

Table 3 *Effects of insulin on arterial tissue*

-
- (2) increased formation of lipid lesions (Abe et al., 1996; Stout, 1970)
- increased lipid synthesis (Stout, 1970)
 - connective tissue synthesis (Abe et al., 1996)
 - proliferation and migration of arterial smooth muscle cells (Larson and Haudenschild, 1988; Pfeifle and Ditschuneit, 1981)
 - increased cholesterol synthesis and LDL binding in smooth muscle cell and monocyte derived macrophages (Krone and Greten, 1984; Krone et al., 1988)
-

1.8.5. Hyperglycaemia

Impaired glucose tolerance (IGT), a category that falls between normal glucose tolerance and diabetes, can be experienced for years before overt diabetes and hyperglycaemia develop. Nondiabetic subjects with IGT have about a twofold increase in the risk of macrovascular disease. The risk starts at levels of glycaemia considerably lower than the threshold for the diagnosis of diabetes (Bonora et al., 2000; Laakso, 1999).

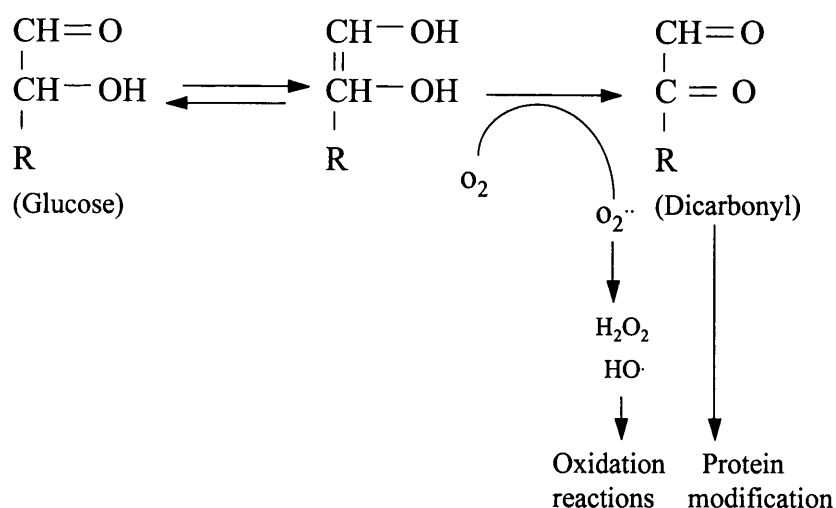
One mechanism by which glucose may influence atherosclerosis is the formation of advanced glycation end products (AGEs), due to prolonged incubation of proteins with glucose. In its open chain form glucose has an aldehyde group that reacts with lysine residues of proteins forming fructoselysine, 'the amadori product'. A protein with the amadori product can be cross-linked to other proteins and lipoproteins through its' reactive carbonyl

group (Semenkovich and Heinecke, 1997) (see figure 1.7). These AGEs are thought to promote vascular disease.

Glycation of apolipoproteins in all classes of circulating lipoproteins has been found in diabetes. Glycated LDL interacts poorly with the LDL receptor, therefore its residence time in the plasma and in the extracellular space of the artery wall, where oxidative modification can occur, is increased. Macrophage scavenger receptors recognise proteins modified by AGEs and mediate their uptake and degradation, and thus, may take up AGE modified LDL at an increased rate. This process has been shown to stimulate the release

Figure 1.8 *Set of reactions which glycated proteins may undergo:*

Dicarbonyls covalently modify proteins and promote cross-linking reactions and reduced oxygen species trigger the oxidation of proteins and lipids



of cytokines and growth factors (Vlassara et al., 1988). The macrophage scavenger receptor-mediated endocytotic uptake of AGEs can be accelerated by insulin through a PI 3-kinase dependent pathway (Sano et al., 1998). Also, glycated HDL shows impaired ability to stimulate cholesterol efflux from cells (Chait and Brunzell, 1996). All of these effects of glycation may play a role in increased atherogenesis. Also the reactive oxygen species, superoxide, hydrogen peroxide and hydroxyl radical produced during glucose autooxidation (see figure 1.7) will contribute to lipoprotein oxidation and foam cell formation. Hyperglycemia has also been shown to promote leukocyte adhesion to the endothelium through upregulation of the expression of cell surface adhesive proteins (Morigi et al., 1998). Again, this process is an important early event in atherosclerosis.

Glucose-induced activation of PKC may represent another pro-atherogenic mechanism. Cells transport excess glucose intracellularly using glucose transporters, where it is metabolised and alters signal transduction pathways, such as the activation of diacylglycerol (DAG) and protein kinase C (PKC). DAG contents have been shown to be increased in vascular cells when glucose levels were increased from 5 to 22mmol/l. DAG, which activates PKC can be derived from phosphoinositides (PIs) or from the hydrolysis of phosphatidylcholine (PC) by phospholipase C (PLC) or D (PLD). However, studies using labelled glucose have shown that glucose is incorporated into the glycerol backbone of DAG, suggesting that the source of glucose-induced DAG appears to be from the *de novo* pathway (Xia et al., 1994).

As mentioned earlier, glucose can affect blood flow and promote hypertension. PKC activation has been shown to increase the expression of endothelin-1, a potent vasoconstrictor (Koya and King, 1998) and decrease the production of NO, a potent vasodilator, and its second messenger, cGMP (Craven et al., 1994). Hyperglycaemia, like hyperinsulinemia, also reduces Na^+/K^+ -ATPase activity, which affects cellular functions such as contractility, growth and differentiation (Xia et al., 1995).

1.8.6. Obesity

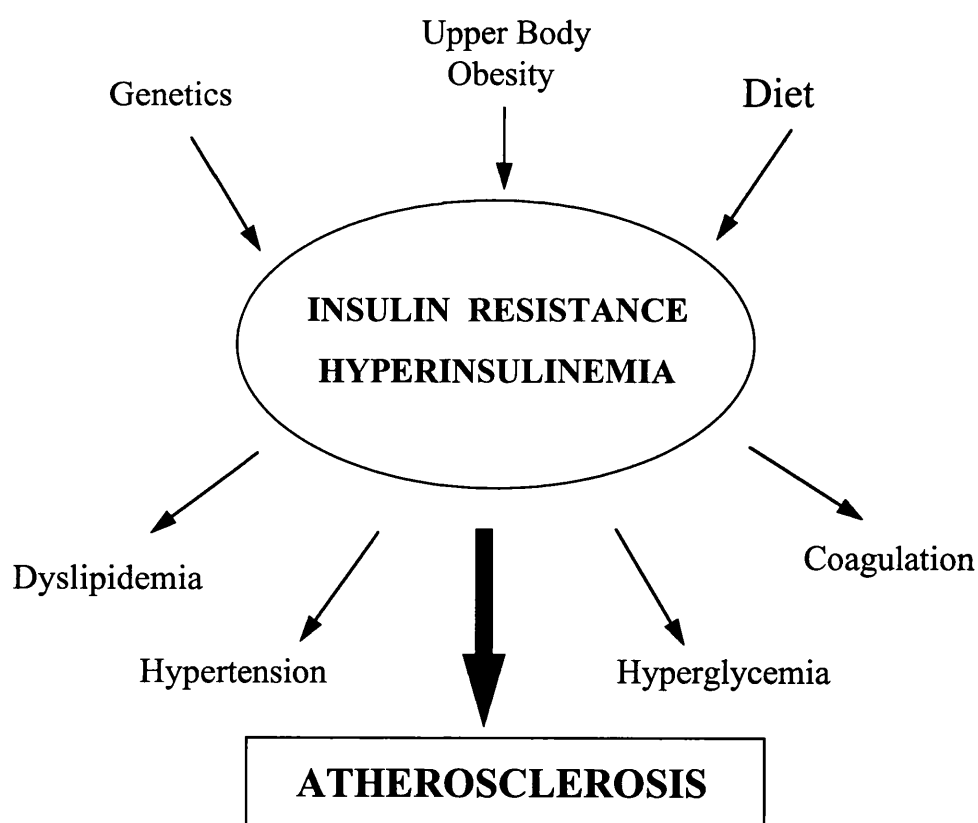
Two major types of obesity can be easily defined. These are upper body (apple) and lower body (Pear) obesity. Upper body, fat distribution is measured as increased waist to hip ratio, and is associated with hypertriglyceridemia, low HDL-cholesterol levels, impaired glucose tolerance and an increased risk of diabetes and atherosclerosis (Stout, 1993). Insulin concentrations, both in the basal state and after a glucose challenge are associated with increasing body weight and are reduced by weight loss. Upper body obesity is associated with large fat cells, which tend to be insulin resistant, suggesting disturbances in abdominal fat lipolysis (Klannemark et al., 1998).

The following model has been proposed to explain the mechanism of obesity-associated cardiovascular disease:

Initially increased intra-abdominal fat leads to increases in circulating free fatty acids, accompanied by the development of insulin resistance. This combination increases the secretion of VLDL from the liver and hepatic lipase activity

resulting in the dyslipidemia of central obesity; hypertriglyceridemia, with the generation of small dense LDL and reduced HDL cholesterol. In addition to this change in lipid profile, impaired glucose tolerance develops from insulin resistance, leading to hyperglycaemia. Finally increased arterial blood pressure accelerates atherogenesis further (Schwartz and Brunzell, 1997).

Figure 1.9 *Diagram illustrating some of the factors involved in the insulin resistance syndrome that might account for the observed increase in atherosclerosis*



1.9 The obese (ob) gene and its product leptin

The ob/ob and db/db mutant mice were identified over thirty years ago as having genetic obesity syndromes (Hummel et al., 1966). Body weight of the ob/ob mouse could be normalised with the blood of a lean animal, suggesting that the ob/ob mouse was deficient in a circulating factor. However, db/db mice did not exhibit body weight normalisation suggesting that these mice were unable to respond to the circulating factor, perhaps due to a defect in the receptor, (reviewed in (Coleman, 1978)). As recently as five years ago the obese gene product was identified. The product is a 16kDa protein expressed predominantly in adipose tissue, named leptin. Production of this cytokine by adipose tissue is a negative feedback mechanism which acts at the brain to reduce food intake and increase energy expenditure (Auwerx and Staels, 1998). Leptin is transported through the blood/brain barrier and acts at its receptor in the hypothalamus, resulting in reduced expression of effectors such as neuropeptide Y (Mantzoros, 1999), a potent stimulator of food intake, decreasing appetite and increasing energy expenditure by altering sympathetic and parasympathetic tone (Collins et al., 1996). In obesity, leptin mRNA expression and circulating levels of leptin are increased, but appetite is not suppressed, suggesting resistance to leptin action (Frederich et al., 1995).

1.9.1. The leptin Receptor

The leptin receptor (OB-R) was first revealed as a single membrane-spanning receptor of the class I cytokine receptor family (Auwerx and Staels, 1998), which shows most similarity to the gp130 and G-CSF receptors. The extracellular

domain of this originally identified receptor was quite large while the intracellular domain was fairly short, about 34 amino acids. However, further screening of cDNA libraries revealed that there were multiple forms of OB-R in both mice and humans, including a long form (OB-RL) with an intracellular domain of 303 amino acids (Chen et al., 1996; Lee et al., 1996; Tartaglia et al., 1995). The extracellular domains of the short and long forms are identical as RNA splicing occurs at the most C-terminal exon, resulting in OB-R intracellular domains with different length and sequence composition (Chen et al., 1996; Lee et al., 1996). Additional short intracellular domains have now been identified which terminate shortly after amino acid 29 of the intracellular domain. A transcript encoding a soluble form with no transmembrane domain has also been identified (Lee et al., 1996).

Expression of OB-R was found in multiple tissues. In the mouse the highest levels of OB-R are found in the choroid plexus, lung and kidney and lower levels in nearly all tissues. The vast majority are the short forms (OB-R_S) with the long form being much less abundant, expressed in most tissues but at very low levels (Ghilardi et al., 1996). To date, only the hypothalamus has been found to express more long form than short form (Mercer et al., 1996). This pattern of tissue distribution suggested that leptin might exert cellular effects on tissues other than the brain.

1.9.2. Role of leptin in the regulation of lipid storage

Leptin was found to have adipose reducing effects beyond what could be accounted for by a reduction of food intake, and a series of findings support the

idea that leptin has direct effects on adipose tissue. Firstly, leptin directly alters TAG lipolysis in adipocytes. Leptin stimulation increases basal lipolysis in ob/ob mice and lean mice, but not in db/db mice, which have a mutation in the OB-R gene (Fruhbeck et al., 1998; Fruhbeck et al., 1997; Siegrist-Kaiser et al., 1997). Recently, it has been reported that this is a novel form of lipolysis, where hydrolysis of TAG, results in glycerol release but not FFA release (Wang et al., 1999). Chatecholamine-induced lipolysis increases the release of both, therefore suggesting that leptins effects on lipolysis are not due to adrenergic stimulation. The mechanism of leptin-induced lipolysis remains to be elucidated. It is not known if leptin, like chatecholamines, increases cAMP levels or HSL activity.

In addition to the effects on lipolysis leptin is responsible for its own downregulation (MacDougald et al., 1995) and also regulates the expression of other genes involved in lipid metabolism. It was found to reduce mRNA of fatty acid synthase and increase mRNA of peroxisome proliferator-activated receptor (PPAR)- α , carnitine palmitoyl transferase-1 (CPT-1) and acyl CoA oxidase (ACO) (Wang et al., 1999). The up-regulation of ACO and CPT-1 support the idea that FFAs are oxidised inside the adipocytes rather than exported to the liver for oxidation to ketoacids.

It is proposed that an important function of leptin is to limit the storage of TAG in non-adipocytes. The TAG content of non-adipocytes normally remains within a narrow range so the cells are protected from lipotoxicity. When leptin receptors are dysfunctional, the TAG content in non-adipocytes such as islets can increase 100 fold. This together with the fact that leptin administration

depletes TAG, suggests that leptin is important in TAG homeostasis in these cells (Unger et al., 1999).

1.9.3. Effects of leptin on other peripheral tissues

1.9.3.1 *Macrophages*

The leptin receptor was found to be abundant on the surface of macrophages. In transfection experiments the long form but not the short form was capable of signalling for differentiation of monocytes into macrophages (Gainsford et al., 1996). Leptin also enhances the production of cytokines and increases the attachment and receptor-mediated phagocytosis by mature macrophages (Gainsford et al., 1996; Loffreda et al., 1998). Studies in rodents with abnormalities in leptin or the leptin receptor revealed reduced macrophage phagocytosis and expression of pro-inflammatory cytokines. Increased macrophage production of TNF α , IL-6 and IL-12 is a fundamental aspect of the immune response and is induced by bacterial lipopolysaccharide (LPS). Leptin enhances the synthesis of these proinflammatory cytokines when cells are treated with LPS (Loffreda et al., 1998). There is also evidence that leptin has an important role in hematopoietic and immune system development (Mikhail et al., 1997; Umemoto et al., 1997). Leptin stimulates the proliferation of murine myelocytic progenitor cells and the proliferation of primitive hematopoietic progenitors *in vitro* (Mikhail et al., 1997). The role of leptin in the immune response may explain the suppression of this response and susceptibility to infectious diseases during nutritional deprivation. Low levels of leptin are associated with low body weight and impaired cell-mediated immunity. Leptin appears to cause T-cells to switch from a regulatory phenotype to a pro-

inflammatory phenotype (Lord et al., 1998). It induces the expression of adhesion molecules on the surface of T-cells and stimulates proliferation and IL-2 and IFN γ , pro-inflammatory cytokine, production (Lord et al., 1998). Therefore it seems that maintaining leptin levels is important for a proper immune response, but equally important is the fact that excess leptin may contribute to inappropriate recruitment and activation of leukocytes that can lead to diseases such as atherosclerosis.

1.9.3.2. Endothelial cells

It has recently been reported that leptin can induce oxidative stress in human endothelial cells (Bouloumie et al., 1999). Leptin causes the accumulation of reactive oxygen species which act as secondary messengers enhancing the expression of monocyte chemotactic protein-1 (MCP-1) (Bouloumie et al., 1999). MCP-1 stimulates the transendothelial migration of monocytes. Therefore obesity-associated hyperleptinemia may contribute to atherogenesis by causing oxidative stress in endothelial cells.

1.9.3.3. Myotubes

It has been reported that leptin stimulates glycogen synthesis and Glut 4 recruitment in myotubes. Pre-incubation of cells with leptin did not effect insulins ability to activate glucose transport and glycogen synthesis but leptin itself stimulated 80-90% of the insulin effect (Berti et al., 1997).

1.9.3.4. *Pancreas*

Studies have revealed both OB-R_L and OB-R_S in pancreatic islets (Tanizawa et al., 1997). As mentioned above, a major role of leptin is thought to be to limit TAG accumulation in non-adipocytes. TAG homeostasis in islet cells is vital for maintaining cell function. Islets from rats with defective leptin receptors show increased TAG accumulation compared to normal islets (Unger et al., 1999). As the fat content rises the insulin production from β -cells rises. However, when the fat content exceeds 50 times that of a normal β -cell lipoapoptosis occurs and insulin secretion ceases leading to diabetes. Excess leptin on the other hand depletes islet cells of fat and they become functionally paralysed with a low rate of insulin production and complete unresponsiveness to glucose (Unger et al., 1999). Hormone-sensitive lipase has been identified in β -cells (Mulder et al., 1999) but it has not been determined if leptins actions on TAG homeostasis are mediated by the regulation of this enzyme. Leptin has also been reported to directly regulate insulin secretion from islet cells. In the presence of elevated blood glucose, cAMP stimulates insulin secretion from pancreatic B cells and leptin has been shown to inhibit insulin secretion by reducing cAMP levels (Zhao et al., 1998).

1.9.4. Leptin signalling mechanisms

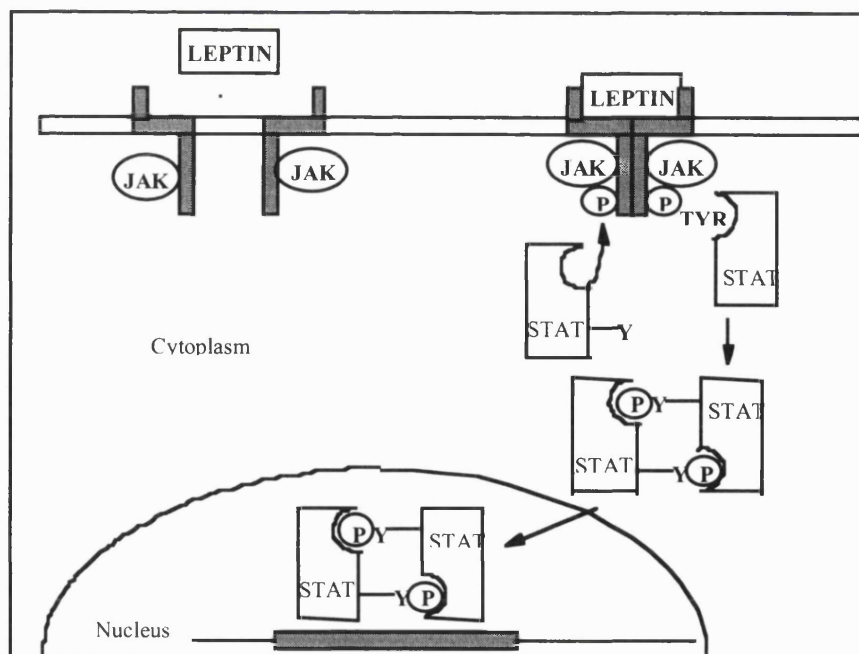
1.9.4.1. *STAT signalling*

The first signalling pathway found to be associated with the leptin receptor was the 'JAK/STAT' pathway, capable of modulating gene transcription (Baumann et al., 1996). Janus kinase-2 (JAK-2) associates with the leptin receptor and is required for activation of the receptor. Upon ligand binding the receptor is

induced to homo- or heterodimerise and JAKs become tyrosine phosphorylated and are activated as tyrosine kinases. The intracellular tail of the receptors are then phosphorylated on one or more tyrosine residues allowing binding via the SH₂ domains on STATs (signal transducers and activators of transcription) (Baumann et al., 1996; Darnell, 1996; White et al., 1997). The attached STATs are themselves phosphorylated by JAK kinases and then dimerise and translocate to the nucleus and bind to specific DNA sites in certain gene promoters, regulating transcription. Leptin has been shown to activate STAT3, STAT5 and STAT 6, a STAT subset known as the 'fat STATs', due to their association with lipid metabolism (Darnell, 1996). In the non-functional mutant form of OB-R found in db mice, both putative STAT-binding sites are missing (Ghilardi et al., 1996).

Figure 1.10. *Leptin receptor signalling via janus kinase-2 and STATs*

(signal transducers and activators of transcription)



1.9.4.2 *Other signalling proteins employed by leptin*

It has previously been shown that leptin is able to impair insulin signalling. In NIH3T3 adipocytes (Kroder et al., 1996) and HEPG2 cells (Cohen et al., 1996) leptin has been shown to impair autophosphorylation of the insulin receptor and tyrosine phosphorylation of IRS-1. Contrary to these findings, leptin was shown to mimic insulin effects in C₂C₁₂ myotubes (Berti et al., 1997). The leptin-stimulated glucose transport and glycogen synthesis in these cells is inhibited by wortmannin and H7, suggesting PI 3-kinase and PKC dependent signalling (Berti et al., 1997). It should be noted that the inhibitory effects of leptin on insulin signalling were only observed at high concentrations of leptin and the insulin-like effects of leptin were only observed at low concentrations. This might suggest that the high leptin concentrations observed in obesity could contribute to insulin resistance. Further investigation into the signalling mechanisms employed by leptin revealed that leptin can activate PI 3-kinase via JAK-2, which phosphorylates IRS-2, but not IRS-1, and recruits PI 3-kinase (Kellerer et al., 1997). It was claimed that these effects were mediated by the short leptin receptor isoform, as the long form could not be detected in C₂C₁₂ myotubes. In contrast a more recent report detected both the long and short forms in C₂C₁₂ myotubes (Berti and Gammeltoft, 1999). This report shows that leptin-induced activation of GLUT4 recruitment and glucose uptake requires extracellular signal-regulated kinase 2 (ERK2) and PI 3-kinase, whereas insulin-stimulated glucose transport requires PI 3-kinase but not ERK-2.

At least 3 different MAP kinase classes have been identified; ERK kinases, JNK-SAPK (NH₂-terminal c-Jun kinase/stress activated protein kinase) and p38. In endothelial cells, leptin has been shown to stimulate ERK 1/2 resulting in enhanced cell proliferation (Bouloumie et al., 1998)]. Leptin also activates the JNK-SAPK pathway in these cells (Bouloumie et al., 1999). It was mentioned earlier that stimulation of endothelial cells with leptin leads to an accumulation of reactive oxygen species. This effect is associated with the activation of JNK/SAPK pathway and the transcription factor NF- κ B, which results in enhanced expression of MCP-1 (Bouloumie et al., 1999).

Another signalling mechanism employed by leptin is PI 3-kinase dependent activation of PDE 3B, resulting in a reduction of cAMP levels (Zhao et al., 1998). Leptin suppresses glucose-stimulated insulin secretion from islet cells by activation of these signalling molecules. This is another example of leptin using the same signalling pathway as insulin, as this is how the antilipolytic effect of insulin is mediated in adipocytes (see section 1.6). The leptin effect on insulin secretion was found to be biphasic, with the maximal effect being observed at 2nM, and higher concentrations being less efficient (Zhao et al., 1998).

The majority of investigations of leptin function have concentrated on its role and signalling mechanisms in the hypothalamus, where it mediates important physiological effects on appetite. However, the effects of leptin in peripheral tissues remain controversial but several of the responses detailed above could be physiologically important so will no doubt be a main focus for future research.

The effects of leptin on macrophages are of particular interest to the current thesis.

1.10. Treatments for Type 2 Diabetes

The goal of effective antidiabetic therapy is to achieve as closely as possible normal blood sugar levels while avoiding serious hypoglycemia. The first-line therapy for type 2 diabetes is diet and weight loss, however compliance is poor for most patients. A range of drug therapies are currently available to treat hyperglycemia, although side effects are commonly experienced and attempts at improving current therapies and developing new ones are ongoing.

1.10.1. Sulfonylureas

Sulfonylurea therapy is a common treatment for type 2 diabetes. These agents stimulate release of insulin from pancreatic β -cells by causing changes in the ATP sensitive K^+ channels in the β -cell membrane (Dean et al., 1975; Meissener and Atwater, 1976). Tissues such as the brain need glucose constantly and low blood glucose can cause seizures, loss of consciousness and death (Shepherd and Kahn, 1999). The most common and significant side effect of sulfonylurea use is hypoglycemia and the elderly are more vulnerable to this side effect (Asplund et al., 1983; Brodows, 1992). Data indicates that more than 85% of sulfonylurea-related hypoglycemia occurs in patients over 60 years of age and patient fatality rates are as high as 10%. Sulfonylureas can also cause weight gain and hyperinsulinemia, which in turn may be related to an increased risk of atherosclerosis in patients with type 2 diabetes (Amatruda, 1996).

1.10.2. α -Glucosidase Inhibitors

A new alternative therapy is the α -glucosidase inhibitor, acarbose, which is commercially available and is approved for treatment in 57 countries. Acarbose reduces postprandial glucose and insulin by retarding the digestion of carbohydrates in the small intestine, delaying glucose absorption. Starch is digested to oligosaccharides by amylase and is then further digested by α -glucosidases to glucose. The delay in carbohydrate digestion by inhibition of α -glucosidase activity results in blunting of blood glucose and insulin peaks after a meal and a smoothing of the daily insulin and glucose profiles (Amatruda, 1996). Acarbose does not cause weight gain, like sulfonylurea and insulin therapy, which is most likely related to the decrease in postprandial insulin concentrations in contrast to the increase in insulin concentrations associated with sulfonylurea treatment (Coniff et al., 1995).

1.10.3. Fatty Acid Oxidation Inhibitors

For some time elevated FFA concentrations and fatty acid oxidation (FAO) rates have been implicated as a cause of insulin resistance (Boden et al., 1994; Roden et al., 1996). As a result, the potential use of fatty acid oxidation inhibitors as a treatment for type 2 diabetes has recently been recognised. Liver is the major regulator of glycemia. After a meal glucose production is reduced and glucose utilisation by the liver is increased. Excess glucose escapes regulation by the liver and is utilised via insulin-mediated uptake, mainly by muscle (Mitrakou et al., 1990). Glucose and FFA are competing sources of fuel by muscle, and FFA is the preferred substrate. In diabetes, high lipolysis rates at fasting insulin concentrations leads to increased FFA levels and increased FAO. This results in

reduced glucose oxidation and ultimately the accumulation of free glucose in the cell. As glucose transporters work by a process of facilitated diffusion (Shepherd and Kahn, 1999), this leads to reduced net glucose transport. Also the high FFA concentration and elevation in FAO in NIDDM will lead to increased gluconeogenesis and hepatic glucose production (Groop et al., 1989; Taskinen et al., 1989).

Fatty acids are transported across the mitochondrial membrane by the carnitine palmitoyl transferase system prior to β -oxidation (Woeltje et al., 1990). CPT-1 is the rate-limiting step in FAO, therefore CPT inhibitors were recognised as having great potential for the treatment of diabetes-related disorders. Early CPT inhibitors were found to have side-effects on the heart, however SDZ CPI 975 is a liver-selective inhibitor which has entered the late stages of clinical trials (Foley and Anderson, 1996).

1.10.4. Thiazolidinediones in the treatment of insulin resistance and type 2 diabetes

Much attention is now focussed on a novel class of drugs, the thiazolidinediones (TZDs). These drugs directly decrease insulin resistance by enhancing insulin action, without increasing insulin secretion. The underlying mechanisms of action of the drugs are not fully understood as these compounds were initially isolated by screening experiments and not by rational drug design. The first reported TZD was ciglitazone in 1982, since then many analogues have been produced including troglitazone, pioglitazone and more recently rosiglitazone, or BRL-49653 . If the mechanism of action of TZDs was better understood this could also lead to better understanding of the pathophysiology of type 2

diabetes and the drugs could more easily be modified to reduce side effects. Therefore, in recent years a large amount of research has focussed on the mode of action of TZDs.

1.10.4.1. PPAR- γ thiazolidine receptor

The most clearly defined receptor for TZDs is the peroxisome-proliferator activated receptor γ (PPAR γ) (Young et al., 1997). The PPAR family of ligand-dependent transcription factors bind to specific DNA sequences termed PPAR response elements (PPRE) and regulate the expression of a number of genes, many of which are involved in lipid metabolism (Martin et al., 1998). Target genes for PPAR γ , and thus TZDs, include TNF α and leptin, the expression of which are reduced, and also Glut4, lipoprotein lipase, fatty acid binding protein (aP2) (Spiegelman, 1998).

The PPARs are members of the nuclear hormone receptor superfamily and on isolation from cells are found to exist as heterodimers with the retinoid X receptors (RXR). There are three known PPAR isoforms, PPAR α , PPAR δ and PPAR γ , and they are differentially expressed in tissues. PPAR γ is found mainly in adipose tissue (Fajas et al., 1997), where it plays a crucial role in adipocyte differentiation and fat storage, whereas PPAR α , the main PPAR subtype in liver, plays a role in lipid oxidation (Braissant et al., 1995). In addition there are two splice variants of PPAR γ , PPAR γ 1 and PPAR γ 2. PPAR γ 2 is specifically found in adipocytes and PPAR γ 1 is found in low levels in other tissues.

The high level of PPAR γ in adipose tissue points to the important function of this transcription factor in adipogenesis and adipocyte gene expression. Adipogenic stimuli activate the transcription factors C/EBP β and γ , both of which induce the expression of PPAR γ . PPAR γ subsequently stimulates the expression of adipocytes specific genes such as aP2, . The adipogenesis-promoting effects of TZDs raised concerns surrounding the clinical use because of the potential weight gain in patients. However clinical use in humans has not shown these drugs to induce weight gain. It is thought that this is because TZDs produce more fat cells of smaller average size. Smaller cells are more insulin-sensitive resulting in reduced lipolysis and reduced FFA levels. As mentioned earlier this would improve glucose uptake and oxidation in muscle.

Interestingly, not only are PPARs responsible for regulating genes involved in lipid metabolism but, conversely, lipids are also shown to regulate PPAR activity. PPAR γ and PPAR α are activated by naturally occurring arachidonic acid metabolites, for example, prostaglandin J2 is a ligand for PPAR γ and 5,8,11,14-eicosatetraenoic acid (ETYA) activates PPAR α , and also PPAR γ at higher levels . Another way of regulating PPAR function is by phosphorylation. PPAR γ activity can be depressed by mitogen-activated protein kinase-mediated phosphorylation of a serine residue at position 114. In contrast MAP kinase phosphorylation of PPAR α at two serine residues (positions 12 and 21) leads to activation (Juge-Aubry et al., 1999).

1.10.4.2. Effects on insulin signalling

There is evidence that much of the insulin sensitisation caused by TZDs is due to actions on skeletal muscle, which express little PPAR γ . It is possible therefore, that TZDs may enhance insulin action by mechanisms other than PPAR γ activation. For example, TZDs have also been shown to have acute effects on insulin signal transduction pathways. In cultured CHO cells, expressing the human insulin receptor and L6 myotubes pre-incubation for 1 hour with the TZD, CP-86 325, followed by a 5 minute insulin stimulation showed increased PI 3-kinase recruitment and activity (Zhang et al., 1994). The mechanism for this is unclear. Some TZD-treated cells show a limited increase in insulin binding and/or the number of insulin receptors while others have shown no change (Zhang et al., 1994). The effect on IRS-1 also appears to be contradictory with some reports showing no change in the tyrosine phosphorylation state, whilst treatment with a TZD derivative JTT-501 stimulated autophosphorylation of the insulin receptor and phosphorylation of IRS-1 (maegawa et al., 1999).

1.10.4.3. Improvement in insulin sensitivity in animal models of diabetes

TZDs decrease plasma glucose, insulin and TAG in insulin-resistant animals including ob/ob, db/db and the Zucker fa/fa rat (Fujita et al., 1983). TZDs increase insulin-induced glucose uptake, glycogen synthesis and glycolysis while reducing hepatic glucose output due to decreased gluconeogenesis in the liver (Bowen et al., 1991; Oakes et al., 1994). However, in normal animals glucose levels are unaffected although plasma insulin and TAG levels are lowered (Stevenson et al., 1991). This highlights an important advantage of TZD action, insulin sensitisation without hypoglycemia. Treatment of obese mice

with rosiglitazone results in increased glucose transport in adipocytes both by increasing insulin receptor number and increasing GLUT4 translocation (Young et al., 1995). Hepatocytes from streptozotocin-induced diabetic rats show a significant decrease in the activation of glycogen synthase. These hepatocytes are also characterised by a 50% decrease in the activity of protein phosphatase-1, the enzyme which dephosphorylates and activates glycogen synthase. Pioglitazone enhanced the long-term stimulatory effect of insulin to ameliorate the decrease in PP1 activity in these cells (Pugazhenthir and Kandelwahl, 1998).

TZD treatment increases plasma HDL levels and reduces LDL, VLDL, TAG and FFA levels in diabetic rodent models. These effects are observed in both insulin-resistant and insulin deficient animals suggesting that these actions are not a result of sensitisation to insulin action (Kraegen et al., 1989; Stevenson et al., 1991). Hypertriglyceridemia is an important risk factor for atherosclerosis, especially in type 2 diabetes. The mechanism by which TZDs reduce TAG is not clear, it may be a direct effect on VLDL production by the liver or an indirect effect resulting from the reduction in plasma insulin. TZDs increase lipoprotein lipase activity so it appears that the decrease in TAG is probably a result of both enhanced clearance and reduced synthesis (Saltiel and Olefsky, 1996). Currently little is known about the effects of TZD treatment on the processes that result in conversion of macrophages to foam cells. However, in isolated monocytes/macrophages OLDL has been shown to activate PPAR γ resulting in promotion of monocyte/macrophage differentiation, regulation of macrophage gene expression and increased uptake of OLDL. These findings suggest an unexpected role for nuclear receptors in the pathogenesis of atherosclerosis.

PDE 3B is known to be a key enzyme in the antilipolytic action of insulin in adipocytes, which results in reduced output of FFA. Recently, the expression of this enzyme was found to be reduced in the fat tissues of obese diabetic KKAY mice and pioglitazone treatment increased protein levels and activity of PDE 3B in these tissues (Tang et al., 1999).

Although TZDs in general do not modulate insulin secretion in islet cells, improvement of insulin resistance restores the responsiveness of desensitised islets to external stimuli. It is thought that this beneficial effect is due to glucose lowering as glucose toxicity is thought to be responsible for attenuating glucose-dependent insulin secretion (Fujiwara et al., 1991). TZD treatment also suppresses hypertension in obese zucker rats (Yoshioka et al., 1993) and high fructose-fed rats (Lee et al., 1994). This may be due to the insulin-lowering effects of TZDs.

A possible mechanism by which TZDs improve insulin resistance could be the reduction in TNF α and leptin mRNA and protein, caused by treatment with these drugs (Vos et al., 1996). Both TNF α (Uysal et al., 1997) and leptin (Kroder et al., 1996; Cohen et al., 1996) have been reported to interfere with insulin signalling

1.11 Summary and Aims

In summary, the diabetic population have at least a two fold increased risk of developing heart disease. The majority of diabetics suffer from type 2 diabetes, which is generally preceded by prolonged periods of insulin resistance. Insulin

resistance is associated with a series of abnormalities including hyperinsulinemia, postprandial hyperglycaemia and obesity, collectively described as 'syndrome X'. There is evidence to suggest that these conditions could promote atherosclerosis. The bulk of the earliest lesion of atherosclerosis is made up of lipid filled macrophages, called foam cells. We aim to investigate how factors associated with syndrome X could affect foam cell formation.

HSL is the rate-limiting enzyme in triglyceride lipolysis and its activity is reduced by insulin in adipocytes. Leptin is a hormone raised in the circulation of obese individuals. Leptin has been shown to directly increase lipolysis in adipocytes, potentially by regulation of the enzyme HSL. HSL is also the enzyme responsible for the breakdown of CE in macrophages, however, little is known about HSL regulation in macrophages, only that the activity of the enzyme is increased by cAMP, which is also the case in adipocytes.

Therefore the main aims of this work are;

(i) to determine if insulin and leptin acutely regulate HSL activity in macrophages. If this is the case dysregulation of HSL activity due to resistance to the actions of these hormones may be important for atherosclerosis.

(ii) to investigate the chronic effects of insulin and leptin on HSL expression and activity. These studies will be carried out on cells maintained in media containing high or low levels of glucose, to determine the importance of hyperglycaemia on HSL regulation

(iii) to investigate how glucose levels, insulin and leptin effect the loading of macrophages with cholesterol esters.

(iv) Finally the signalling mechanisms by which HSL is regulated in macrophages will be investigated.

2. MATERIALS AND METHODS

2.1 MATERIALS

3T3-L1 adipocytes were obtained from the American tissue culture Collection (ATCC CCL 92.1) and J774.2 macrophages were kindly provided by Professor S.J.Yeaman, Newcastle-upon-Tyne. Anti-HSL antibody, (Kraemer et al., 1993) was provided by Dr Raj Beri, AstraZeneca Pharmaceuticals, anti-p110 α antibody was supplied by Dr Bart Vanhaesebroeck, Ludwig Institute, London, anti-JAK-2 and anti-C-terminal leptin receptor antibodies were supplied by Dr Martin Myers, Joslin Diabetes Center, Boston, anti-phosphotyrosine antibody (Py99) was obtained from Santa Cruz biotechnology and thiazolidinedione drugs were provided by Smithkline Beecham. [125 I]Protein A (cat no, IM 144), cholesterol[1- 14 C]oleate (CFA 256), [1- 14 C]oleic acid (CFA 243) and [γ - 32 P]ATP (AA 0068) were obtained from Amersham. RPMI 1640 (52400-041) and New born calf serum (NBCS, 16010-076) were obtained from Gibco. Foetal calf serum (FCS, F7524) and DMEM (D-6046 and D-5796) were from Sigma. All chemicals were from Sigma, unless stated otherwise.

2.2 METHODS

2.2.1 Cell Culture Systems

The cell culture model to be used for the studies in adipocytes are 3T3 L-1 adipocytes, clonally selected from a 3T3 mouse embryo cell line (Green and

Kehinde, 1973). They initially exist as fibroblasts, but once they become confluent and contact inhibited they are able to differentiate. During differentiation the cells develop an adipocyte-like morphology by becoming rounded and forming lipid droplets (Green and Meuth, 1974). 3T3 L-1 adipocytes are very insulin responsive and show increased glucose transport and glycogen synthesis and reduced lipolysis upon insulin stimulation. 3T3 L-1 adipocytes exhibit differences in morphology compared to true adipocytes, for example, TAG is stored in multiple droplets, as opposed to one large fat droplet in animal and human adipocytes (Green and Meuth, 1974). These cells have however proved to be invaluable in the study of insulin signalling pathways.

The chosen cell line for the macrophage studies was the J774 macrophage-like cell line (Ralph et al., 1975) which have the macrophage properties of adherence, morphology, receptors for immunoglobulin, and antibody-dependent lysis of target cells (Ralph et al., 1976). These cells grow partly adherent and partly floating with both populations of cells showing identical properties (Ralph and Nakoinz, 1975). J774 macrophages have been used for previous studies on HSL (Jepson et al., 1996) and are also commonly used as a foam cell model (Avart et al., 1999; Bernard et al., 1991; Miura et al., 1997).

2.2.2 Tissue Culture

Reagents were prewarmed to 37°C and all procedures were carried out in a laminar flow hood, providing a sterile environment. All cells were maintained in a 37°C, 5% CO₂ gassed incubator.

2.2.2.1 J774.2 macrophages

The murine macrophage cell line J774.2 were grown in 10 ml RPMI medium (2g glucose/l), supplemented with 10% FCS and 1% antibiotic-antimycotic, per 10cm dish (NUNC, Gibco) or 75 cm² flasks (Falcon, Marathon labs). The medium was removed and replaced with fresh media every 48 hours. To passage the cells the medium was removed and cells were scraped from the bottom of the flask. The appropriate amount of pre-warmed media was used to resuspend and split the cells between the corresponding number of dishes.

2.2.2.2 3T3-L1 Adipocytes

3T3-L1 fibroblasts were grown in DMEM containing 4.5g/l glucose, 10% NBCS and 1% antibiotic-antimycotic. Stock flasks of fibroblasts were split at sub-confluency to avoid contact inhibition. Medium was removed and fibroblasts were washed with 2 ml PBS (without calcium or magnesium) before addition of 1ml trypsin/EDTA per 75cm² flask (TPP). This was incubated for 5-10 minutes at 37°C. The flask was tapped lightly to dislodge cells from the surface. The appropriate amount of media was then added and cells were split into 10cm² dishes (TPP) for differentiation or flasks for stock fibroblasts.

2.2.2.3 Differentiation of adipocytes

Fibroblasts were differentiated two days after confluence had been reached. Method of differentiation was that of Frost and Lane,(Frost and Lane, 1985). Cells were incubated for two days in DMEM (4.5g/l glucose) containing 10%FCS, 1%antibiotic-antimycotic, 1µg/ml insulin, 0.25µM dexamethasone and 0.5mM IBMX (3-isobutyl-1-methyl-xanthine). At day 2 of differentiation the

medium was then changed to DMEM containing 10%FBS, 1% antibiotic-antimycotic and 1µg/ml insulin. From day 4, cells were fed every 48 hours with DMEM containing FCS and antibiotic-antimycotic only. Adipocytes could be used after day 7 and before day 12.

2.2.2.4 Freezing of cells

Cells were removed from the surface of the dish by trypsinisation or scraping (see above). and the cell suspension was transferred in 10 ml medium to a sterile falcon tube. The cells were pelleted by centrifugation at 800rpm for 7 minutes at room temperature in a Sorvall RT7 centrifuge. The supernatant was gently poured away and the pellet tapped to resuspend the cells. Ice cold freezing medium (for 3T3-L1s; 10% DMSO, 70% DMEM, 20% FCS, for J774.2s; 10% DMSO, 50% RPMI, 40% FCS) was added to give a cell density of $1-2 \times 10^6$ cells/ml. Cells were then put on ice immediately and aliquoted into sterile cryotubes before being frozen in a cryo freezing container (Nalgene cat no. 5100-0001) in a -70°C freezer, this allows cooling at a rate of 1°C/ minute. Cells were transferred to liquid nitrogen storage after several days.

2.2.2.5 Stimulation of cells

J774.2 macrophages were serum starved overnight in RPMI media without FCS containing 0.2% BSA (fatty acid free). This was replaced with fresh RPMI/BSA before stimulation. 3T3-L1 adipocytes were washed twice with 1ml DMEM (1g/l glucose) containing 5mg/ml BSA (fatty acid free) before being incubated in DMEM/BSA for 2 hours. This media was replaced with DMEM/BSA containing 20mM HEPES prior to stimulation

2.2.3 Protein Isolation

2.2.3.1 Antibody Purification

Rabbits were immunised with the following peptides corresponding to different regions in the p110 catalytic subunit isoforms of PI 3-kinase:

p110 α	N-terminal	(C)GGS ₇₂ VTQEAEREFFDETTR ₈₈
	PIK	(C)K ₇₇₆ RPLWLNWENPDIMSE ₇₉₁
p110 δ	N-terminal A	(C)P ₈ MEFWTKEENQS ₁₉
	N-terminal B	(C)I ₇₃ NQTAEQQELEDQRR ₈₈
	C-terminal δ	(C)KVNWLAHNVSKDNRQ ₁₀₄₄
p110 β	C-terminal	(C)K ₁₀₅₀ VNWMHAHTVRKDYRS ₁₀₆₄

For purification of antibodies raised to the peptides above ACTIGEL-ALD resin was used (Sterogene SN2701-S-1). The matrix contains aldehyde groups which couple to the amine group of proteins. The manufacturers instructions were followed with minor modifications.

2.2.3.1.1 Coupling of peptide to actigel

10mg of peptide was dissolved in 1.5ml of coupling buffer, 100mM phosphate buffer pH 7.8 (may need to dissolve in a small volume of DMSO and then dilute in phosphate buffer). 4.5 ml of ACTIGEL was washed 5 times in phosphate buffer. 0.9 ml (1/10 final reaction volume) of coupling solution (1M NaCNBH₃) was added and mixed on a rotating wheel overnight at 4°C. Beads were then washed in 10 volumes of 100mM TRIS-HCL pH 8.0 - 500mM NaCl and 100mM

TRIS-HCL pH 8.0. Beads were incubated for 2-4 hours in 100mM TRIS-HCL pH 8.0 to block uncoupled sites.

To check coupling of peptide: 2 column volumes of 100mM DTT and Guanidinium HCL (1g/ml) and 5 volumes of 100mM TRIS - HCL pH 8.0 were passed through the column to remove any unbound peptide. Cysteine residues can then be titrated by the addition of 0.38mM dithiobisnitrobenzoic acid (DTNB) in TRIS pH 8.0. A yellow colouration in the column indicates good coupling. Wash with TRIS until colour has gone.

Column can be stored at 4°C in 50mM TRIS pH 8.0 + 0.02%NaN₃.

2.2.3.1.2 Column loading

See figure 2.1 for summary of column loading and elution.

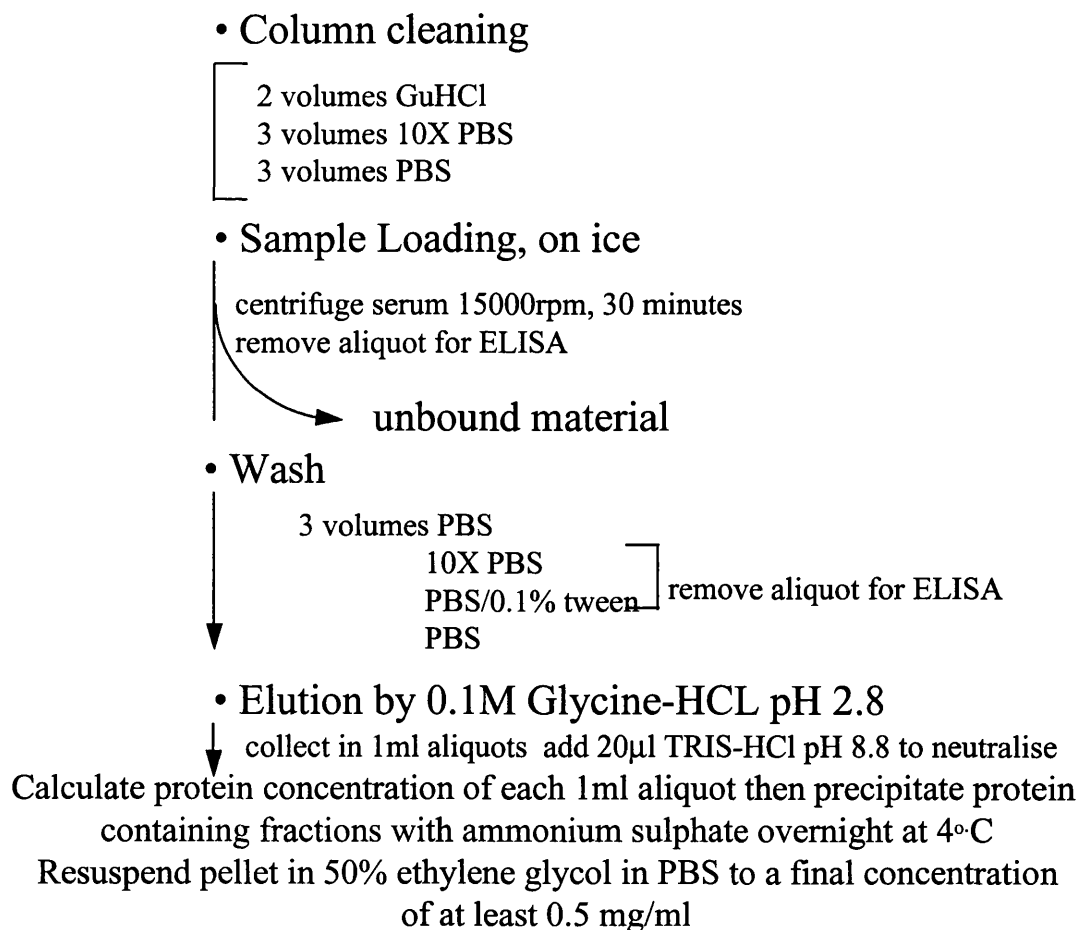
2.2.3.1.3 ELISA assay

ELISA was used to test peptide binding of proteins in; the original serum, the eluted serum, 10X PBS wash, PBS/tween wash and purified antibody.

Peptide was dissolved in PBS (10µg/ml) and 50ul was added per well in columns with even numbers, PBS was added to odd columns of a 96 well plate. After incubation for 2 hours at room temperature the peptide solution was removed and wells were washed once with PBS. 5% skimmed milk solution containing 0.05% NaN₃ was added to each well and incubated overnight, or longer, at 4°C. The cells were washed with PBS before addition of 50ul of antibody test solution in (titrations of the test solutions were assayed). This was incubated for 1 hour at room temperature before wells were washed 4 times in PBS and 50ul of α- rabbit IgG peroxidase in PBS/skimmed milk (1:5000) was

added to each well. Again the plate was incubated for 1 hour and washed 4 times with PBS before the addition of 100ul of colouration mix (0.25g urea peroxidase, 10mg o-phenylenediamine and 10 ml buffer containing 25mM citric acid and 50mM Na_2HPO_4 , pH 4.7) per well. After 5-15 minutes the reaction was stopped with 2N H_2SO_4 . The absorbance was then read in a platereader at 450nm.

Figure 2.1 Loading of sample for affinity purification of antibody from serum



2.2.3.2 *Immunoprecipitation of proteins*

After stimulation cells were washed once with ice cold PBS (calcium and magnesium free) and lysed in lysis buffer (1 ml per 10cm dish) containing 1% Triton X100, 10mM Tris base, 5mM EDTA, 0.1mM sodium orthovanadate, 1mM PMSF, 10µg/ml leupeptin, 1µg/ml pepstatin and 200 KIU aprotinin. Cells were allowed to lyse for approximately 5 minutes and then harvested by scraping and transferred to a pre-chilled Eppendorf. Unlysed material and nuclei were removed by centrifugation in a microfuge at 14000rpm for 10 minutes at 4°C. To isolate the protein of interest a defined volume (usually 800µl/10cm dish) of cell lysate was incubated at 4°C with an antibody raised to the particular protein of interest at the required concentration (usually 1:100) for 2 hours in a rotating wheel. Protein A-agarose was washed three times in lysis buffer before the addition of 30 µl/tube and a further 1 hour incubation rotating at 4°C. Antibody bound proteins complexed with the protein A-agarose were collected by centrifugation for 10 seconds at maximum rpm (14,000) at 4°C. The pellet was washed extensively. The washing protocol varied depending on whether the protein/bead complex was subsequently loaded onto a gel or used in an enzyme assay.

2.2.3.3 *SDS Polyacrylamide gel electrophoresis (PAGE)*

To visualise proteins SDS PAGE was performed essentially as described by Maniatis et al (p18.47-18.54). Whole cell lysates or immunoprecipitated proteins were run on SDS PAGE gels (8% running gel, 4% stacking gel, 16 x 16cm) using Hoefer electrophoresis apparatus. For cell lysates the appropriate amount of 4X Western loading buffer was added (20% glycerol, 4% SDS, 200mM DTT, 0.2M

Tris buffer, pH6.8). Immunoprecipitates were washed 3 times with lysis buffer and 50µl of 2X western loading buffer was added to samples which were boiled for 5 minutes before loading. 50 µl of immunoprecipitate and 150 µl of whole cell lysate were loaded and run overnight at 55 volts. Running buffer contains : 3.03 g/l Tris base, 14.42 g/l glycine and 1 g/l SDS.

2.2.3.4. Wet transfer and and western blotting

Samples were then transferred onto polyvinylidene fluoride blotting membrane (PVDF, Gelman Sciences), which had been activated by wetting with methanol. The stacking gel was removed and a corner of the gel removed for orientation. Transfer buffer contained 0.05M Tris base, 2.5M glycine, 0.05% SDS and 20% methanol. The gel was assembled in a gel holder with the membrane held in place by sponges soaked in transfer buffer. The gel holder was placed in a buffer filled transfer tank set at 35v for 2 hours then 70v for 2 hours. The membranes were removed and blocked in PBS containing 0.05% Tween 20 (Sigma) and 5% milk (Marvel) for 1 hour. The membrane was then incubated with the primary antibody, diluted appropriately in PBS-Tween containing 1% milk, overnight at 4°C. The membrane was then washed 5 times for 5 minutes in PBS-Tween and incubated with a secondary reagent diluted in PBS-Tween containing 1% milk. The secondary reagent was either ¹²⁵I-protein A (0.1µCi/ml) or goat anti-rabbit gamma globulin coupled to horseradish peroxidase (1:1000). After incubation for 2 hours the membranes were washed with PBS-Tween 5 times for 10 minutes. ¹²⁵I labelled Proteins were visualised using a Fuji BAS2000 phosphorimager and HRP labelled proteins by using the ECL reagent kit

(Amersham Cat. No. RPN 2106) and a luminescent image analyser (Fujifilm LAS1000).

2.2.3.5 Gel fixing and drying

For gels which contained radioactively labelled proteins (e.g. samples from an in vitro kinase assay, see 2.2.5.1) the gel was fixed by incubation in a solution of 7% acetic acid and 25% methanol for 2 hours at room temperature. The gel was then washed in cold distilled water before drying in a gel dryer (Hoefer model SE1160) set at 70-80°C for 1 hour. Proteins were then visualised using a phosphoimager.

2.2.4 Enzyme Assays

2.2.4.1 Protein kinase assays

(a) In vitro kinase assays PKB , P110, A-Kinase

When assaying immunoprecipitates they were washed twice in lysis buffer and twice in assay buffer. Protein kinase assays were generally carried out in a reaction volume of 50µl. To measure p110 protein kinase activity the buffer used contained; 50mM HEPES, pH7.4, 150mM NaCl, 10mM MnCl₂. For all other kinases activity was measured in 100mM Tris-HCl, pH7.5 and 20mM MgCl₂. Reactions were begun by the addition of 100µM ATP (0.5uCi of [γ^{32} P]ATP) followed by 20 minutes incubation at room temperature and stopping by the addition of 10ul of WLD. Samples were run on SDS-polyacrylamide gels, the gels were fixed and dried (see 2.2.3.5), and the products of the kinase reactions were visualised by autoradiography.

(b) Phosphocellulose cation-exchange paper assay

Phosphocellulose paper was used for measuring the phosphorylation of synthetic peptides by protein kinases. P81 cation exchange paper was used so that the basic residues of peptides can bind at low pH while free [γ - 32 P]ATP does not, and can be washed away.

To measure p110 protein kinase activity the buffer used contained; 50mM HEPES, pH7.4, 150mM NaCl, 10mM MnCl₂. For all other kinases activity was measured in 100mM Tris-HCl, pH7.5 and 20mM MgCl₂. The peptides (0.5mM) were mixed with assay buffer and enzyme in a total volume of 45 μ l before the addition of 5 μ l 100 μ M ATP (0.5 μ Ci of [γ - 32 P]ATP) to each tube with 20 second intervals. Samples were vortexed and placed in a water bath set at 30°C. After 20 minutes a 30 μ l aliquot was removed from each tube, at 20 second intervals, and spotted on to a 2x2 cm P81 paper square and dropped into a wire cage in a 500ml beaker, containing approximately 200ml 75mM orthophosphoric acid. When all reactions were finished the first wash was poured into a radioactive waste container and 3 more washes were carried out with 75mM orthophosphoric acid (500ml per wash for 5minutes). Papers were washed once with ethanol before being dried. Papers were then counted in a scintillation counter in 10ml organic scintillant.

2.2.4.2 PI3-kinase assay***(a) Assay procedure***

Cells were stimulated, lysed and immunoprecipitated, as described previously, using antiphosphotyrosine antibody (1:100). The assay was performed as

described previously (Nave et al., 1996). The beads were washed twice in lysis buffer and twice in 2X kinase assay buffer (100mM HEPES, pH 7.4, 200mM NaCl, 2mM DTT) excess buffer was removed using a hamilton syringe and resuspended in 25µl 2X assay buffer and 10ul PI (1mg/ml) and incubated on ice for 10 minutes. 15µl ATP/Mg mix (100µM ATP, 1µCi [γ P]ATP, 5mM MgCl₂) was then added and incubated for 20 minutes at room temperature. The reaction was terminated by the addition of 100µl of 0.1 M HCl and 200µl of chloroform/methanol (1:1). The mixture was vortexed and centrifuged at 10,000rpm for 2 minutes at room temperature. The upper phase was discarded and the lower organic phase washed with 80µl methanol, 1M HCl (1:1). After centrifugation the upper phase was again discarded and the lower phase evaporated to dryness.

(b) Analytical methods

Dried phospholipids were resuspended in 30µl chloroform/methanol (4:1) and spotted onto a Silica Gel-60 coated aluminium backed 20x20cm TLC plates (Merck, 5554). The TLC plates had been pretreated by dipping into a solution of 1% oxalic acid, 1mM EDTA in water/methanol (6:4) and baking at 150°C for 2 hours. TLC plates were developed in chloroform/methanol/4M ammonia (9:7:4).

2.2.4.3 HSL / neutral cholesterol ester activity assay

(a) Cell preparation. To measure the neutral cholesterol esterase activity in macrophage lysates the cell monolayers were washed with PBS before being scraped into 200ul of assay buffer (5mM imidazole pH 7.0, 30% glycerol, 50mM

NaCl, 20mM EDTA, 5mM sodium pyrophosphate, 0.1mM benzamidine, 1mM DTT, 5 µg/ml leupeptin, 1µg/ml pepstatin, 200KIU aprotinin). Cells were lysed with 4 rounds of freeze-thawing followed by centrifugation at 14000rpm at 4°C. 25µl aliquots of the cell lysate (approximately 0.15 mg protein) were assayed. To directly measure HSL activity the protein was first isolated from cells by immunoprecipitation with anti-HSL antibody (see 2.2.3.2). The immunoprecipitate was washed 2 times in lysis buffer followed by 2 washes with assay buffer. All buffer was removed with a Hamilton syringe before the immunoprecipitate was resuspended in 25µl of assay buffer.

(b) Substrate preparation and assay procedure. The assay was performed as previously described (Khoo et al., 1976). Briefly, the substrate (3.5ml volume, enough for 19 assays) is prepared by drying down a mixture of Cholesterol[¹⁴C]oleate (2.5µCi, 30µg) and cholesterol oleate (400µg) under nitrogen. The lipid was then resuspended in 100ul ethanol and added to; 100mg BSA (sigma A60093), 1ml 0.2M sodium phosphate buffer, pH7 and 2.4 ml distilled water in a flask which was being swirled swiftly in constant motion at room temperature. 175µl substrate was added to 25µl sample and incubated for 50 minutes at 37°C. To stop the reaction 0.5ml of stopping buffer was added (0.1M potassium tetraborate, 0.1M potassium carbonate, pH 10.5) followed by 1.6 ml extraction buffer (methanol (1.41)/ chloroform (1.25)/ heptane(1)) containing 0.3%(w/v) of unlabeled oleic acid as carrier. The mixture was vortexed vigorously and then centrifuged at 3000rpm for 10 minutes at 10°C. 0.5ml of the upper layer containing [¹⁴C]oleic acid was transferred to vials containing 5 ml of scintillation fluid. 1 unit of enzyme activity catalyses the

release of 1 μ Mole oleate per minute. (see figures 2.1, 2.2, 2.3. for characterisation of assay)

2.2.4.4 Cholesterol cell loading assay

(a) Lipoprotein preparation:

Lipoproteins were isolated by density gradient ultracentrifugation in a vertical rotor, as previously described (Chung 1980, Chung 1986). LDL (1ml in the range of 8-16 mg/ml) was acetylated by the addition of 1ml saturated sodium acetate solution followed by the addition of acetic anhydride (Basu, 1976) (at a volume in μ l 1.5 times the number of mg LDL protein). Acetic anhydride was added in 2-4 μ l aliquots over an hour, then incubated for a further 30 minutes at 4°C. Acetylation of lipoprotein was confirmed by loading samples on 0.85-1% agarose gel at pH8.6 (acetylated LDL runs faster than unmodified LDL). Acetylated LDL was then de-salted using PD10 columns (Pharmacia, 17-0851-01) and concentrated (vivaspins, vivascience VS 1521) before filter sterilising.

(b) Freezing of lipoproteins

Cryopreservation of LDL in 10% sucrose (w/v) has previously been shown to prevent structural and functional changes. Sucrose was added by direct addition of a stock solution (50% sucrose, 150mM NaCl, 0.24mM EDTA, pH7.4). Prior to all experimental procedures, sucrose was removed by dialysis at 4°C against saline (150mM NaCl, 0.24mM EDTA, pH7.4). Dialysis buffer was changed 3 times over a 24 hour period.

(c) Preparation of [¹⁴C] oleate label

12g of BSA (fatty acid free) was added to 35 ml of solution 1.(150mM NaCl, 50mM Tris-HCl pH7.4) in 2g aliquots over a 5 hour period, stirring continuously at room temperature. The pH was adjusted to 7.4 and the volume made up to 50ml with solution 1, this is solution 2 (24% BSA solution). To make solution 3 (sodium oleate complexed with 12% BSA), 90mg of oleic acid was dissolved in 2 ml ethanol and 100µl 5M NaOH. The lipid was then dried under nitrogen and then 10 ml of solution 1 was added. This was heated to 60°C for 3-5 minutes before addition of 12.5ml ice-cold solution 2 and stirring for 10 minutes at room temperature. The final volume was adjusted to 25ml with solution 1. Finally [¹⁴C]oleate-0.12% BSA was prepared by drying 250µCi [¹⁴C]oleic acid (specific activity 50-60mCi/mMol) under nitrogen and resuspending in 4.35 ml solution 3. 0.8ml solution 2 was added followed by 0.8ml solution1. and was stirred gently for 4-6 hours at room temperature. The solution was stored at -20oC.

(d) Cell Incubations:

Cell incubations and analysis was carried out as described previously (Graham et al., 1996). Cells were plated into 6-well (35mm) plates. Prior to loading cells were incubated overnight in RPMI 1640 medium containing 2mg/ml BSA and no serum. Cells were washed twice in PBS before the addition of RPMI containing the relevant amount of glucose, insulin/leptin, 100µg/ml acetylated LDL and 2µCi/ml [¹⁴C]oleate. Cells were then incubated for 24 hours.

(e) Analytical methods:

Cells were washed twice in PBS and harvested into 0.5ml PBS. 0.5ml of chloroform/methanol (2:1) was added followed by vortexing for 10 seconds before centrifugation at 1800g for 10 minutes at 10°C. The lower organic phase was transferred to a glass vial and the upper phase was washed with chloroform/methanol as before. Again the lower organic phase was transferred to the glass vial. Chloroform was removed by drying under nitrogen. Lipids were resuspended in isopropanol and separated by thin layer chromatography on silica gel plates developed in hexane/ethyl ether/acetic acid (80:20:1 v/v/v). Radioactive bands were detected using a Fuji FLA2000 phosphoimager and were quantitated.

2.2.5 Statistical Analysis

Graphs illustrate the mean and standard errors of multiple experiments (see individual figure legends for the number of experiments, n). Results were evaluated, in some cases, by the student's paired *t* test.

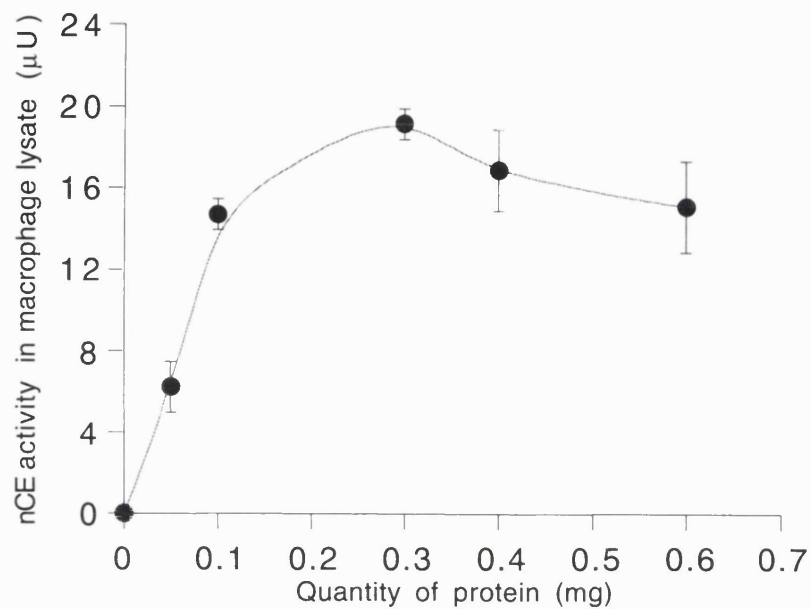


Figure 2.2. Neutral cholesterol esterase activity assay:

Protein concentration dependance

Macrophages were lysed as described (2.2.5.3.1) and the protein concentration of the extracts was determined using the BCA protein assay reagent kit (PIERCE). Aliquote of lysate containing differing amounts of total protein were assayed for neutral cholesterol esterase activity. The graph shows that activity increases linearly with increasing protein to approximately 250μg. Therefore all assays were performed on samples with a protein content in the range of 100-200μg.

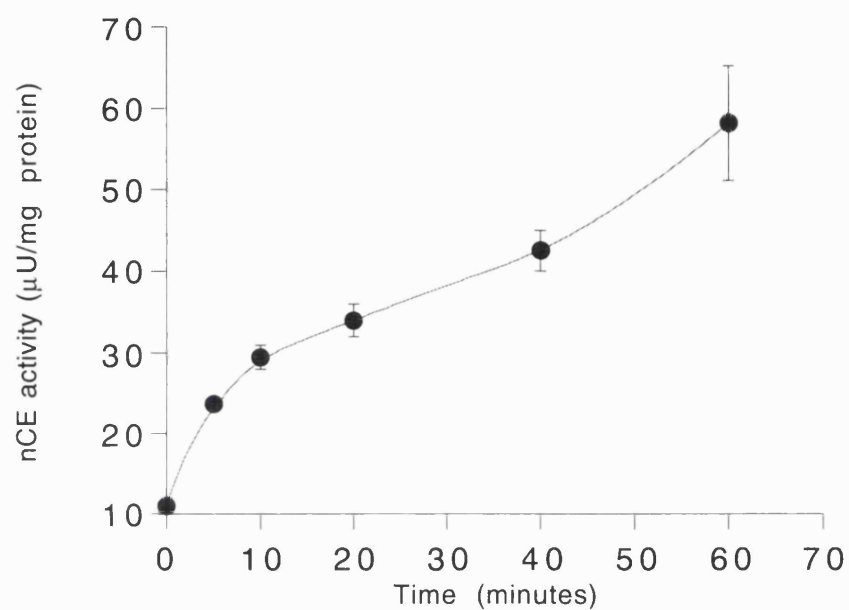


Figure 2.3 Neutral cholesterol esterase activity assay: Timecourse

Samples of macrophage extracts containing 0.15mg of protein were assayed for various lengths of time and the neutral cholesterol esterase activity against cholesterol[¹⁴C]oleate was determined. Activity was found to have a linear relationship with time up to 60 minutes.

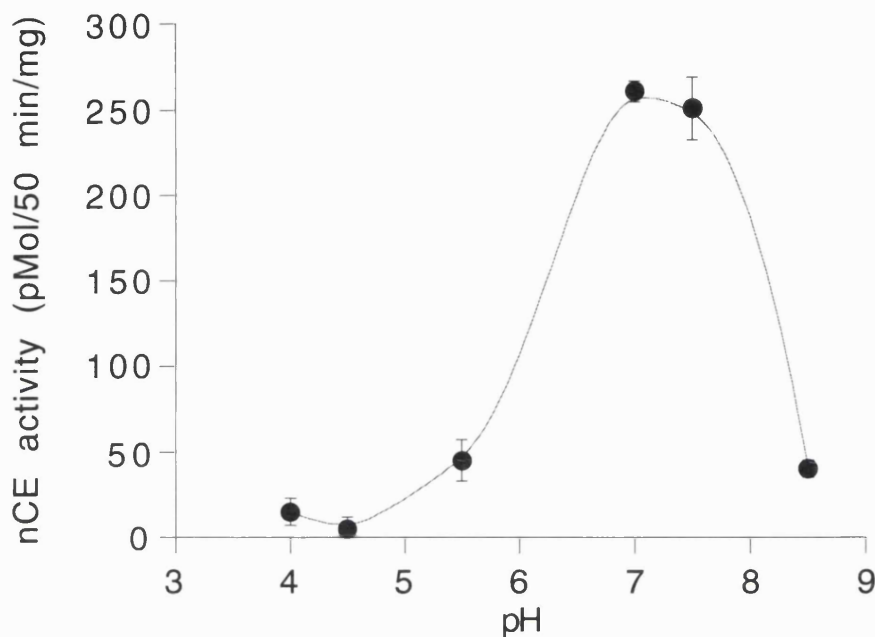


Figure 2.4 Neutral cholesterol esterase activity assay:

pH dependence

All buffers were made up to 200mM. Sodium acetate was used at pH 4.0 to pH 6.0, and sodium phosphate was used at pH 6.0 to 8.0. Stock substrate was made up as described in 2.2.5.3.(b) without 200mM sodium phosphate.

Cholesterol esterase activity in macrophage lysates peaks at pH 7.0 . There is very little detectable activity at pH 4.0 and activity rises sharply at around pH 6.0 , indicating that there is no lysosomal acid hydrolase activity being measured.

3. Effect of Insulin on HSL Regulation in Macrophages and Adipocytes

3.1 Summary

The aim of this section was to assess the effects of insulin and glucose on the neutral cholesterol esterase (nCE) activity of HSL in macrophages. The purpose of these experiments was to determine whether the conditions that characterise insulin resistance and diabetes do indeed affect HSL in a way which might promote foam cell formation.

To investigate the effect of insulin on HSL activity an assay was developed whereby HSL was isolated from cell lysates by immunoprecipitation before measuring activity and this activity was compared to the nCE activity measured in lysates. Insulin was found to acutely reduce cAMP stimulated HSL activity in macrophages as it does in adipocytes. Also insulin was shown to acutely downregulate basal nCE activity in macrophage lysates and HSL immunoprecipitates.

HSL immunoprecipitates from macrophages and adipocytes were subject to gel electrophoresis and blotted with anti-HSL antibody. Adipocyte HSL was consistently observed as two bands of approximately 85 and 80kDa, whereas macrophage HSL was seen as a single protein of 85kDa. Chronic treatment (24 hours) of cells with insulin had different effects depending on the concentration

of glucose in the media. Insulin caused an increase in HSL activity in adipocytes in media containing high levels of glucose (20mM) and a slight reduction in activity after 24 hours insulin treatment in low glucose (5mM) media. The opposite was found in macrophages where high glucose and insulin caused a reduction in HSL activity after 24 hours. The same conditions were used to determine if there was any effect on HSL protein levels. High glucose and insulin caused increased protein levels in 3T3 L-1 adipocytes and reduced protein levels in J774 macrophages.

Finally, an increased accumulation of cholesterol esters (CE) and triglycerides was observed in macrophages loaded in high glucose media containing insulin. Therefore a novel mechanism has been identified by which conditions associated with insulin resistance may increase cholesterol ester accumulation and this could contribute to foam cell formation and increased incidence of CAD in diabetics.

3.2 Introduction

Insulin resistance and type 2 diabetes (Lehto et al., 2000) are well recognised risk factors for coronary heart disease and the mortality rate due to coronary heart disease is at least doubled in the diabetic population (Bressler et al., 1996; Steiner, 1997; Steiner, 1994). The reason for accelerated atherogenesis in diabetics remains unclear. These subjects have alterations in their triglyceride and HDL profiles which might partially explain the increased risk of atherosclerosis. Also the hyperinsulinemia and elevated glucose associated with insulin resistance are likely to contribute to accelerated plaque formation.

An important early event in atherosclerosis is the formation of foam cells (Ross, 1995; Ross and Glomset, 1976; Stary et al., 1992). The bulk of the lipid which accumulates in macrophage foam cells is in the form of cholesterol ester. A cycle exists in macrophages where cholesterol is esterified by Acyl-CoA-cholesterol-acyltransferase (ACAT) and hydrolysed by a neutral cholesterol esterase (nCE). Hydrolysis of CEs is critical for cholesterol excretion from the cell and CE accumulation represents conditions where the rate of esterification exceeds that of hydrolysis. Several lines of evidence suggest that hormone-sensitive lipase (HSL) is the enzyme responsible for CE hydrolysis in macrophages (Khoo et al., 1993; Osuga et al., 1997; Reue et al., 1997; Small et al., 1989). The importance of HSL in regulating cellular levels of CEs is emphasised by the finding that downregulation of HSL expression in macrophages correlates with an increase in cellular levels of CE (Jepson et al., 1996) and that overexpression of HSL stimulates hydrolysis of CEs in macrophage foam cells (Escary et al., 1998).

HSL is the rate-limiting enzyme in triglyceride lipolysis in adipocytes. It is activated by agents that raise cAMP (Garton et al., 1988; Stralfors and Belfrage, 1983) and it is well documented that insulin reduces cAMP stimulated lipolysis in adipocytes (Elks and Managaniello, 1985; Stralfors and Honnor, 1989). Surprisingly, while it is known that macrophages respond to insulin (Costa Rosa et al., 1996) and that insulin promotes atherosclerosis (Joron and Webb, 1991) there have been no studies to investigate how insulin might affect cholesterol ester deposition in macrophages.

Therefore the following will be investigated in this chapter; (1) the acute effects of insulin on HSL regulation in macrophages compared to adipocytes, (2)

chronic effects of insulin and glucose on HSL expression and activity in macrophages and adipocytes and (3) the effect of insulin and glucose on cholesterol ester loading of macrophages.

3.3 Results

3.3.1. *Acute Regulation of HSL activity in macrophages compared to adipocytes*

In the current study we have used a direct immunoprecipitate (IP) assay to determine the neutral cholesterol esterase activity of HSL in 3T3-L1 adipocytes and in J774.2 macrophages. In unstimulated 'basal' macrophages the amount of neutral cholesterol esterase activity in cell lysates is in the range of 150-400 mU/mg (approx 15-40 μ U/ aliquot of lysate used for each sample, that is, an eighth of a whole dish of cells) and in immunoprecipitates it is in the range of 3.75-7.5 μ U/immunoprecipitate (HSL immunoprecipitated from a whole dish). Therefore, neutral cholesterol esterase activity measured in each sample of cell lysate is on average 40 fold more than that measured in immunoprecipitate samples. This is likely to be due to IP efficiency, i.e not all HSL protein has been isolated, and/or that antibody binding reduces overall enzyme activity or that the nCE activity measured in macrophage lysates is not due entirely to HSL. To address this, the amount of HSL protein and nCE activity was measured in cell lysates after HSL immunoprecipitation (fig 3.1). The western blot revealed that 42% of the HSL protein was remaining after immunoprecipitation and the activity assays showed 46% of the total nCE activity was still present. Therefore due to the fact that the reduction in HSL protein directly reflects the reduction in the nCE activity it appears that HSL is responsible for the majority, if not all, of

nCE activity in these cells and that the lower activity observed in IPs is most likely due to antibody binding and to a lesser extent IP efficiency. Because of the reduced nCE activity in IPs however, it was important to verify that the effects observed in IPs were the same as those for nCE activity in whole cell lysates.

It was found that HSL activity in J774.2 macrophage cells is increased by CPT-cAMP (fig 3.2), a cell permeable cAMP analogue, by around 70% which agrees with previous data where partially purified HSL was activated directly with cAMP (Khoo et al., 1981) and agents which increase cAMP were added to intact WEHI cells (Small et al., 1991) also increased HSL activity approximately two fold. The stimulation of HSL activity was similar in lysates and IPs, again suggesting that HSL activity directly reflects total cellular nCE activity. Activity measured in immunoprecipitates from 3T3 L-1 cells is in the range of 50-125 μ U, approximately 14-15 fold more activity than macrophages. While it is well known that insulin reduces cAMP stimulated glycerol release/lipolysis in adipocytes, a parallel effect of insulin directly on HSL activity has not previously been demonstrated in this cell type. Isoprenaline, which increases intracellular cAMP caused a significant increase in HSL activity in 3T3-L1 adipocytes and this is counteracted by insulin (fig. 3.3), confirming that the effect of insulin on triglyceride lipolysis in these cells is, at least in part, due to direct effects on HSL activity. We then conducted the first investigation of the effects of insulin on HSL activity in macrophages. Maximally stimulated HSL activity in macrophages was blocked completely by insulin, as it was in the adipocytes (Fig. 3.3).

In addition to the antagonistic effects of insulin towards cAMP stimulated HSL activity, data in figure 3.4 shows that insulin acutely inhibits the nCE activity in HSL IPs from serum starved macrophages in the absence of cell permeable forms of cAMP. This effect was paralleled by a reduction in nCE activity measured directly in macrophage lysates confirming that the effects seen in nCE activity from IPs directly reflects regulation of nCE activity in the whole cell lysate. A similar effect on basal HSL activity was observed in immunoprecipitates from adipocytes (fig. 3.5). There was variation in the timecourse of the acute inhibitory effects of insulin on HSL activity from experiment to experiment. However, HSL activity always returned to basal levels after 30 minutes of insulin stimulation in macrophages, whereas in adipocytes a more prolonged reduction in activity was observed. Most experiments were carried out at a maximal dose of 1 μ M insulin, but a more physiological insulin dose of 10nM also causes similar effects on HSL activity in macrophages (Figs. 3.6 and 3.3).

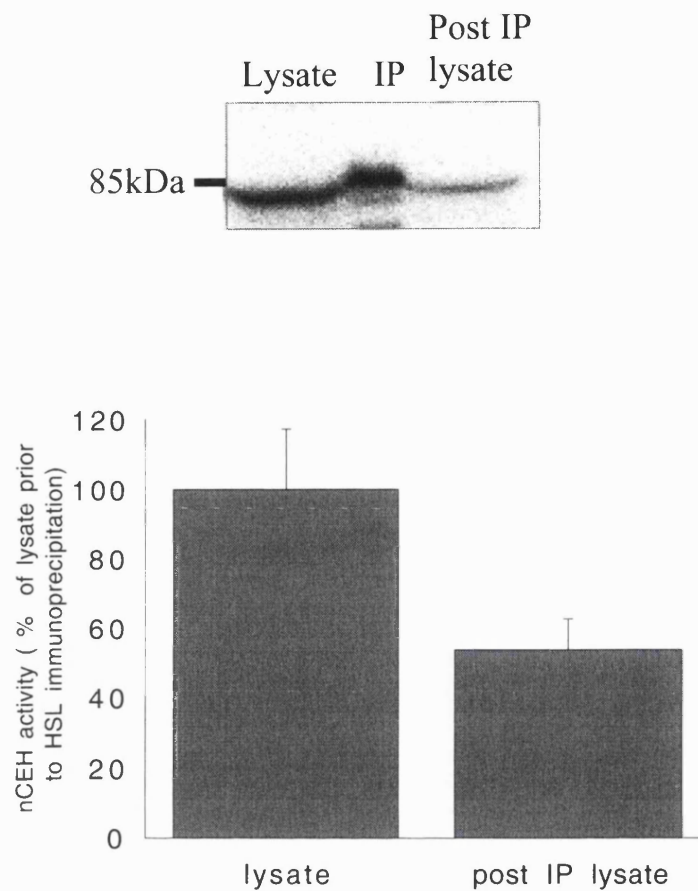


Figure 3.1. Efficiency of anti-HSL immunoprecipitation

J774.2 macrophages were harvested and lysed by freeze thawing. Two aliquots of an equal amount of lysate was taken, one of which was subject to anti-HSL immunoprecipitation. Protein A-agarose beads were pelleted and the remaining lysate assayed for neutral cholesterol esterase activity together with the original lysate. To determine how much of the total cellular HSL protein is being immunoprecipitated, the pre and post immunoprecipitation lysates were subject to SDS PAGE and western blotted for HSL. Data represents the mean ($\pm$ SEM) of two independent determinations, each performed in triplicate

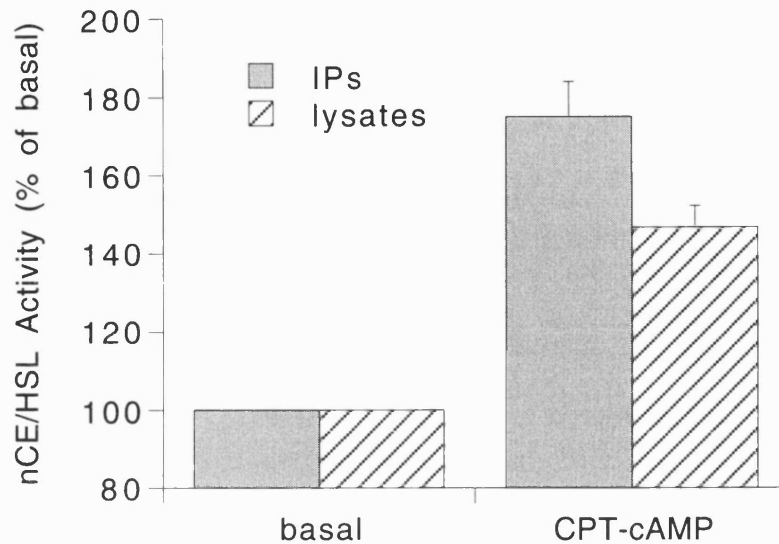


Figure 3.2. Stimulation of neutral cholesterol esterase activity by cAMP analogues occurs in macrophage lysates and HSL immunoprecipitates

J774.2 macrophages were serum starved overnight before stimulation with 1mM 8-(4-chlorophenylthio)-adenosine3':5'-cyclic monophosphate (CPT-cAMP) for 20 minutes. nCE activity was measured either directly in macrophage lysates or in HSL immunoprecipitates, as described in materials and methods (2.2.5.3). Data represents the mean ($\pm$ SEM) of three independent determinations, each performed in triplicate.

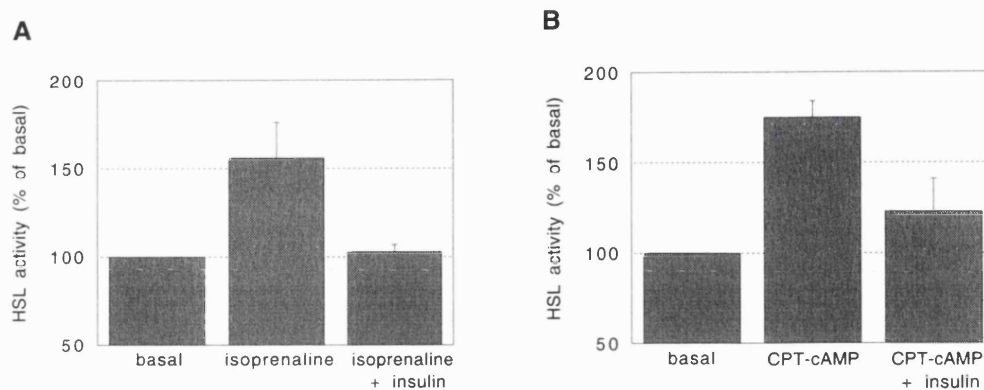


Figure 3.3 Effect of insulin on cAMP stimulated HSL activity

3T3 L-1 adipocytes were treated with 10 μ M isoprenaline (**A**) and J774.2 macrophages with 1mM 8-(4-chlorophenylthio)-adenosine3':5'-cyclic monophosphate (CPT-cAMP) (**B**) alone for 15 minutes or with the addition of 10nM insulin for the last 5 minutes. Cell lysates were prepared and HSL was immunoprecipitated. nCE activity was measured in the immunoprecipitates, as described in materials and methods. Data represents the mean ($\pm$ SEM) of five independent determinations, each performed in duplicate.

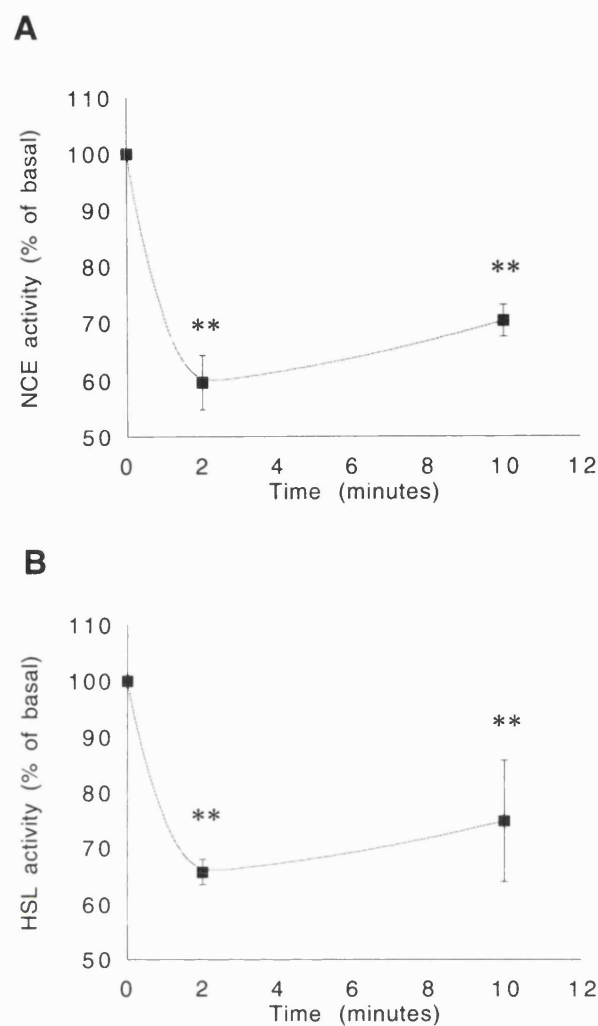


Figure 3.4. Effect of insulin on basal HSL activity in macrophages

Macrophages were serum starved overnight in serum free RPMI +0.2% BSA. Neutral cholesterol esterase activity was measured directly in lysates (**A**) and HSL immunoprecipitates (**B**) from J774.2 macrophages upon stimulation with 1 μ M insulin. Data represents the mean ($\pm$ SEM) of four independent determinations, each performed in at least duplicate. The differences in nCE activity from basal were significant (** $P = <0.01$) for lysates and immunoprecipitates.

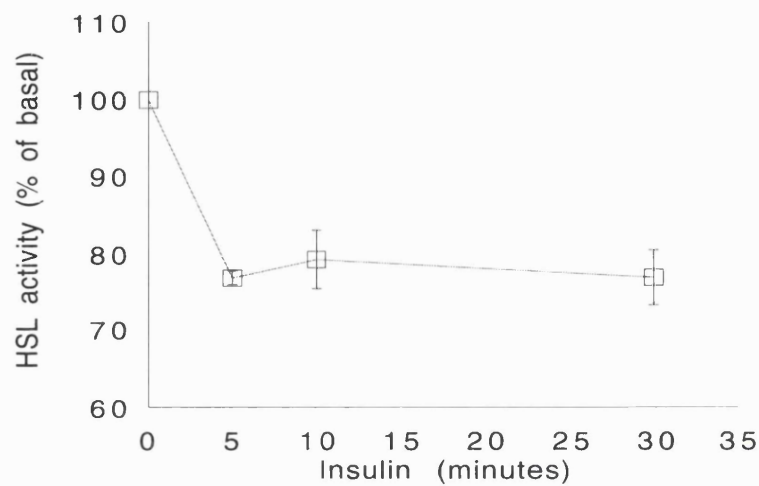


Figure 3.5. Effect of insulin on basal HSL activity in adipocytes

3T3 L-1 adipocytes were serum starved for 2 hours in DMEM containing 5 mg/ml BSA. Cells were stimulated with 1 μ M insulin. At various timepoints cells were lysed and HSL was immunoprecipitated and assayed for neutral cholesterol esterase activity. Data represents the mean ($\pm$ SEM) of two independent determinations, each performed in triplicate

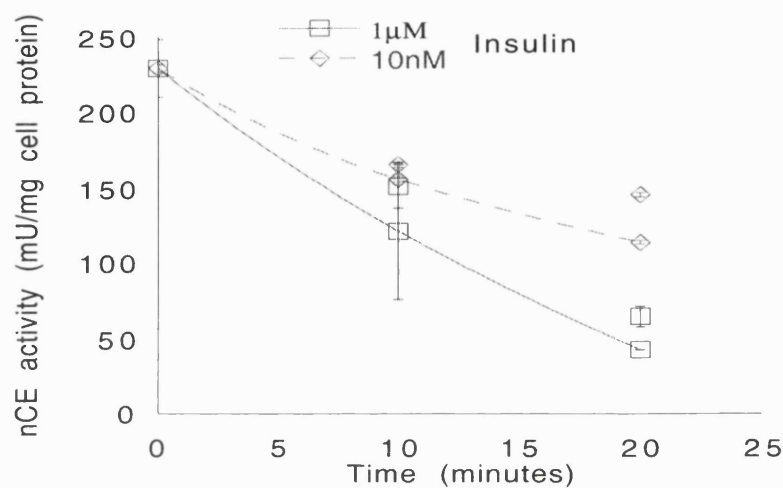


Figure 3.6. Dose response of insulin on HSL activity in Macrophages

Macrophages were serum starved overnight prior to stimulation with either 1µM insulin or 10nM insulin and assaying for nCE activity in the lysates. Graph shows data from a single experiment. Graph shows duplicate samples for each timepoint, each of which was assayed in triplicate.

3.3.2. Chronic effect of insulin and glucose on HSL activity in macrophages and adipocytes

It has been demonstrated that insulin can acutely regulate HSL activity in macrophages, however the long term effects of insulin may be just as important. Many type 2 diabetics have been insulin resistant for prolonged periods, maybe even decades, prior to diabetes, therefore tissues in the body would be exposed to higher levels of insulin and glucose than is normal. To investigate the chronic effects of insulin and glucose levels on HSL activity in adipocytes and macrophages, cells were exposed for varying times to 1 μ M insulin in the presence of either 5 mM or 20 mM glucose. These glucose concentrations were chosen to represent normal fasting blood glucose and an extreme concentration observed in uncontrolled diabetes. Incubation in high and low glucose media without insulin did not alter activity significantly. In 3T3-L1 adipocytes insulin had very little long term effect on HSL activity in cells maintained in normoglycemic media (Figure 3.7) and similar effects were seen in J774.2 macrophages. However, when maintained in high glucose media insulin caused a sustained increase in HSL activity in the 3T3-L1 adipocytes, which rose by approximately 40% after 24 hours incubation (Figure 3.6). J774.2 macrophages maintained in high glucose media with insulin showed a similar increase in HSL activity after one hour, however this increase was rapidly lost and a marked decrease in the neutral cholesterol esterase activity of 25% was observed after 24 hours (Figure 3.7).

3.3.3 Chronic effects of insulin and glucose on HSL expression

To determine whether the changes in HSL activity were due to changes in the level of HSL protein we performed western blotting experiments in cells treated in the same way as those used for the activity experiments. In 3T3-L1 adipocytes we consistently find that the HSL antibodies identify two bands of 85 and 80 kDa although in the J774 macrophages only a single band corresponding to an 85 kDa protein was observed (figure 3.8). It is possible that the 80kDa band identified in 3T3-L1 adipocytes represents the recently reported catalytically inactive splice variant of HSL, only observed previously in human adipocytes (Laurell et al., 1997). Incubation of both macrophages and adipocytes in media with different glucose concentrations did not alter HSL protein levels (Figure 3.8). In normoglycemic media insulin had very little effects on HSL expression in 3T3 L-1 adipocytes (Fig. 3.9 A). However, at high levels of glucose insulin caused a prolonged increase in expression of the two proteins recognised by the HSL antibody. Expression of the 85kDa protein had increased by 50% after 6 hours incubation and remained at this level of expression for 24 hours, whereas, the 80kDa followed the expression of the 85kDa isoform closely for 6 hours but then gradually increased so levels were 80% more than basal after 24 hours incubation. The magnitude of the increase in expression of the 85kDa isoform, which we suspect represents the catalytically active isoform, parallels the increase in HSL activity at 24 hours much more closely than the 80kDa isoform. The effects of insulin on HSL expression in macrophages also broadly followed the effects on activity with insulin (figure 3.9 B), causing a 25 % decrease in HSL expression after 24 hours incubation with insulin in high glucose media. Low glucose and insulin caused an increase in HSL expression at early timepoints,

which was not reflected in the activity measurements, with protein levels returning almost to basal levels at 24 hours.

3.3.4. Effect of glucose and insulin on cholesterol ester loading in macrophages

Decreased HSL/nCE activity in macrophages would be predicted to increase accumulation of cholesterol esters in macrophages. We therefore loaded J774.2 macrophages with labelled cholesterol using acetylated human LDL and [^{14}C]-oleate and measured the amount of label incorporated into both the cholesterol ester and triglyceride (TAG) pools (figure 3.10) in cells incubated with insulin in high or low glucose media. Figure 3.11 shows that without insulin glucose does not affect CE/TAG accumulation. In low glucose media, insulin caused an average decrease of 15% in the accumulation of CE (figure 3.12). In high glucose conditions however, insulin caused a marked increase in CE and TG accumulation (figure 3.12). These results therefore clearly demonstrate that the incubation with insulin in high glucose media causes a marked increase in the accumulation of cholesterol esters and triglycerides compared to low glucose media (figure 3.13).

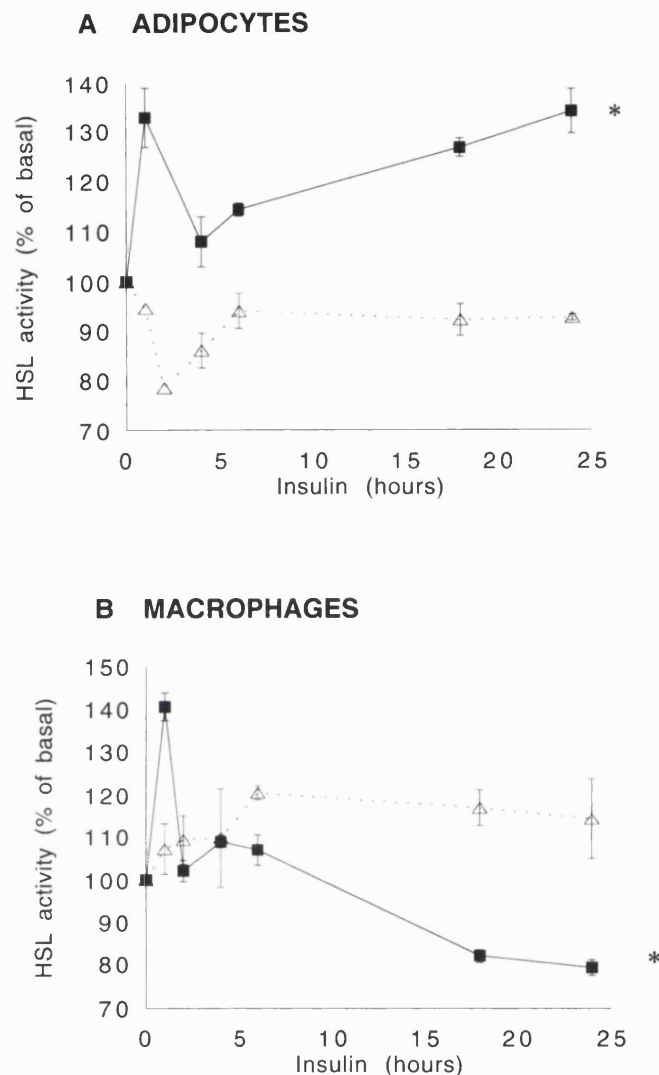


Figure 3.7. Chronic effect of insulin and glucose on HSL activity.

3T3-L1 adipocytes (**A**) and J774.2 macrophages (**B**) were incubated in media containing 1 μ M insulin, 2% FCS and either 5mM (triangles) or 20mM (squares) glucose. HSL was immunoprecipitated and cholesterol esterase activity was determined. Data represents the mean ($\pm$ SEM) of five independent determinations, each performed at least in duplicate. The differences in HSL activity from basal were significant (* $P = <0.05$) at 24 hours for high glucose data.

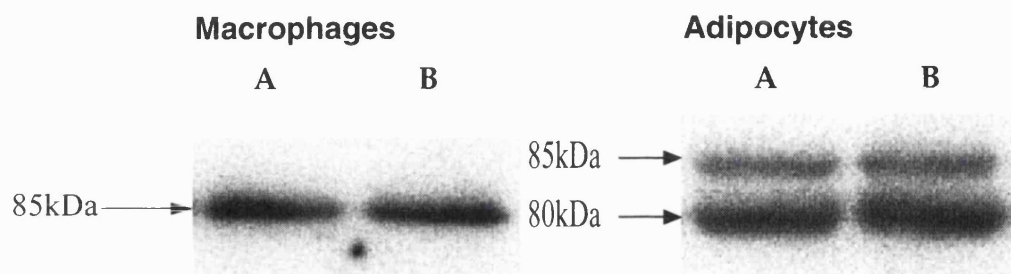


Figure 3.8. Effect of glucose alone on HSL expression

J774 macrophages and 3T3 L-1 adipocytes were incubated with media containing 2% FCS and either 20mM glucose (high, A) or 5mM glucose (low, B) for 24 hours. HSL was immunoprecipitated and blotted with anti-HSL antibody and levels of proteins were quantitated using a phosphoimager. No significant difference in protein level was observed.

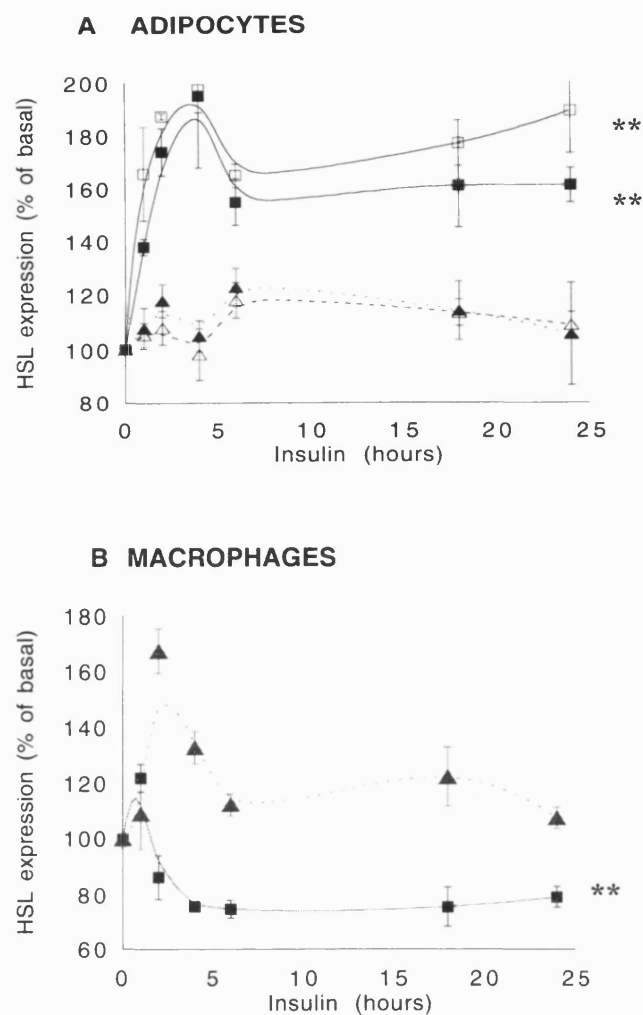


Figure 3.9. Effect of insulin and glucose on HSL expression in macrophages and adipocytes

3T3 L-1 adipocytes (**A**) and J774.2 macrophages (**B**) were incubated in media containing 2% FCS, 1 μ M insulin and either 5mM (triangles) or 20mM glucose (squares) prior to immunoprecipitation and blotting with anti-HSL antibody. Levels of proteins were quantitated using a phosphoimager. The 85kDa (filled symbols) and 80kDa (open symbols) proteins in adipocytes were quantitated separately. Data represents the mean ($\pm$ SEM) of five independent determinations, each performed at least in duplicate. The change in expression in high glucose was significantly different (** $P = <0.01$) from basal levels.

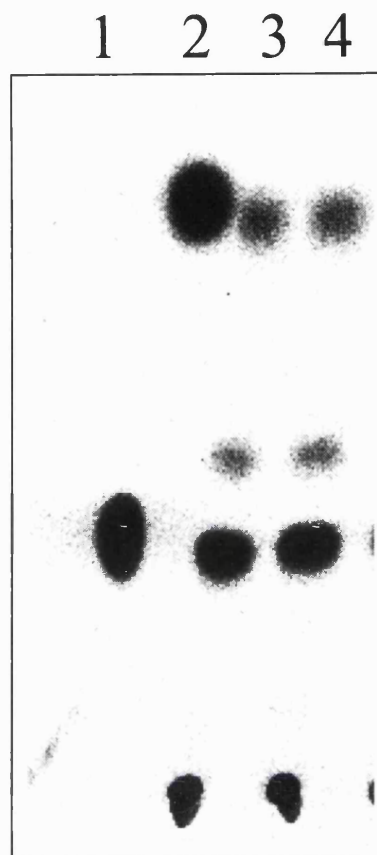


Figure 3.10. Identification of lipids extracted from loaded macrophages using radiolabelled standards

J774.2 macrophages were incubated with acetylated LDL and [^{14}C]oleate, as described (see 2.2.5.4 c). Lipids from the cells were extracted and separated by thin layer chromatography (see 2.2.5.4 e) together with lipid standards. Lane 1, glycerol tri- ^{14}C oleate, Lane 2, cholesterol ^{14}C oleate, Lanes 3 and 4, J774.2 macrophage lipid extracts.

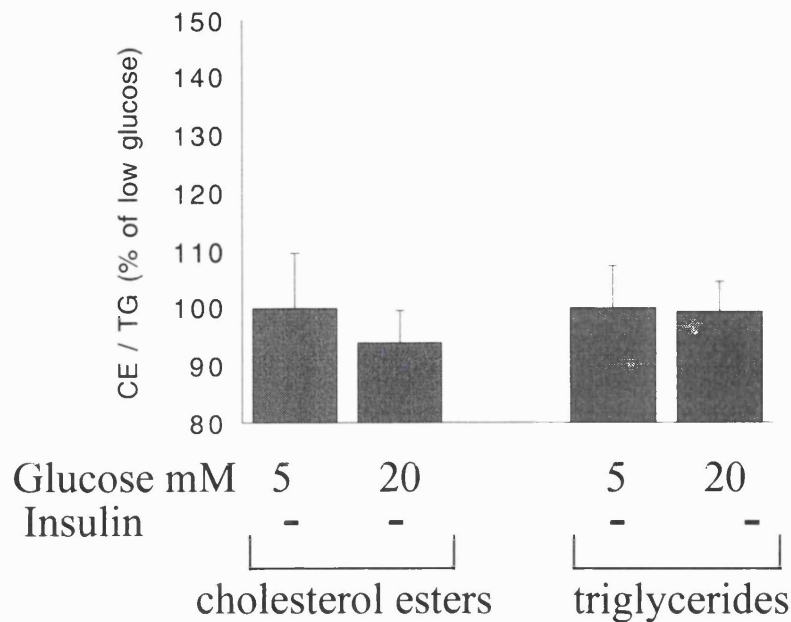


Figure 3.11. Effect of glucose on accumulation of cholesterol esters and triglycerides in macrophages

Macrophages were incubated for 24 hours with acetylated LDL and [^{14}C]oleate in media containing either 5mM (low) or 20mM (high) glucose. Lipids were extracted from cells after 24 hours and [^{14}C]-labelled cholesterol esters and triglycerides were separated by thin layer chromatography. Graphs show the effect of 5mM glucose and 20mM glucose on cholesterol ester and triglyceride accumulation. Basal values were in the range of 3-6 nM CE / mg cell protein. Data represents the mean ($\pm$ SEM) of four independent experiments, each performed in at least duplicate.

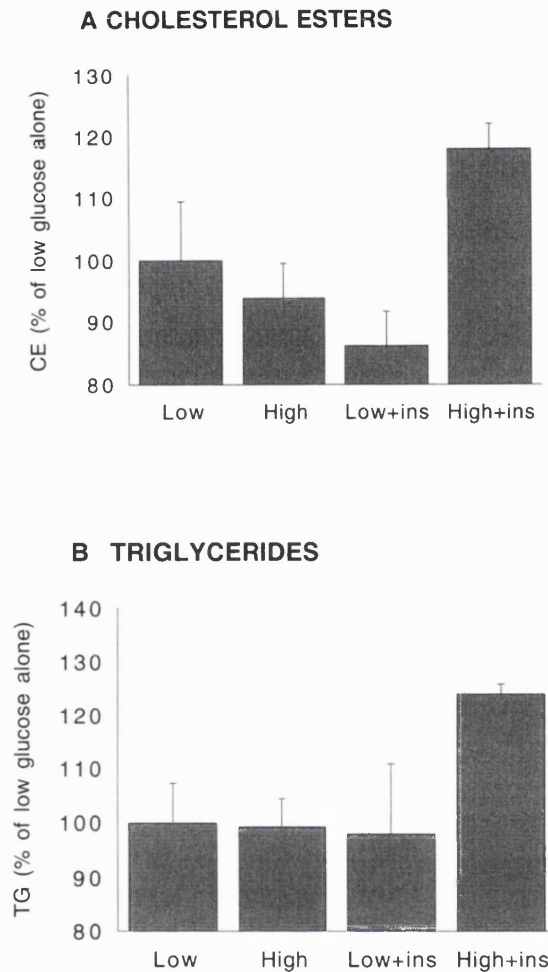


Figure 3.12. Lipid accumulation in macrophages upon incubation with glucose and insulin

Macrophages were incubated for 24 hours with acetylated LDL and [^{14}C]oleate in media containing either 5mM (low) or 20mM (high) glucose and 1 μM insulin. Lipids were extracted from cells after 24 hours and [^{14}C]-labelled cholesterol esters (**A**) and triglycerides (**B**) were separated by thin layer chromatography. Basal values were in the range of 3-6 nM CE / mg cell protein. Data represents the mean ($\pm\text{SEM}$) of four independent experiments, each performed in at least duplicate.

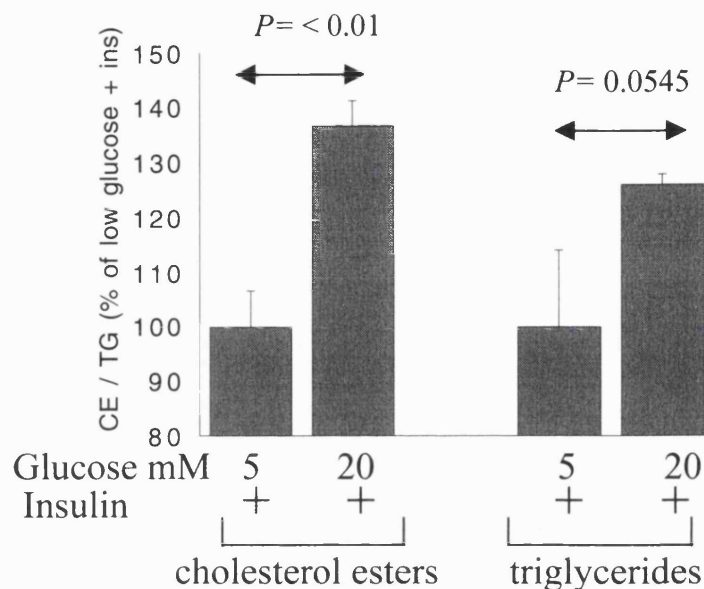


Figure 3.13. Lipid accumulation in macrophages with insulin in high glucose media compared to low glucose media

Macrophages were incubated for 24 hours with acetylated LDL and [^{14}C]oleate in media containing either 5mM (low) or 20mM (high) glucose and 1 μM insulin. Lipids were extracted from cells after 24 hours and [^{14}C]-labelled cholesterol esters and triglycerides were separated by thin layer chromatography. Basal values were in the range of 3-6 nM CE / mg cell protein. Data represents the mean ($\pm\text{SEM}$) of four independent experiments, each performed in at least duplicate.

3.4 Discussion

3.4.1. *Acute effects of insulin on HSL regulation*

Insulin is known to counter the lipolytic effects of agents which raise intracellular cAMP. Insulin achieves this by attenuating HSL activity in adipocytes and if a similar effect occurred in cholesterol accumulating cells in the intima it may potentiate the development of atherosclerotic plaques. It has previously been reported that nCE activity is activated by cAMP in macrophages (Khoo et al., 1981; Small et al., 1991) and the HSL IP assay has confirmed that this activation is due to an increase in HSL activity. The effect of insulin on cAMP-stimulated HSL activity has not been reported, even though it is well established that macrophages express insulin receptors and that insulin can effect macrophage metabolism and function (Costa Rosa et al., 1992; Costa Rosa et al., 1996). There is already evidence that insulin downregulates nCE activity in smooth muscle cells and it has been suggested that this contributes to the development of the cholesterol laden smooth muscle that accumulates during atherogenesis (Abe et al., 1996; Fujiwara et al., 1995).

The results presented in this chapter have demonstrated that insulin does regulate HSL activity in macrophages and that the acute effect is similar to that in adipocytes. The fact that insulin downregulates HSL activity stimulated by a cAMP analogue suggests that it is not an effect on adenylate cyclase itself. It is possible, therefore, that macrophages may utilise the same mechanism for reducing HSL activity as that which has been elucidated in adipocytes, i.e PI 3-kinase mediated activation of PDE3B resulting in reduced levels of cAMP and cAMP dependent protein kinase (A-kinase) activity. Another potential

mechanism may be via a naturally occurring protein inhibitor of macrophage nCE activity that has been reported (Shand et al., 1993). The inhibitor activity increased when J774 macrophages were incubated with oleate for 24 hours which suggests that this mechanism for negatively regulating nCE activity could have important implications in foam cell formation. Activation of this inhibitor by insulin is another possible mechanism for the reduction in HSL activity. However, the fact that the inhibition is observed in HSL immunoprecipitates as well as lysates suggests that this mechanism is unlikely unless the inhibitor co-immunoprecipitates with HSL.

3.4.2. Chronic regulation of HSL by insulin and glucose

As insulin resistance develops in muscle and fat, more insulin is produced by the pancreas to compensate for the resistance to insulin action in these tissues. For tissues that are responding normally to insulin however, exposure to high insulin concentrations for longer periods could have adverse effects. This prompted us to investigate the effect of longer incubations with insulin and glucose on HSL activity. The increase in HSL activity and expression in 3T3 L-1 adipocytes with insulin and high glucose agrees with previous reports where rat fat cells incubated with insulin and 10mM glucose showed increased lipolysis (Kang et al., 1993), glucose deprivation reduces HSL expression in adipocytes (Raclot et al., 1998), HSL levels are greatly increased in adipocytes from hyperglycemic rats (Sztalryd and Kraemer, 1995) and very recently it has been reported that insulin and glucose together but not alone enhance lipolysis in fat cells (Botion and Green, 1999). Our findings confirmed and extended these previous findings. The upregulatory effect of insulin was not observed in normoglycemic media which suggests that glucose may be the major regulatory

feature of HSL expression in adipocytes. It has been reported that activators of PKC and A-kinase exert a negative control on HSL expression in adipocytes (Plee-Gautier et al., 1996) and the effect observed with insulin and glucose could be due to downregulation of these PKC/A-kinase activating pathways. Another factor to be considered is that insulin, under conditions of high glucose, promotes synthesis of leptin in adipocytes. Therefore these effects could be directly due to leptin, which is known to alter expression of genes involved in lipid metabolism.

In contrast to the results in adipocytes the combination of hyperglycemia and hyperinsulinemia caused a decrease in HSL activity and expression in macrophages over 24 hours. The only previous report of altered HSL expression in macrophages is the reduction in HSL protein and activity in sterol ester laden J774 macrophages (Jepson et al., 1996). Conversely, during adipocyte differentiation there is a massive accumulation in lipid but also a 19 fold increase in HSL activity. Therefore lipid accumulation decreases HSL expression in macrophages but not in adipocytes. This is another example of differential regulation of HSL expression in macrophages and adipocytes other than our observations with chronic insulin treatment.

Interestingly the presence of sterol regulatory element 1 (SRE-1) has been detected in the 5' flanking region that controls expression of the HSL gene in different tissues (Talmud et al., 1998). Three members of the SREBP (sterol regulatory element binding protein) family of transcription factors, which bind SRE sites, have been identified. SREBP-1a and SREBP-1c, the most abundant form, are encoded by a single gene and SREBP-2 is derived from a different

gene. The SREBPs were originally identified as being involved in the regulation of genes according to cellular cholesterol availability (Brown and Goldstein, 1996). They enhance transcription in the absence of sterols but not when they are present (Goldstein and Brown, 1990). SREBPs are bound in a precursor form to the endoplasmic reticulum and nuclear membranes when the concentration of cholesterol is high. When the concentration of cholesterol decreases, the precursor form is cleaved by a complex mechanism involving two proteolytic cleavages and a protein sensor for the cholesterol concentration (Brown and Goldstein, 1997; Sakai et al., 1998). The mature form migrates inside the nucleus where it binds to the SRE, (e.g. 5'-ATCACCCAC-3'), on the promoters of genes involved in cholesterol, fatty acid and triglyceride metabolism (Brown and Goldstein, 1997; Goldstein and Brown, 1990). Recently SRE-1 has also been identified as the cis element by which insulin, IGF-1 and PDGF exert some of their effects on gene expression (Streicher et al., 1996; Wade et al., 1988). It has been reported that insulin can positively regulate SREBP-1a, -1c and -2 (Fajas et al., 1999; Foretz et al., 1999; Shimomura et al., 1999) and this involves signalling via the MAP kinase pathway (Kotzka et al., 2000). Furthermore Insulin-induced transcription of the LDL receptor can even be seen in the presence of cholesterol levels that would completely suppress LDL receptor gene expression (Streicher et al., 1996). SREBPs are able to bind both to SREs and also to glucose response elements, (5'-CACGTG-3', E-boxes) (Foretz et al., 1999) but the role of glucose in the regulation of SREBPs remains unclear. In hepatocytes SREBP-1c has been reported to be necessary for the activation of expression of lipogenesis-related genes, such as fatty acid synthase, in response to glucose (Foretz et al., 1999), however it appears that expression of these genes are both insulin and glucose-dependent. It is thought that insulin stimulates the transcription of SREBP-1c

and that high glucose levels result in activation of SREBP-1c, either by increased proteolytic cleavage or through activation of the mature form (Foretz et al., 1999; Foretz et al., 1999). It has been suggested that SREBPs can also mediate downregulation of transcription and insulin-induced repression of transcription occurs where SREBP levels increase (Brown and Goldstein, Keystone symposium unpublished). Therefore due to the presence of SRE sites in the HSL promoter and the combined effect of glucose and insulin on HSL expression it is possible that SREBPs are involved in mediating these effects on HSL expression.

Alternatively the HSL promoter contains a peroxisomal proliferator response element (PPRE) to which PPAR γ will bind (Talmud et al., 1998). PPAR γ is expressed at high levels in the nuclei of macrophage foam cells within atherosclerotic lesions and mounting evidence suggests that this factor may play a role in the control of gene expression and lipid metabolism in macrophages (Tontonoz and Nagy, 1999). A number of potential targets for PPAR γ in macrophages have recently been identified, some of which are induced by PPAR γ ligands and others which are repressed. Insulin has been shown to cause a significant increase in PPAR γ protein levels in adipocytes but the mechanism of action of insulin is unknown (Rieusset et al., 1999). SREBPs are known to regulate PPAR γ expression in adipocytes (Fajas et al., 1999) so it is possible that insulin is positively regulating PPAR γ via these transcription factors. Insulin has been shown to acutely upregulate PPAR γ -1 and PPAR γ -2 mRNA and protein levels twofold in adipocytes (Rieusset et al., 1999). PPAR γ -2 is rapidly (1-2h) and transiently increased and our studies also show a rapid and transient increase in HSL protein and activity in this time frame. This isoform is currently thought to

be exclusive to adipocytes, but no studies have specifically looked for this isoform in macrophages. Therefore the possible regulation of HSL gene expression by PPAR γ is a matter that should be investigated in future studies.

The differential regulation of HSL gene expression by insulin and glucose in macrophages and adipocytes is most probably due to the different proteins and enzymes expressed in these two cell types, such as trans-activating factors and their ligands. It is interesting to note that insulin and glucose treatment gave an increase in HSL activity in both macrophages and adipocytes in the first few hours of treatment, and it appears that it is the longer incubations of more than 6 hours which give the differential effects. In adipocytes it is known that while PPAR γ 2 is increased rapidly by insulin, PPAR γ 1 is induced slowly over 6 hours (Rieusset et al., 1999). Therefore maybe HSL is regulated by PPAR γ in both cell types but the transcriptional activity of PPAR γ 1 is being inhibited in the macrophages.

3.4.3. Effect of insulin and glucose on cholesterol ester and triglyceride accumulation in macrophages

It would be predicted that the insulin and glucose-stimulated downregulation of HSL activity would result in an increased accumulation of cholesterol esters in macrophages. In agreement with this we find that hyperinsulinemia and hyperglycemia cause increased loading of cholesterol esters in macrophages. There are several ways insulin could cause this but most can be ruled out in this case. It is unlikely that changes in ACAT are responsible as insulin does not upregulate ACAT levels (Yang et al., 1994). It has been reported that insulin increases the number of LDL receptors (Krone et al., 1988) but this is not likely to contribute to the observed CE accumulation because acetylated LDL is not recognised by the LDL receptor.

Modified LDL is taken up rapidly by scavenger receptors in macrophages and it is possible that insulin may be affecting scavenger receptor expression/activity. Insulin has been shown previously to enhance scavenger receptor-mediated uptake of ligands in liver cells . In these experiments insulin did not cause an increase in the number of cell surface scavenger receptors. Evidence suggests that activation of PPAR γ facilitates induction of transcription of the class B scavenger receptor, CD36, and the cellular uptake of oxLDL in macrophages (Tontonoz and Nagy, 1999; Torra et al., 1999). Therefore, due to the fact that insulin upregulates PPAR γ in adipocytes (Rieusset et al., 1999), a possible explanation for the CE accumulation observed with insulin in macrophages is PPAR γ activation and increased modified LDL uptake. Lipoxxygenases within the macrophage can act on linoleic and arachadonic acids to generate oxidized fatty acid ligands of PPAR γ (Huang et al., 1999) resulting in further induction of

PPAR γ (Nagy et al., 1998). However, if this was the sole reason for CE accumulation we would expect to see the same amount of accumulation at high and low glucose concentrations. One possibility for the observed difference in accumulation is that insulin-induced PPAR γ expression is mediated by SREBP transcription factors. SREBPs are reported to regulate PPAR γ expression in adipocytes (Fajas et al., 1999) and are regulated themselves by both insulin and glucose.

A model can therefore be suggested to account for the cholesterol ester accumulation observed with insulin and glucose (see figure 3.14):

1. Insulin/glucose increase SREBP activity (Foretz et al., 1999) which will increase PPAR γ expression (Fajas et al., 1999) and cholesterol and triglyceride synthesis and reduce HSL expression.
2. PPAR γ increases the synthesis of scavenger receptors which results in more modified LDL uptake (Tontonoz and Nagy, 1999).
3. Oxidised lipids activate PPAR γ (Huang et al., 1999) and downregulate HSL expression .
4. The decrease in HSL expression would result in less neutral cholesterol esterase activity leading to increased accumulation of cholesterol esters. The storage of cholesterol esters will then reduce HSLmRNA further (Jepson et al., 1996) or activate a cytosolic HSL protein inhibitor (Shand et al., 1993), promoting yet more CE accumulation in a self-perpetuating cycle.

3.5. Conclusion

The work described in this chapter demonstrates that under conditions of high glucose and high insulin, HSL expression is downregulated. These conditions also promote CE accumulation in macrophages. This work therefore identifies a novel mechanism that is likely to contribute to the accelerated foam cell formation and atherogenesis seen in insulin resistant subjects.

4. Effect of Leptin on HSL Regulation In Macrophages

4.1. Summary

Insulin resistance is often accompanied by obesity, which is associated with high circulating levels of the hormone leptin. The purpose of the following experiments was to determine if leptin can regulate HSL activity in macrophages, which will allow us to predict if obesity-associated hyperleptinemia is contributing to foam cell formation.

Leptin was found to acutely increase HSL activity in macrophages at physiological concentrations. This effect was maximal at 15 minutes leptin stimulation. Leptin stimulation increased HSL activity by the same amount as 1mM CPT-cAMP, a cAMP analogue, and the combined effects of leptin and CPT-cAMP were additive.

In macrophages, chronic leptin treatment in high glucose conditions results in a reduction in HSL expression and activity. In contrast, incubation with leptin and low glucose for 24 hours causes an increase in HSL expression and activity.

When macrophages were loaded with acetylated LDL and labelled [^{14}C]-oleate we found increased cholesterol ester (CE) accumulation with high glucose and leptin and decreased CE accumulation with low glucose and leptin. The

situation with triglycerides (TAG) was slightly different, TAG accumulation increased by approximately 20% in cells maintained in both high and low glucose containing leptin.

Therefore a novel leptin response in macrophages has been identified and results suggest that obesity, a condition associated with the insulin resistance syndrome, could promote foam cell formation by increasing cholesterol ester and triglyceride accumulation in macrophages.

4.2. Introduction

Obesity is a well-recognised risk factor for heart disease [Tuomilehto, 1987 #321; [Wickelgren, 1998 #324] and is associated with insulin resistance (Shoji et al., 1997; Stout, 1993). Leptin (OB) is an adipose-derived secreted hormone that circulates in the serum at levels which reflect the amount of energy stored in adipose tissue . Leptin plays an important role in regulating body weight and was first identified as a negative regulator of appetite by acting at its receptor in the hypothalamus . Leptin injected into rodents with genetic or diet-induced obesity results in normalisation of body weight (Campfield et al., 1995), however, high levels of leptin are often observed in obese individuals and animal models of obesity (Frederich et al., 1995; Maffei et al., 1995). This suggests resistance to the physiological effects of leptin and a compensatory increase in plasma leptin levels.

There are multiple forms of the leptin receptor (OB-R) including a long form (OB-R_L) with an intracellular domain of 303 amino acids . RNA splicing at the

most C-terminal exon produces several forms of OB-R with shorter intracellular tails . Evidence indicates that leptin only signals inside the cells when the long form (OB-R_L) is expressed (Baumann et al., 1996). Expression of OB-R_L is very high in specific cell types in the hypothalamus, but appears to be very low in other tissues, certainly in relation to the short form of the receptor . Although both the long and short forms of the leptin receptor are widely distributed in the body the entire spectrum of physiological roles of leptin remains poorly understood. Not surprisingly leptins interactions with its receptors in the hypothalamus have been most extensively studied and no evidence has yet been uncovered that explains why defective leptin action is so frequently associated with diabetes or heart disease.

As well as signalling to the CNS, leptin has direct effects on adipose tissue. Leptin has been shown to increase basal lipolysis and TNF α production (Fruhbeck et al., 1998; Fruhbeck et al., 1997; Siegrist-Kaiser et al., 1997) and alter the expression of several genes involved in fatty acid metabolism (Wang et al., 1999) as well as downregulating its own expression . Another important role of leptin is thought to be to limit lipid accumulation in non-adipocytes, such as β cells (Unger et al., 1999). Evidence for this is provided by the fact that when leptin receptors are dysfunctional, the TG content of islet cells can increase 100 fold. It is possible that leptins' effects on lipolysis in adipocytes and lipid homeostasis in β cells are mediated by regulation of HSL activity, but this has not yet been determined.

The leptin receptor has been found to be abundant on the surface of primary macrophages (Gainsford et al., 1996, Bennet, 1996 #403) and leptin has profound effects on macrophage function, such as, enhanced phagocytosis and production of pro-inflammatory cytokines including m-CSF (Gainsford et al., 1996; Loffreda et al., 1998).

Therefore due to the fact that macrophages are known to respond to leptin and leptin is known to have effects on lipid metabolism we have investigated whether leptin affects HSL activity and lipid accumulation in macrophages

4.3. Results

4.3.1. *Acute effects of leptin on HSL activity*

Leptin is known to directly increase lipolysis in adipocytes. The mechanism for activation of lipolysis is currently unknown but it is possible that leptin is regulating HSL activity. Therefore we investigated whether leptin is able to regulate HSL in macrophages.

Leptin was found to acutely increase nCE activity in HSL IPs from J774.2 macrophages by approximately 40% (figure 4.1). At 2nM leptin, this effect was maximal after 15 minutes stimulation. Dose response data (figure 4.2) indicates that the effect is maximal at a dose of 2nM leptin and activity appears to be returning back to basal levels at 5. Leptin binding activates OB-R, by causing dimerisation. A possible reason for this reduction in activity with higher concentrations is inhibition of receptor dimerisation, with increasing concentration of ligand. This dose response curve is similar to that seen for the

inhibition of insulin secretion by leptin in the pancreas, where the maximal effect was also observed at 2nM, with higher concentrations being less efficient . The stimulation of HSL activity by leptin is of a similar magnitude to the approximate 50% increase observed after CPT-cAMP stimulation (Figure.4.3). This agrees with previous reports on the stimulation of nCE activity by cAMP in macrophages (Small et al., 1991). Incubation with CPT-cAMP and leptin together gave an additive effect, almost doubling basal HSL activity.

4.3.2 Chronic effects of leptin on HSL expression and activity

It has been determined that leptin can regulate HSL activity acutely in macrophages and, as leptin is known to regulate the expression of genes, we continued these studies by investigating if chronic leptin treatment affects HSL expression and activity.

Results presented in chapter 3 showed that glucose, while having no effect on HSL expression alone, can influence expression in combination with other agents. Therefore the chronic effects of leptin were investigated in macrophages maintained in high and low glucose conditions. Incubation of macrophages with normoglycemic media containing 10nM leptin results in a significant increase (40-50%) in HSL expression (figure 4.4) and activity (figure 4.5) after a 24 hour incubation. Similarly, Sarmiento et al (Sarmienmto et al., 1997) found that leptin caused an increase in HSL mRNA in adipocytes from lean mice, which would be normoglycemic. However J774.2 cells maintained in high glucose show a reduction in HSL expression (figure 4.4) and activity (figure 4.5) by approximately 30% after leptin treatment for 24 hours. Therefore cells

incubated with leptin for 24 hours in low glucose will have approximately twice as much HSL activity as identical cells incubated with the same amount of leptin in high glucose media.

4.3.3. Effect of leptin and glucose on lipid accumulation in macrophages

Experiments reported in chapter 3 demonstrated that insulin had effects on HSL activity in macrophages and that this was reflected in the amount of cholesterol esters that accumulated over a 24 hour period in lipid loading experiments (see 3.3.4). Therefore we have repeated the loading experiments to determine if the effect of leptin on HSL expression/activity will give the predicted effect on lipid accumulation in these cells. Figure 4.6 shows that without the addition of leptin the accumulation of CEs and TAGs does not alter significantly in cells maintained in either high or low glucose media. With the addition of 10nM leptin, however, cholesterol ester accumulation is reduced in low glucose and increased in high glucose (figure 4.6A) and TAGs show a small increase in accumulation in both high and low glucose (figure 4.6B). Figure 4.7 shows the same data but is represented to show that the major significant difference in CE accumulation is seen between cells incubated with leptin for 24 hours in different glucose concentrations. Approximately 60% more CEs accumulate in high glucose compared to low glucose but similar amounts of TAG accumulate at high and low glucose concentrations.

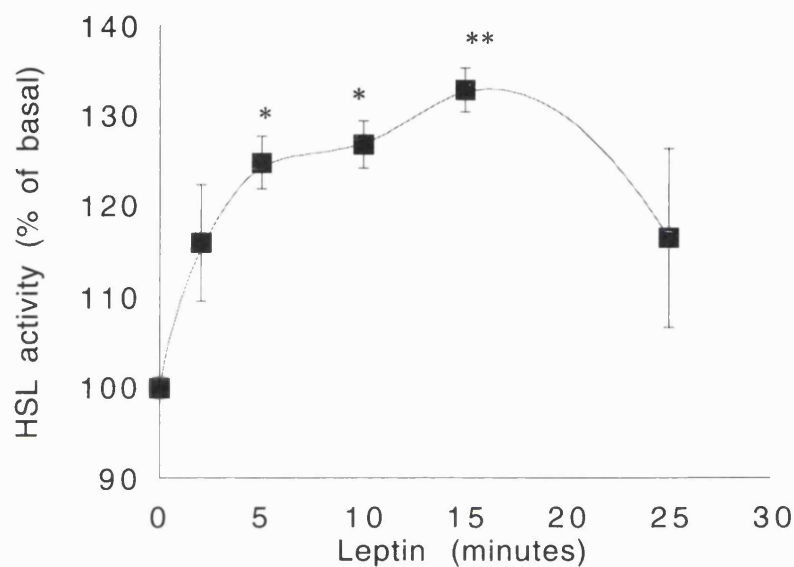


Figure 4.1. Timecourse for leptin stimulation of HSL activity in macrophages

Macrophages were serum starved overnight in serum free RPMI + 0.2% BSA. Cells were stimulated with 2nM leptin and lysed at different timepoints. HSL was immunoprecipitated and neutral cholesterol activity was determined, as described in materials and methods. Data represents the mean ($\pm$ SEM) of three independent determinations, carried out at least in duplicate. * P = <0.05, ** P = <0.01 compared to basal.

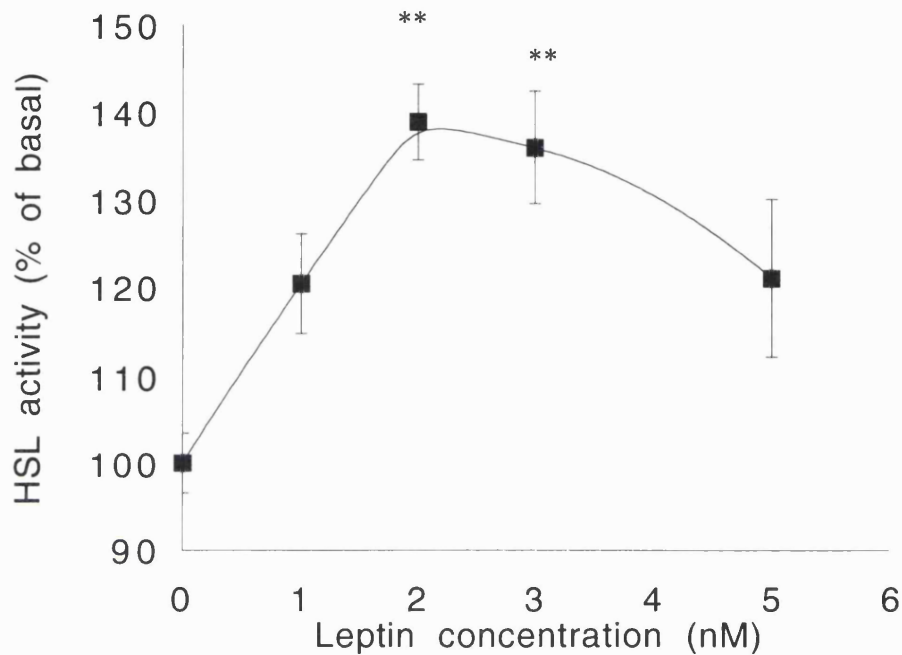


Figure 4.2. Dose response for leptin on HSL activity in macrophages

J774 macrophages were serum starved overnight prior to stimulation with various concentrations of leptin for 15 minutes. Cells were harvested and lysed and nCE activity was measured in HSL immunoprecipitates. Data represents the mean ($\pm$ SEM) of three independent determinations, each carried out in at least duplicate. ** $P = <0.01$ compared to basal.

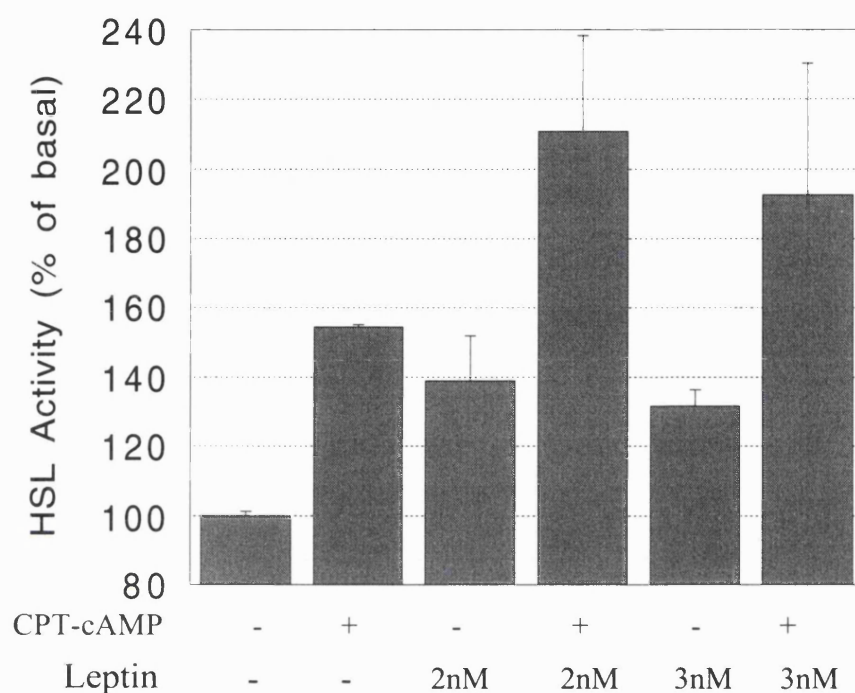


Figure 4.3. The effects of leptin and cAMP in macrophages are additive

J774.2 macrophages were serum starved overnight prior to stimulation with 1mM 8-(4-chlorophenylthio)-adenosine3':5'-cyclic monophosphate (CPT-cAMP) alone for 15 minutes or with the addition of 2nM leptin. Cells were lysed and HSL was immunoprecipitated. nCE activity was measured in the immunoprecipitates, as described in materials and methods. Data represents the mean ($\pm$ SEM) of two independent determinations, each carried out at least in duplicate.

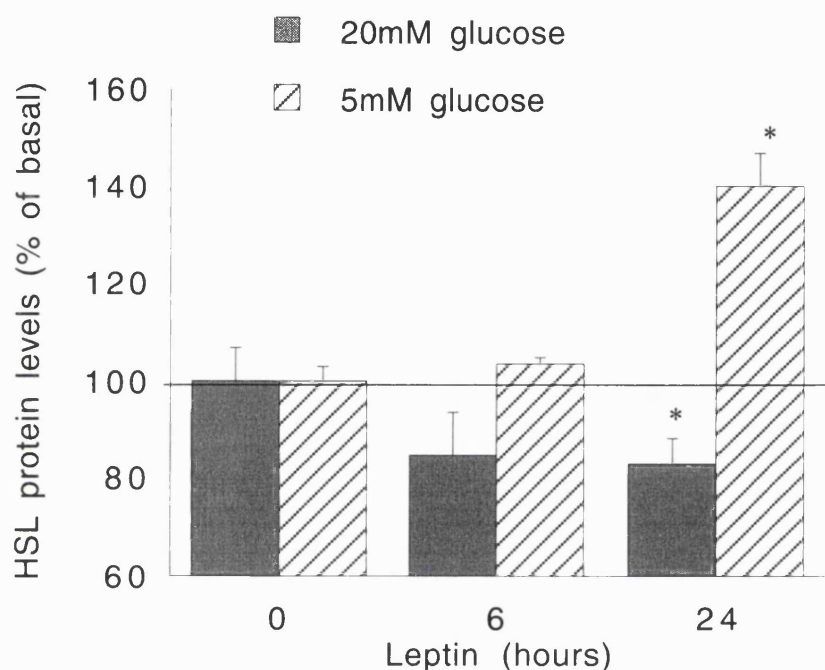


Figure 4.4 Effect of glucose and leptin on HSL expression in macrophages

J774.2 macrophages were incubated in RPMI media containing 2% FCS, 10nM leptin and either 5mM or 20mM glucose prior to immunoprecipitation with anti-HSL antibody. nCE activity in the HSL immunoprecipitates was then measured. Data represents the mean ($\pm$ SEM) of three independent determinations, each carried out in at least duplicate. * $P < 0.05$ compared to basal.

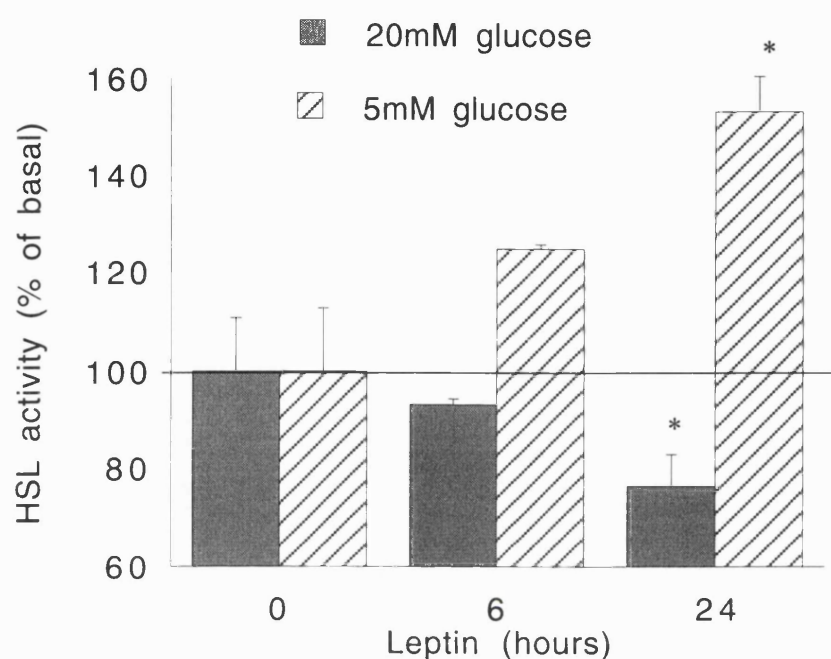


Figure 4.5. Effect of glucose and leptin on HSL activity in macrophages

J774.2 macrophages were incubated in RPMI media containing 2% FCS, 10nM leptin and either 5mM or 20mM glucose prior to immunoprecipitation and western blotting with anti-HSL antibody. HSL protein levels were quantitated using a phosphoimager. Data represents the mean ($\pm$ SEM) of three independent determinations, each carried out in at least duplicate.* $P = <0.05$, compared to basal.

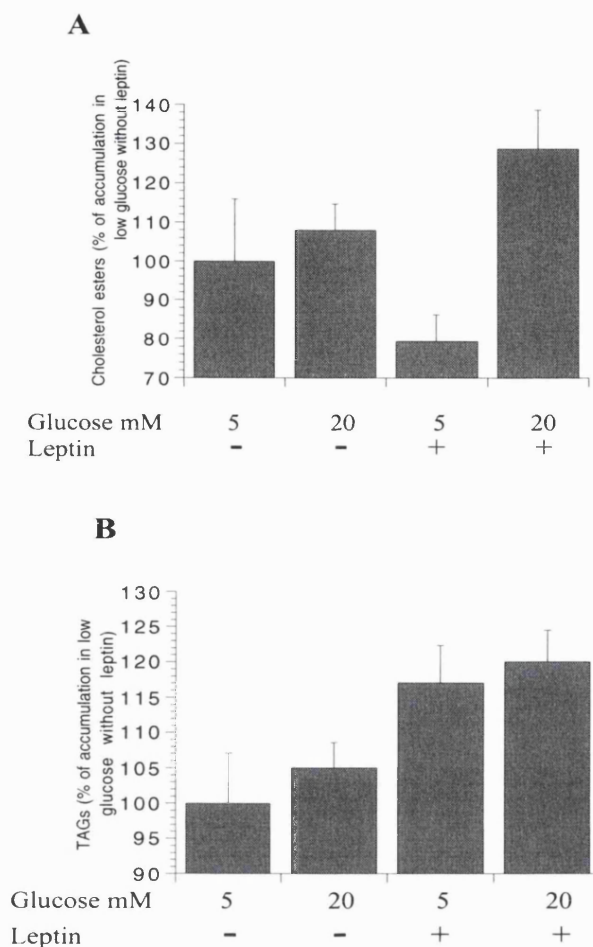


Figure 4.6. The effect of glucose and leptin on the accumulation of lipid in macrophages

Macrophages were incubated for 24 hours with acetylated LDL and [^{14}C]oleate in media containing either 5mM (low) or 20mM (high) glucose and 10nM leptin. Lipids were extracted from cells after 24 hours and [^{14}C]-labelled cholesterol esters (**A**) and triglycerides, (TAGs), (**B**), were separated by thin layer chromatography. Graphs show lipid accumulation as a % of that which accumulates in cells incubated in low glucose media without leptin. Data represents the mean ($\pm$ SEM) of four independent determinations, each carried out in duplicate. See figure 4.7 for statistical significance of high glucose compared to low glucose.

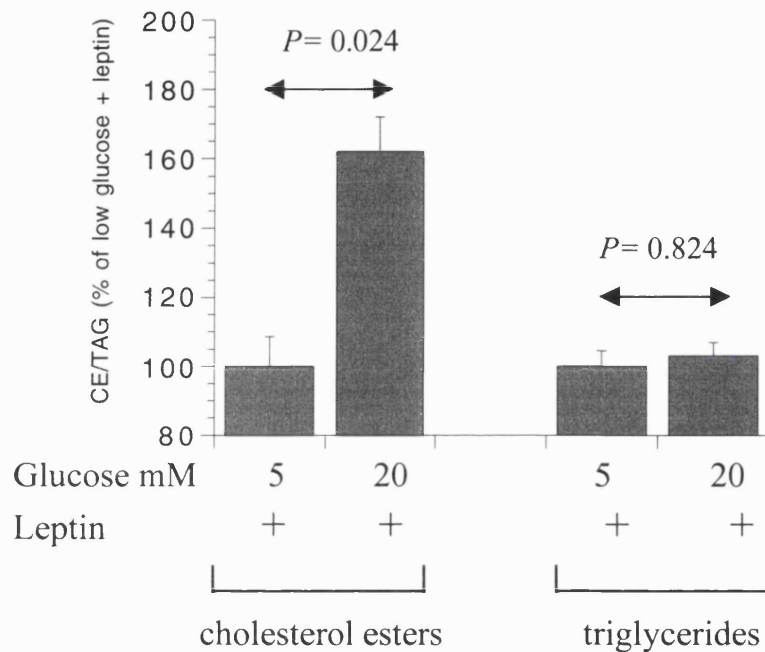


Figure 4.7. Lipid accumulation in high glucose media with leptin compared to low glucose media with leptin

Lipid loading of J774 macrophages was carried out as described in materials and methods. The data is the same as that shown in figure 4.7. but is presented to show how glucose affects loading of lipids with leptin. Incorporation of [^{14}C]oleate into the triglyceride pool does not significantly alter with a change in glucose levels from 5mM to 20mM, as it does into the cholesterol ester pool.

4.4. Discussion

Obesity is reaching epidemic proportions around the globe and the obesity-related increased risk for diabetes and CAD is a big health care problem. Given that few people lose weight and keep it off, understanding the molecular mechanisms linking obesity to CAD could be important in the preventative treatment of this disorder.

Leptin is an adipocyte derived cytokine which is increased in the serum of obese individuals and reduced in humans under conditions of nutritional deprivation. Leptin increases adipocyte lipolysis which suggests that it may directly regulate HSL activity. If leptin can regulate HSL activity in adipocytes, it is also possible that it may regulate HSL in macrophages. This could be important from the point of view of leptin resistance/hyperleptinemia affecting CE homeostasis in macrophages and consequently foam cell formation. Therefore we determined if leptin is capable of HSL regulation in macrophages.

4.4.1 *Acute effects of leptin on HSL regulation*

The studies detailed in this chapter clearly demonstrate that leptin, at physiological concentrations, acutely increases HSL activity in macrophages. The mechanism by which leptin may be increasing HSL activity is discussed further in chapter 5. The acute increase in HSL activity was not altogether unexpected, considering the hypothesis that a major role of leptin is to limit the accumulation of TAG in non-adipocytes (Unger et al., 1999). Data in β cells, which also contain HSL, shows that leptin administration depletes TAG (Unger et al., 1999). It is also hypothesised that leptin is an insulin counter-regulatory

hormone. Leptin is produced by WAT under conditions of high glucose and insulin stimulus and acts to oppose the effects of insulin, for example insulin drives adipose tissue to store energy in the form of fat whereas leptin acts to increase lipolysis and prevent this storage. It has been reported in previous chapters that insulin acutely reduces HSL activity in macrophages, therefore this acute stimulation of activity by leptin fits with the hypothesis that leptin opposes the effects of insulin. Thus, if leptin resistance exists in macrophages, as evidence suggests that it does in the hypothalamus, then the loss of acute HSL activation may contribute to CE accumulation and foam cell formation.

4.4.2. Chronic regulation of HSL by leptin and glucose

High circulating levels of leptin are often a feature of insulin resistance-associated obesity (Maffei et al., 1995; Shoji et al., 1997). It has been suggested that the short form of the leptin receptor, which is expressed in the liver, functions in the removal of leptin from the circulation (Gainsford et al., 1996). A possible explanation for hyperleptinemia is that leptin resistance is occurring in this tissue or that the hyperinsulinemia and high glucose seen in insulin resistance causes overproduction of leptin (Shoji et al., 1997). Whatever the cause of high circulating levels of leptin, chronic exposure of non-resistant cells to leptin may be having pathological effects. Therefore we have investigated chronic effects of this hormone on HSL in macrophages.

Leptin is known to activate the JAK/STAT pathway (Darnell, 1996; White et al., 1997) and regulate gene expression, particularly of proteins involved in glucose and lipid metabolism, such as uncoupling proteins (UCP), lipoprotein lipase

(LPL), fatty acid synthase (FAS) and glucose transporter (GLUT4) . In our experiments chronic leptin treatment in macrophages caused a significant decrease in HSL expression and activity in hyperglycemic conditions. Contrary to this, leptin treatment in normoglycemic conditions results in a significant increase in HSL activity and expression. This suggests that under normal conditions leptin acts to limit lipid accumulation in macrophages, but under conditions of elevated glucose this protective effect of leptin is lost. It has already been suggested in chapter 3 that glucose may have a major regulatory role for the effect of insulin on HSL expression. Leptin has been shown to stimulate glucose uptake and oxidation in a similar manner to insulin (Berti et al., 1997; Fruhbeck and Salvador, 2000). Therefore cellular uptake of high levels of glucose in these macrophages may be affecting HSL promoter activity in the same way, when in combination with either hormone. On the other hand, insulin and leptin appear to regulate HSL expression/activity slightly differently under low glucose conditions, where insulin does not alter HSL significantly but leptin causes a significant increase in HSL activity and expression.

It has been established in islets that TAG accumulation is limited due to upregulation of PPAR α in the presence of FA (Zhou et al., 1998). This results in induction of genes including enzymes such as acylCoA oxidase (ACO) and carnitine palmitoyl transferase 1 (CPT-1), which will direct FA into oxidative rather than lipogenic pathways (Torra et al., 1999; Zhou et al., 1998). This homeostasis cannot be maintained however, when leptin receptors are defective suggesting that activation of PPAR α , may be the mechanism by which leptin

protects non-adipocytes from excessive accumulation of TAG during periods of overfeeding. Therefore the increase in HSL protein levels that we see with chronic leptin treatment in normoglycemic conditions could be due to PPAR α . This possibility is further supported by the finding that a PPAR response element (PPRE) has been found in the HSL promoter (Talmud et al., 1998). PPAR α is becoming increasingly recognised as a key regulator of energy homeostasis, body weight control, glucose and lipid homeostasis and inflammation control, all of which are perturbed in insulin resistance, obesity and type 2 diabetes predisposing to atherosclerosis (Torra et al., 1999). As a result attention is now focussing on PPAR α as a potential pharmacological target in the treatment of insulin resistance associated obesity.

In contrast to PPAR α , PPAR γ acts to increase lipid synthesis and storage in non-adipocytes by increasing transcription of genes such as fatty acid synthase and acetyl CoA carboxylase and is also abundant in macrophage foam cells (Tontonoz and Nagy, 1999). The reduction in HSL expression and activity with leptin and high glucose would also drive TG storage so it is tempting to speculate that PPAR γ is involved. To further support this, PPAR γ has previously been shown to negatively regulate genes, such as TNF α and leptin (Spiegelman, 1998). A recent study in obese, lean and type 2 diabetic subjects, revealed that PPAR γ , but not PPAR α or β , protein expression in muscle and fat was increased as glucose disposal rates decreased, that is, as circulating glucose levels increased (Loviscach et al., 2000). We can therefore hypothesise that leptin acts to limit lipid accumulation in macrophages through PPAR α activation but in high glucose conditions leptin promotes PPAR γ activation and a reduction in

HSL expression. As mentioned in chapter 3, the PPAR γ gene is regulated by sterol regulatory element binding protein (SREBP) , the activity of which is regulated by growth factors and glucose. It is therefore possible that these transcription factors may play a role in the observed effects on expression with leptin. Figure 4.8 shows a model of how HSL gene regulation may be controlled by leptin under different glucose conditions.

It is now well established that leptin induces cytokine release from macrophages, such as m-CSF, TNF α and IL-6, (Gainsford et al., 1996; Gainsford et al., 1996; Loffreda et al., 1998; Santos-Alvarez et al., 1999) so it is also possible that any effects on HSL expression and activity upon incubation with leptin could be a secondary effect due to cytokines acting at macrophage cell-surface receptors.

These effects on HSL expression and activity could have important implications in insulin resistance where elevated levels of circulating leptin together with higher than normal glucose concentrations could combine to increase lipid accumulation in macrophage foam cells and increase atherogenesis.

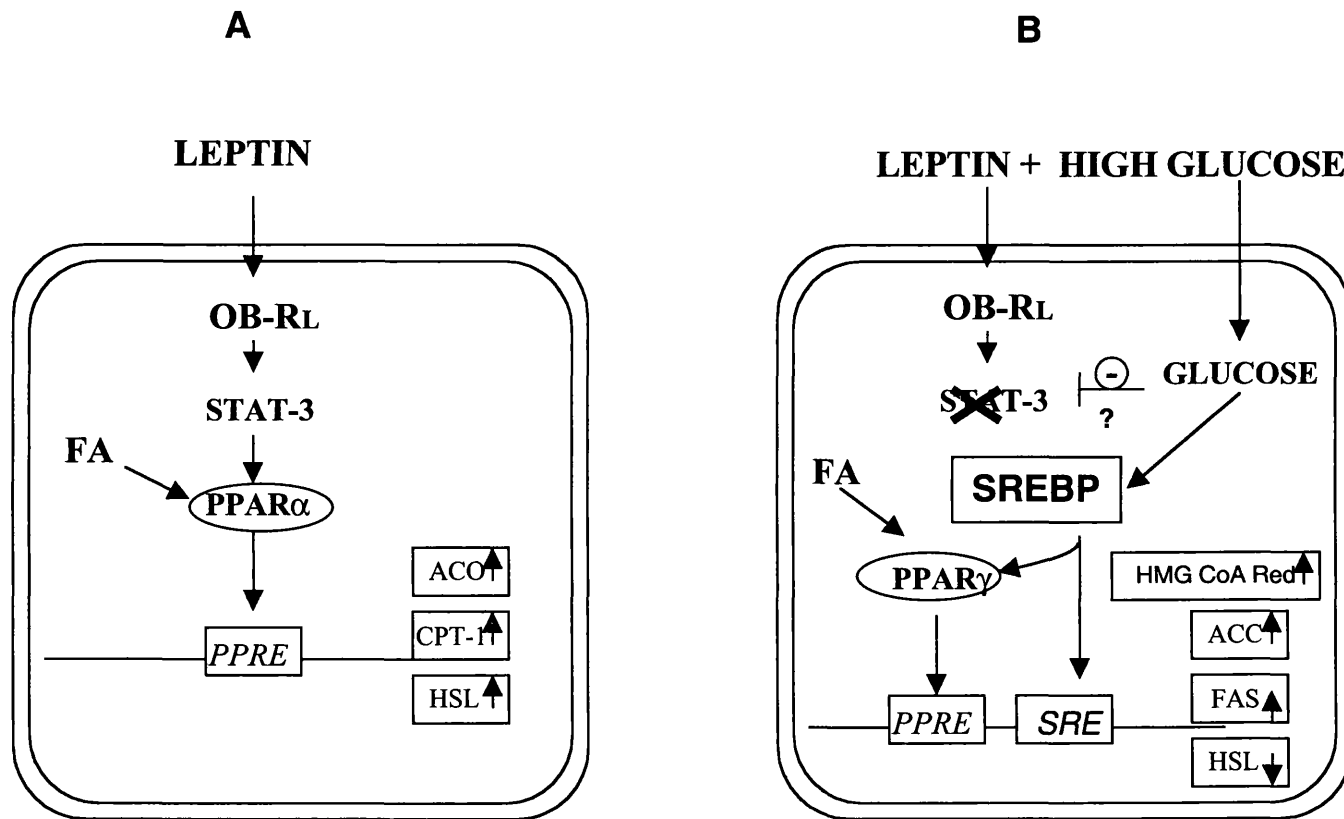


Figure 4.8. (A) Putative cholesterol homeostatic system in macrophages under normoglycemic conditions. PPAR α activation limits lipid formation. Hormone-sensitive lipase and enzymes of lipid oxidation are increased **(B)** in hyperglycemic conditions this homeostatic system is non-functional, SREBP activation and PPAR γ expression results in increased cholesterol ester accumulation, due to increased CE synthesis and reduced CE hydrolysis. Abbreviations: ACO, acyl CoA oxidase, CPT-1, carnitine palmitoylTransferase, HSL, hormone-sensitive lipase, HMG CoA Red, HMG CoA reductase, ACC, acetylCoA carboxylase, FAS, fatty acid Synthase.

4.4.3. Effect of leptin on cholesterol ester and triglyceride accumulation in macrophages

From the expression studies in macrophages we would predict that chronic exposure to leptin and high glucose would increase CE accumulation. To directly test this we loaded macrophages with lipids under different conditions. The finding that leptin tends to reduce CE loading in low glucose media correlates well with the degree to which leptin increases HSL expression in these conditions. Also the increase in CE and TAG loading in high glucose media containing leptin correlates well with the reduction in HSL activity observed under these conditions. All of these results are similar to those observed for insulin (see chapter 3). However, leptin and low glucose conditions resulted in an increase in labelled TAG. This was unexpected as our expression studies and the reduction in CE loading suggests that HSL activity is increased under these conditions. There are several possible explanations for this observation;

(i) A neutral triglyceride lipase distinct from HSL may be expressed in macrophages, which is downregulated by leptin under these conditions, but which is not affected by insulin (see fig. 3.12 for TAG loading with insulin). This is consistent with recent data where mice lacking HSL show that nCE activity is abolished in most tissues but residual TAG lipase activity was still clearly observed (Osuga et al., 2000).

(ii) Alternatively this increase in TAG, but not CEs, could be due to upregulation of the synthesis of TAG. For example, in the liver and adipose the transacylase, diacylglycerolacyltransferase (DGAT), catalyses the synthesis of TAG (Lehner

and Kuksi, 1993; Owen et al., 1997) and high levels of leptin are associated with high DGAT activity and high TAG levels (Smith et al., 1999). Also studies carried out in lean, normoglycemic mice show that chronic leptin treatment increases fatty acid synthase (FAS) and lipoprotein lipase mRNA in adipocytes (Sarmiento et al., 1997). If leptin is regulating these genes in a similar way in macrophages this may contribute to the observed TAG accumulation.

(iii) Recently it has been reported that overexpression of HSL in HEPG2 cells depletes endogenous stores of TAG and that the fatty acids released intracellularly are not removed from the cell, but enter the oxidative pathway (Pease et al., 1999). Similarly, in adipocytes, it has been reported that leptin induces a novel form of lipolysis where glycerol is released from the cell but FFA are not. Again, it was suggested that these FFA are oxidised, as there was an increase in PPAR α expression which in turn increases the expression of lipid oxidation enzymes (Wang et al., 1999). Our results in macrophages suggest that, chronic leptin incubation in the presence of modified LDL and low glucose, causes TAG hydrolysis but released FFAs are re-esterified to form triglycerides rather than entering the oxidative pathway, which in turn may suggest that PPAR α activity is being downregulated. As mentioned in chapter 1, PPAR function can be regulated by phosphorylation. MAP-kinase mediated phosphorylation increases basal and ligand-stimulated PPAR α activity, but reduces PPAR γ activity. Leptin is reported to activate the MAP-kinase pathway, and may upregulate PPAR α by phosphorylation. However, leptin is also known to activate STAT5b which inhibits PPAR α transcriptional activity (Zhou and Waxman, 1999). Therefore leptins' effects on lipid metabolism may depend on

the downstream signalling pathways employed by OB-R under certain conditions. It can be hypothesised that chronic leptin stimulation in the presence of modified LDL results in STAT5 activation and PPAR α inhibition.

Therefore, in summary, chronic leptin treatment acts to promote TAG accumulation in macrophages irrespective of the glucose concentration and CE accumulation in the presence of high glucose levels. A possible explanation for the increased CE accumulation in high glucose conditions is due to the reduction in HSL activity observed under these conditions.

4.5. Conclusion

Leptin acutely increases HSL activity and, in combination with glucose, chronically reduces HSL activity in macrophages. Therefore if these cells become leptin resistant the beneficial acute effect will be lost, and HSL activity reduced. Alternatively if they do not become resistant chronic exposure of macrophages to leptin due to leptin resistance/hyperleptinemia in combination with high glucose levels may contribute to foam cell formation. Therefore a novel mechanism linking diabetes-associated obesity to accelerated atherosclerosis has been identified.

5. Investigation of the mechanisms employed by insulin and leptin to regulate HSL

5.1. Summary

The work described in the previous chapters has established that both insulin and leptin are able to acutely regulate HSL activity in macrophages. Also over longer periods, insulin and leptin synergise with hyperglycaemia to regulate the expression of HSL. This chapter describes a range of preliminary experiments investigating the mechanisms that may be employed by these hormones to exert their effects on HSL.

In adipocytes insulin reduces HSL activity by causing phosphorylation and activation of phosphodiesterase 3B (PDE3B) by a mechanism which is dependent on PI 3-kinase and protein kinase B (PKB) activity. Results presented in this chapter suggest that this is not the case in macrophages as the decrease in HSL activity caused by insulin is not blocked by wortmannin. Insulin is able to increase recruitment of $p85\alpha$, $p110\alpha$ and β subunits of PI 3-kinase and increase PI 3-kinase activity in signalling complexes. It therefore appears that, although insulin is capable in activating the enzyme, it is not utilised in these cells to regulate HSL activity. The lack of PI 3-kinase dependence may lie in the finding that PDE 3B was not found to be present in J774 macrophages.

Like insulin, leptin increases p85 recruitment and in vitro PI 3-kinase activity but, unlike insulin, leptin-induced stimulation of HSL activity is blocked by the PI 3-kinase inhibitor wortmannin. The long form of the leptin receptor (OB-R_L) is present and tyrosine phosphorylated upon leptin stimulation in the macrophages, as is JAK-2. This provides strong evidence that macrophages are a functionally relevant target for leptin.

As the PPAR γ agonists, thiazolidinediones, are widely used as treatments for diabetes, the effect of these drugs on insulin signalling in macrophages compared to adipocytes was investigated. 4 hour pre-treatment of cells with thiazolidinediones, caused increased basal IRS-1 phosphorylation and p85 recruitment in adipocytes. The same pretreatment resulted in reduced HSL activity, possibly due to the increased PI 3-kinase activity. PI 3-kinase recruitment in macrophages was only increased by treatment with rosiglitazone. The effect of this drug on HSL activity in macrophages wasn't clear, but the data suggests that HSL activity may be reduced. This effect of rosiglitazone could potentially exacerbate foam cell formation.

5.2 Introduction

HSL is present in macrophages and, as discussed in detail in chapter 1, evidence suggests that it is responsible for cholesterol ester hydrolysis in these cells (Khoo et al., 1993; Reue et al., 1997; Small et al., 1989). The previous two chapters describe acute and chronic effects of insulin and leptin on HSL in J774 macrophages. Considering the potential implications for foam cell formation, it is important to define how insulin and leptin are signalling to HSL in these cells.

This chapter describes experiments which have given us an insight into the mechanisms which might be used by these factors for the regulation of HSL.

In adipocytes lipolytic stimuli, such as catecholamines, increase cAMP-dependent protein kinase (A-kinase) activity and phosphorylation of HSL (see figure 1.6) (Garton et al., 1988; Stralfors and Belfrage, 1983). The signalling pathway by which insulin exerts its antilipolytic effect in adipocytes is well characterised. The insulin receptor has intrinsic tyrosine kinase activity and insulin binding results in phosphorylation of insulin receptor substrate (IRS) (Sun et al., 1991). The phosphorylated tyrosine residues of IRS then recruit PI 3-kinase through binding to the SH2 domains of the p85 regulatory subunit (Escobedo et al., 1991). PI 3-kinase activation results in PIP_3 production and PKB activation (Burgering and Coffey, 1995; Franke et al., 1995). PKB then directly phosphorylates and activates PDE 3B (Kitamura et al., 1999). PDE 3B hydrolyses cAMP (Degerman et al., 1997), reducing A-kinase activity and A-kinase dependent phosphorylation of HSL, ultimately leading to reduced HSL activity and lipolysis (Degerman et al., 1996). It has previously been reported that HSL activity in macrophages is increased by cAMP, as it is in adipocytes (Khoo et al., 1981; Small et al., 1991) and we have reported in a previous chapter that insulin can acutely reduce HSL activity in macrophages.

In chapter 4 the acute stimulation of HSL activity by leptin in macrophages was reported. Previous studies on leptin signalling in macrophages are limited. There are multiple forms of the leptin receptor (OB-R), including a long form (OB-R_L) with an intracellular domain of 303 amino acids and several short

forms (OB-R_S) with an identical extracellular domain but shorter intracellular domains. The presence of leptin receptors on macrophages has been demonstrated by the binding of ¹²⁵I-leptin to macrophages, but no detailed studies have been carried out investigating which isoforms of OB-R are present. The function of the OB-R_S is unclear and it is generally thought that only the long form of OB-R is functional and capable of intracellular signalling. The relationship between leptin and insulin signalling pathways is also unclear, in some cell types leptin has been shown to mimic the effects of insulin (Berti et al., 1997) and utilise signalling proteins also employed by insulin, such as PI 3-kinase. On the other hand, leptin has also been shown to inhibit insulin signalling by reducing insulin receptor and IRS-1 phosphorylation (Kroder et al., 1996). The experiments reported in this chapter aim to identify some of the signalling molecules employed by leptin and insulin to regulate HSL in macrophages.

Finally, the PPAR γ agonists, thiazolidinediones (TZDs), have recently been reported to have acute effects on signalling. Given the widespread clinical use of this class of drugs, it is important to consider the effect of TZDs in macrophages and the influence this may have on the atherogenic process. Some preliminary experiments comparing the effects of TZD treatment in macrophages and adipocytes are reported here.

5.3 Results

5.3.1 *Leptin signalling in macrophages*

Despite the fact that leptin has been shown to have profound effects on macrophage function, little attention has focussed on the intracellular signalling pathway of leptin in these cells. It is thought that only the long form of the leptin receptor (OB-R_L) is capable of signalling to downstream molecules. Therefore, to confirm that the long form of the leptin receptor was present in J774.2 macrophages, cell lysates were blotted using a polyclonal antibody generated against the C-terminal 100 amino acids of OB-R_L. The predicted molecular masses of the long and short forms of the OB-R in the rat are 130kDa and 101kDa respectively (Szanto and Kahn, 2000). However CHO cells stably transfected with OB-R isoforms have shown discrepancies between the predicted molecular mass and estimated molecular mass on SDS/PAGE, probably because of glycosylation of the receptors and differences in migration. In the stably transfected cells the long form migrates at 210kDa and the short forms between 110-130kDa (Szanto and Kahn, 2000). Figure 5.1, clearly shows that the long form of OB-R is present in J774.2 macrophages and also runs at 210 kDa. This antibody does not recognise the short form of the receptor, so the lower molecular weight bands, seen here at around 90-100kDa, may represent degradation fragments.

OB-R_L has no intrinsic kinase activity but is associated with Janus kinase 2 (JAK-2), which is required for activation of the receptor. Upon ligand binding the receptor dimerises and JAK-2 becomes tyrosine phosphorylated and is

activated as a tyrosine kinase. The intracellular tail of OB-R_L is then phosphorylated on tyrosine residues allowing binding via the SH2 domains on STATs (signal transducers and activators of transcription). The attached STATs are themselves tyrosine phosphorylated, followed by dimerisation and translocation to the nucleus, where they may exert effects on gene transcription. Therefore it was investigated whether the previously identified OB-R_L in these macrophages was indeed tyrosine phosphorylated in response to leptin stimulation. J774 cells were stimulated with 2nM leptin and immunoprecipitated with anti-phosphotyrosine antibody. Anti-phosphotyrosine immunoprecipitates were then western blotted for OB-R_L and JAK-2. Results indicate that there was a 50% increase in recruitment of OB-R_L (figure 5.2) and a 3.5 fold increase in JAK-2 recruitment (figure 5.3) upon leptin stimulation, in a similar time frame at which the maximal effect of leptin on HSL activity was observed (see chapter 4).

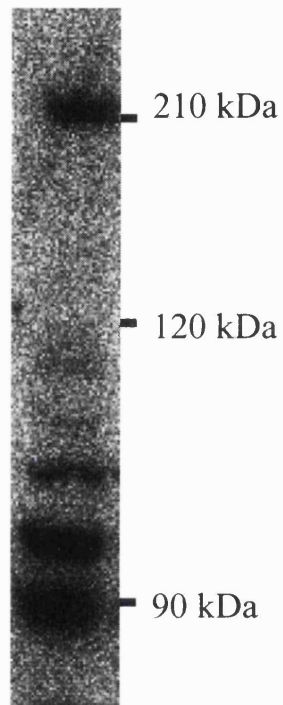


Figure 5.1. Leptin receptor is present in J774.2 macrophage lysates

Unstimulated J774.2 macrophages were lysed and 400 μ g of lysate was subjected to SDS-PAGE. Proteins were transferred and western blotted with antibody recognising the long form of the leptin receptor.

IP: py99

IB: OB-RL

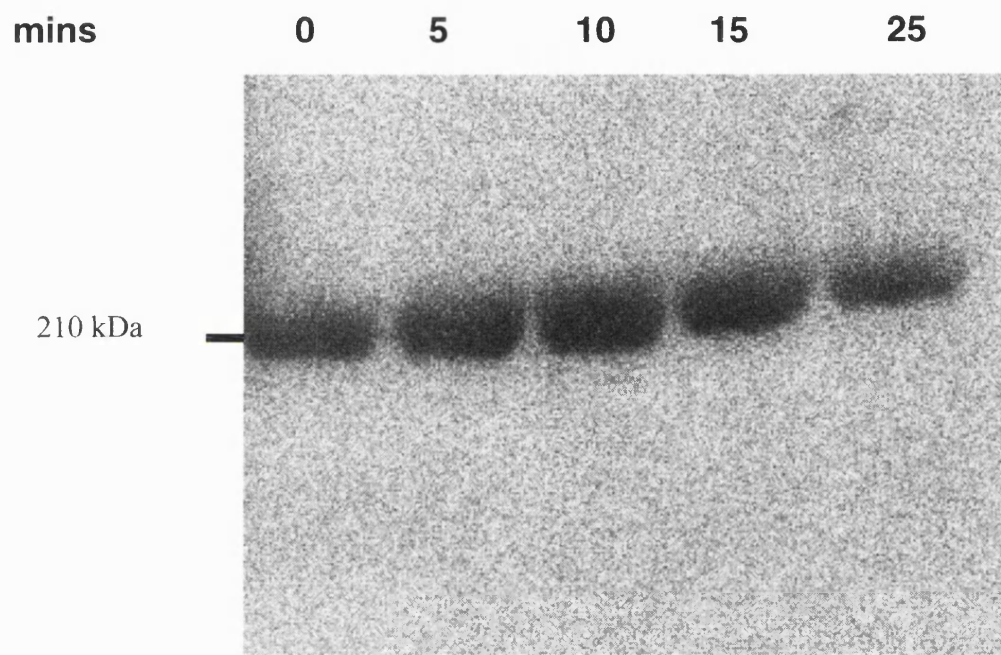


Figure 5.2. Tyrosine phosphorylation of the leptin receptor upon leptin stimulation in macrophages

J774.2 macrophages were serum starved overnight prior to stimulation with 2nM leptin, for the indicated times. Tyrosine phosphorylated signalling complexes were immunoprecipitated with anti-phosphotyrosine antibody, py99, and western blotted with anti-leptin receptor antibody.

IP: py99

IB: JAK-2

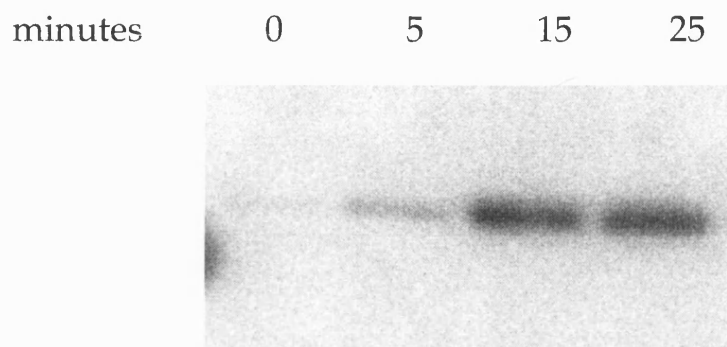


Figure 5.3. Recruitment of Janus kinase-2 upon leptin stimulation

J774.2 macrophages (**A**) were serum starved either overnight (macrophages) or for 3 hours (adipocytes) prior to stimulation with 2nM leptin, for the indicated times. Tyrosine phosphorylated signalling complexes were immunoprecipitated with anti-phosphotyrosine antibody, py99, and western blotted with anti-JAK-2 antibody.

5.3.2. *PI 3-kinase dependence*

The majority of insulins' actions involve PI 3-kinase and in adipocytes this is involved in the regulation of HSL. Also leptin has been shown to regulate this enzyme. Therefore we studied the effect of insulin and leptin on PI 3-kinase recruitment and activity in macrophages.

To determine if insulin and leptin stimulated recruitment of PI 3-kinase to tyrosine phosphorylated signalling complexes, J774.2 macrophages were stimulated and immunoprecipitated with anti-phosphotyrosine antibody. The IPs were then subject to SDS PAGE and blotted with antibody raised against the p85 regulatory subunit of PI 3-kinase. It can be seen that 3nM leptin clearly causes recruitment of p85 (Figure 5.4, A). This recruitment is maximal (more than 3 fold) at 15 minutes stimulation and appears to be returning to basal levels with longer stimulation. 10nM Insulin also increases recruitment of p85 (figure 5.4, B), although to a lesser extent than that seen for 3nM leptin. The maximal increase in recruitment with insulin was approximately 70%, observed after 10 minutes insulin stimulation. It would be expected that increased recruitment of PI 3-kinase into tyrosine phosphorylated signalling complexes would result in increased PI 3-kinase activity. To confirm this, the stimulation of macrophages with insulin and leptin were repeated and the lipid kinase activity in the anti-phosphotyrosine IPs was measured (figure 5.5). As expected both insulin and leptin increased PI 3-kinase activity and 3nM leptin caused a much bigger stimulation of activity compared to 10nM insulin. Mammals have three isoforms of the p110 catalytic isoform of PI 3-kinase. p110 α and p110 β are widely distributed, whereas p110 δ is mainly found in leukocytes. The recruitment of

these different isoforms were examined in J774.2 macrophages upon insulin stimulation using anti-phosphotyrosine IP and western blotting with antibodies specific for each isoform (figure 5.6). The non-immunoprecipitated lysate from basal samples were also loaded to verify the position of p110 on the gel. The p110 α and β specific antibodies also highlighted bands of lower molecular weight, however the bands which run at the same molecular weight as the protein detected in the lysate were presumed to be the true 110kDa band representing the PI 3-kinase catalytic isoforms. It appears that while there is increased recruitment of p110 α and p110 β into tyrosine phosphorylated signalling complexes, there is no increase in the amount of p110 δ recruitment. However, the majority of p110 δ is already associated with tyrosine phosphorylated protein in basal cells, whereas the majority, if not all, of p110 α and p110 β isoforms are still present in the lysate after anti-phosphotyrosine IP, in the basal state.

The SH2 containing inositol phosphatase protein (SHIP) is a 5' phosphatase which is proposed to be involved in downregulating the lipid product of PI 3-kinase, PtdIns(3,4,5)P₃, by hydrolysis to PtdIns (3,4)P₂ (Damen et al., 1996; Lioubin et al., 1996). Previous studies have shown that growth factor stimulation causes tyrosine phosphorylation of SHIP. Therefore we investigated if the same occurred in J774.2 cells. It can clearly be seen in the bottom panel of figure 5.6 that SHIP is not tyrosine phosphorylated at the 10 minute timepoint used in these experiments.

The above studies clearly demonstrate that insulin and leptin are able to activate PI 3-kinase in J774.2 macrophages. Also, it is well documented that regulation of

lipolysis by insulin in adipocytes is dependent on PI 3-kinase activity. Therefore we determined if the effects of insulin and leptin on HSL activity in macrophages required PI 3-kinase activity. This was done using 100nM wortmannin, which at this concentration is a specific inhibitor of PI 3-kinase. Figure 5.7 shows the effect of wortmannin pretreatment on the regulation of HSL by insulin. Experiments carried out in macrophage lysates (A) and HSL immunoprecipitates (B) show that 100nM wortmannin had no effect on the downregulation of HSL activity by insulin. However, the downregulation of HSL activity in HSL immunoprecipitates from adipocytes was blocked by wortmannin. Also the upregulation of HSL activity by leptin in macrophages, was completely blocked by pretreatment with 100nM wortmannin (figure 5.8), clearly indicating that wortmannin is functional in the highly oxidative environment found in macrophages. This data indicates that PI 3-kinase is necessary for leptin effects on HSL.

The thiazolidinedione (TZD) insulin-sensitising drugs have been reported to exert effects on insulin signal transduction pathways. These drugs act as agonists of the transcription factor PPAR γ and are used widely in the treatment of type 2 diabetes. Troglitazone, pioglitazone and rosiglitazone are three TZD drugs which are approved for treatment and their effect on insulin signalling was investigated in macrophages and adipocytes. A four hour treatment with either pioglitazone, troglitazone or rosiglitazone causes an average 50% increase in basal IRS-1 phosphorylation and also slightly increases p85 recruitment in adipocytes (figure 5.9). However the increase in p85 recruitment and IRS-1 phosphorylation in response to insulin is not increased further with TZD pretreatment (figure 5.9). Pioglitazone and troglitazone treatment of

macrophages for four hours did not effect either basal or insulin-stimulated p85 recruitment, however, rosiglitazone did appear to increase p85 recruitment compared to basal cells (figure 5.10). The increased insulin signalling in basal cells might be expected to alter basal HSL activity, particularly in adipocytes where PI 3-kinase is clearly involved in HSL regulation. To investigate this 3T3 L-1 adipocytes were treated for four hours with TZD, or DMSO alone, and HSL activity was measured. Figure 5.11 shows that, as predicted, basal HSL activity was reduced by pioglitazone and troglitazone in adipocytes (rosiglitazone was not tested). The effect of rosiglitazone was tested in macrophages. The experiment was only repeated twice and results were variable. As a result, although it appears that rosiglitazone treatment is causing a reduction in basal HSL activity by approximately the same amount as acute insulin treatment (figure 5.12), the change compared to basal is not statistically significant, due to large errors. Therefore more experiments will be needed to confirm the effect of rosiglitazone on HSL activity in macrophages.

IP: py99

IB: p85

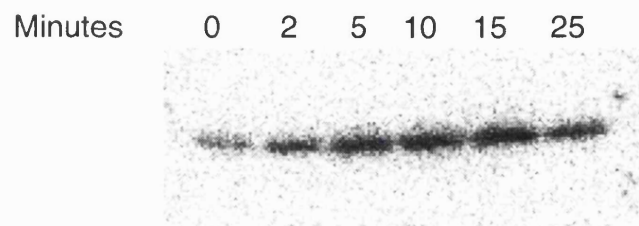
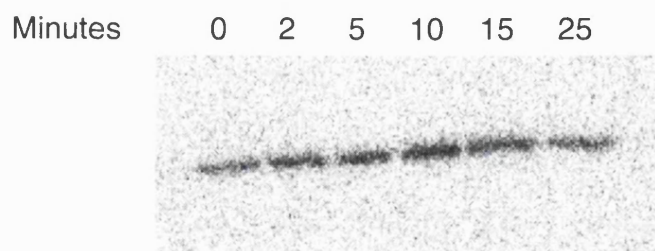
A LEPTIN**B INSULIN**

Figure 5.4. Insulin and leptin stimulate p85 recruitment in macrophages

J774 cells were serum starved overnight prior to stimulation with 3nM leptin (**A**) or 10 nM insulin (**B**) before immunoprecipitation with anti-phosphotyrosine antibody and western blotting with anti-p85 antibody, to determine how stimulation effected recruitment of PI 3-kinase to tyrosine phosphorylated signalling complexes

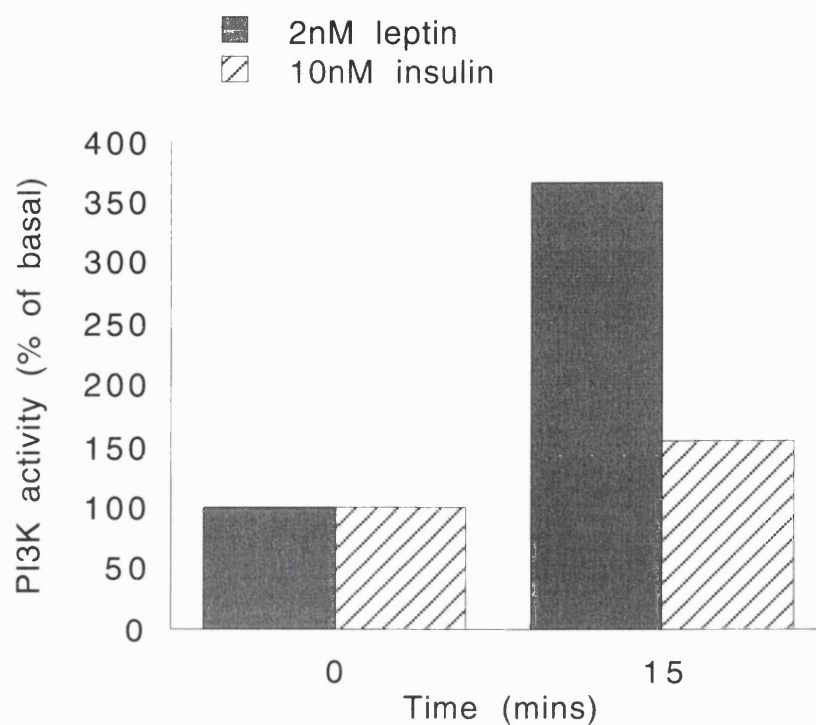


Figure 5.5. Insulin and leptin stimulate phosphatidylinositol 3-kinase activity in macrophages

J774 cells were serum starved overnight prior to stimulation with 10 nM insulin (**shaded bar**) or 3nM leptin (**filled bar**). PI 3-kinase activity was assayed as described in materials and methods. Phosphorylated lipids were separated by thin layer chromatography and quantitated using a phosphoimager. This is a single experiment which reinforces PI 3-kinase recruitment data in figure 5.4.

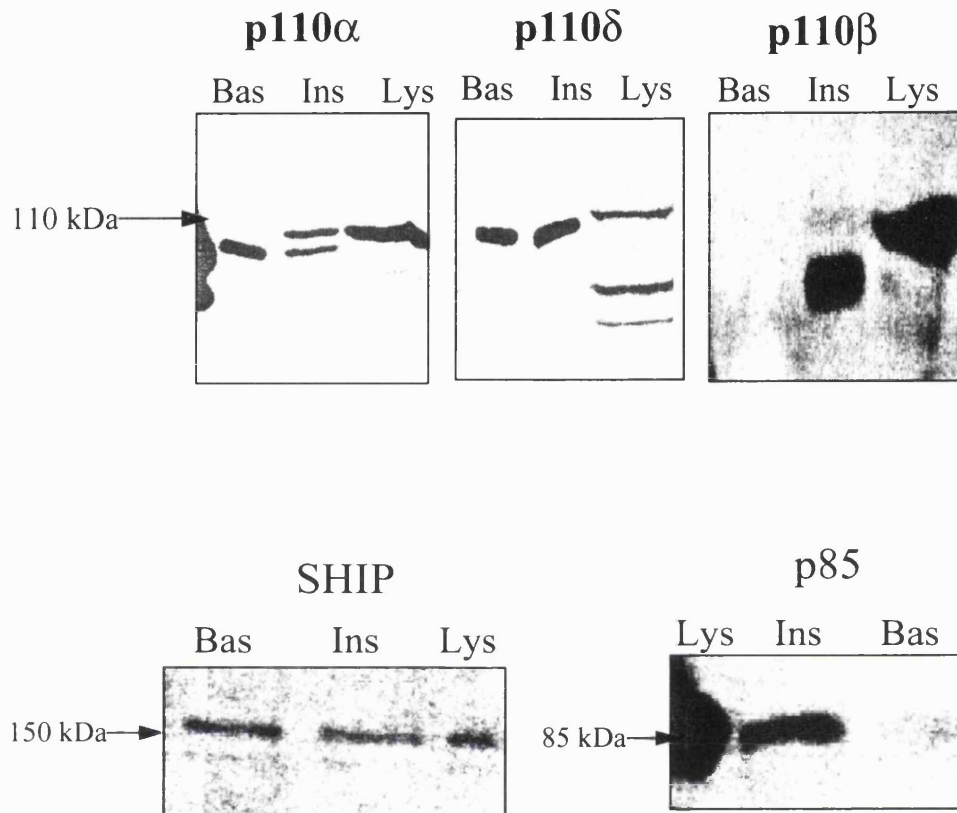


Figure 5.6 Increased recruitment of the α and β but not δ catalytic isoforms of PI 3-kinase in macrophages

J774 macrophages were serum starved overnight. Cells were stimulated with 1 μ M insulin for 10 minutes prior to immunoprecipitation with anti-phosphotyrosine antibody. Immunoprecipitates and also an aliquot of the lysate were subject to SDS PAGE (Immunoprecipitates were from a whole dish of cells, the lysate loaded represents a 6th of a dish). The p110 α , β and δ isoforms of PI 3-kinase, SH2-containing inositol phosphatase (SHIP) and p85 regulatory subunit were western blotted using specific antibodies.

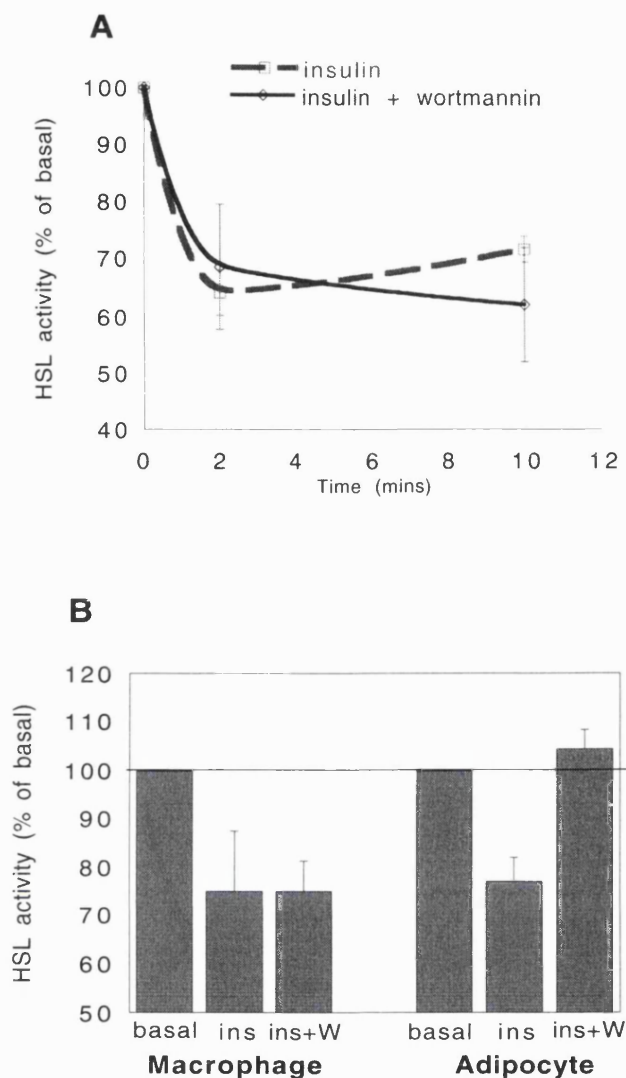


Figure 5.7. Effect of wortmannin on insulin-stimulated reduction in HSL activity in J774.2 macrophages and 3T3 L-1 adipocytes

Serum starved cells were pre-incubated with 100nM wortmannin for 10 minutes before the addition of 10 μ M insulin. Cells were harvested and nCE activity was measured in macrophage lysates (**A**) or HSL immunoprecipitates from the lysates (**B**). Activity was also measured in HSL immunoprecipitates from 3T3 L-1 adipocytes for comparison (**B**). Data represents the mean ($\pm$ SEM) of three independent determinations.

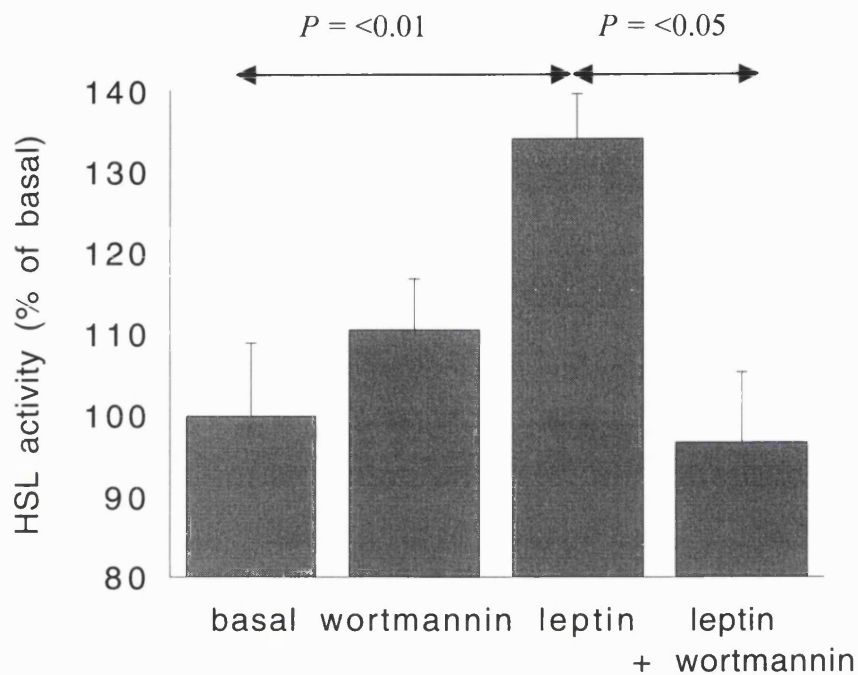


Figure 5.8. Leptin stimulation of HSL activity is blocked by wortmannin in macrophages

J774.2 macrophages were pre-incubated for 10 minutes with 100nM wortmannin, where indicated, prior to stimulation with 3nM leptin for 15 minutes. Neutral cholesterol esterase activity was measured in anti-HSL immunoprecipitates.. Data represents the mean ($\pm$ SEM) of four independent determinations, each performed in at least duplicate.

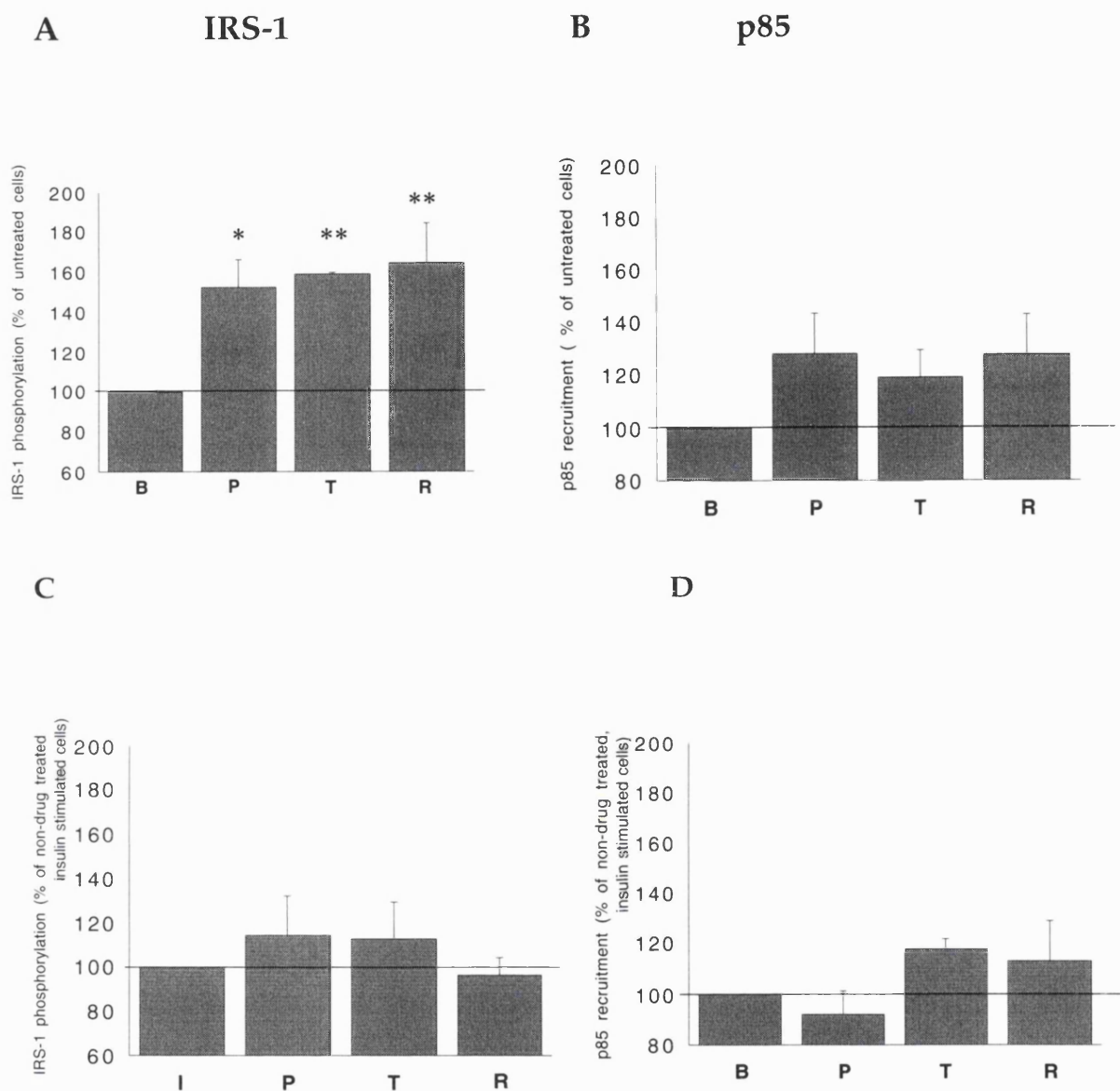


Figure 5.9. Thiazolidinediones increase basal, but not insulin-stimulated IRS-1 and PI 3-kinase recruitment in adipocytes

3T3 L-1 adipocytes were treated for four hours with 10 μ M troglitazone (T) /pioglitazone (P) or 1 μ M rosiglitazone (R) drug before anti-phosphotyrosine immunoprecipitation and blotting for IRS-1 protein (A, C) and p85 subunit of PI 3-kinase (B,D). Results are expressed as percentage of the recruitment of proteins in basal (A,B) and insulin stimulated (C, D), non-drug treated cells. Data represents the mean ($\pm$ SEM) of four independent determinations, each carried out in at least duplicate.

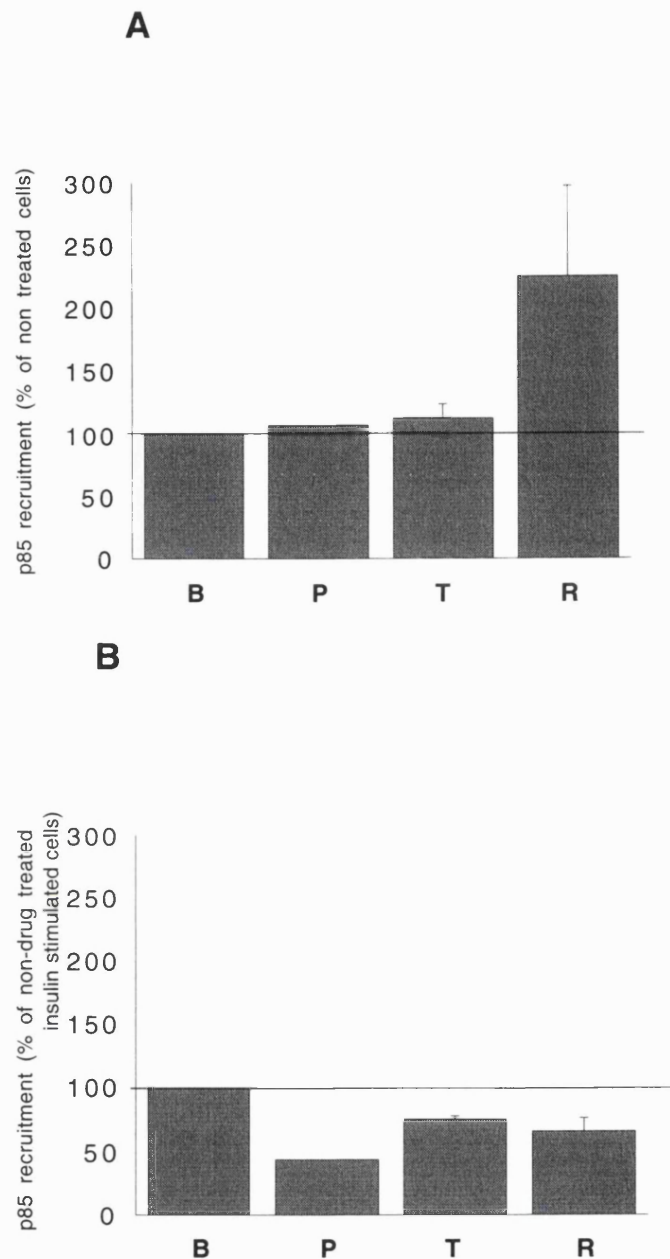
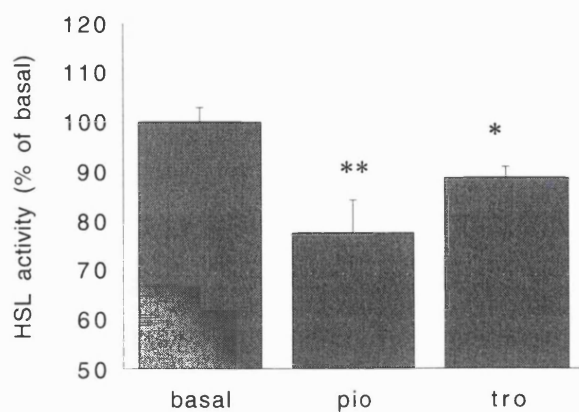
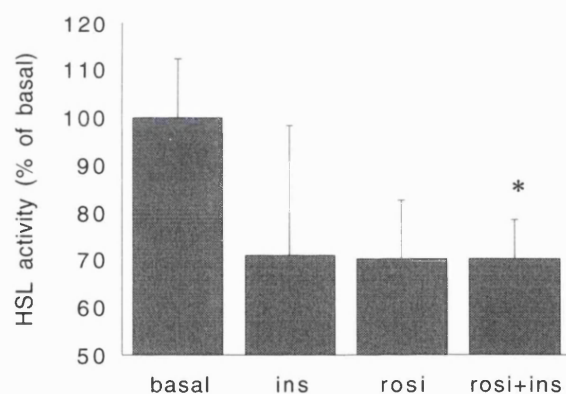


Figure 5.10. Rosiglitazone increases basal PI 3-kinase recruitment in macrophages

J774.2 macrophages were treated for four hours with 10 μ M troglitazone (T) /pioglitazone (P) or 1 μ M rosiglitazone (R) drug before anti-phosphotyrosine immunoprecipitation and blotting the p85 subunit of PI 3-kinase. Results are expressed as percentage of the recruitment of protein in basal (A) and insulin stimulated (B), non-drug treated cells. Data represents the mean ($\pm$ SEM) of four independent determinations, each carried out in at least duplicate.

A ADIPOCYTES**B MACROPHAGES****Figure 5.12. Effect of thiazolidine treatment on HSL activity**

3T3 L-1 adipocytes were treated with 10 μ M troglitazone/pioglitazone (**A**) and J774.2 macrophages (**B**) with 1 μ M rosiglitazone, for four hours. HSL was immunoprecipitated from cell lysates and nCE activity was determined. Data represents the mean ($\pm$ SEM) of two independent determinations, each carried out in at least duplicate. ** P = <0.01, * P = <0.05 compared to basal value.

5.3.3 PDE 3B phosphorylation by PKB

At the commencement of this work it was established that insulin induced phosphorylation of PDE3B was involved in the antilipolytic action of insulin in adipocytes (Rahn et al., 1996). However, the insulin stimulated kinase, downstream of PI 3-kinase, responsible for PDE3B phosphorylation had not been identified. Protein kinase B (PKB) was a likely candidate, especially considering a PKB consensus sequence (RxRxxS) is present in the PDE 3B primary structure, close to Ser302 which is phosphorylated by A-kinase. Therefore *in vitro* experiments were carried out using recombinant PDE3B and catalytically active PKB to determine if PKB could phosphorylate this phosphodiesterase. As expected PKB caused phosphorylation of the recombinant PDE3B seen at 135kDa (Figure 5.13), which is the molecular weight at which PDE3B is observed by western blotting 3T3 L-1 adipocyte lysates (see figure 5.14). A-kinase was assayed as a control and also caused phosphorylation of PDE3B. Having determined that PKB could phosphorylate recombinant PDE3B, peptides were synthesised to test the ability of PKB to phosphorylate two adjacent serine residues within the putative PKB phosphorylation site. Again, as a control a peptide was synthesised corresponding to the A-kinase consensus sequence. The data in figure 5.15 represents results from a single experiment and shows that peptides containing either serine, with the other mutated to an alanine can be phosphorylated by PKB. Soon after these experiments were initiated Kitamura et al, published data confirming that PKB phosphorylated the serine corresponding to our lo2 peptide (Kitamura et al., 1999). However they claim that mutation of this serine to an alanine prevented phosphorylation in response to insulin in intact cells or by PKB *in vitro*, whereas

our data using synthetic peptides would suggest that even when this serine is mutated to an alanine PKB can phosphorylate the adjacent serine. It can be seen in Figure 5.15 that we also tested the ability of p110 α protein kinase activity to phosphorylate Ser302, as it has been claimed that this site is phosphorylated in response to both insulin and A-kinase activation . No p110 α phosphorylation of the lo3 peptide, containing Ser302 was observed. A potentially important finding in the course of these studies is that PDE3B is undetectable in J774.2 cells (figure 5.14), which means that PDE3B is not likely to be involved in HSL regulation in these macrophages.

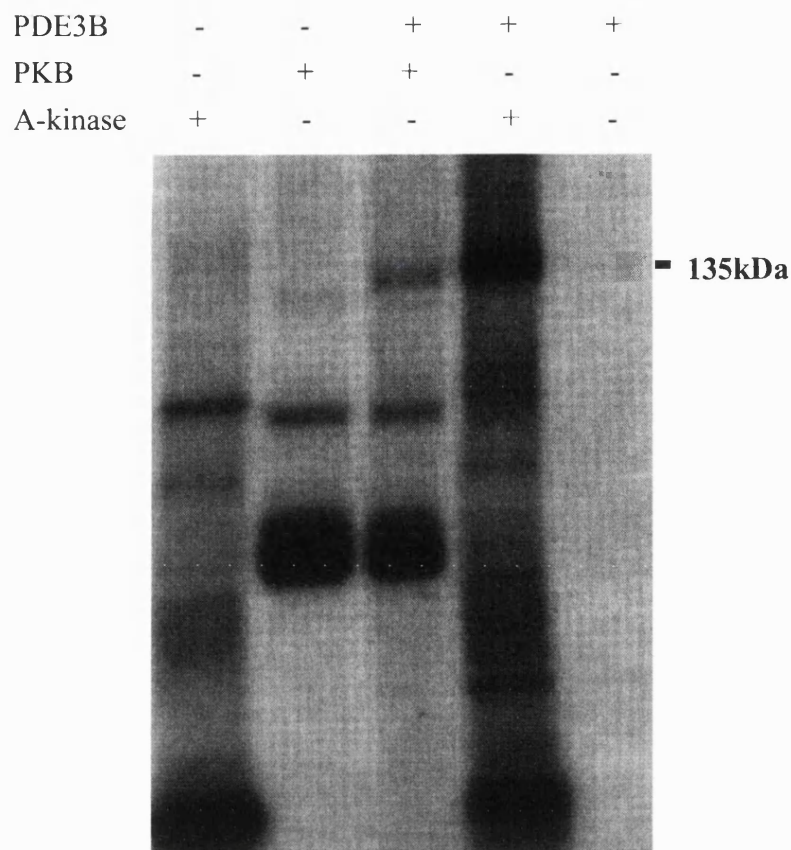


Figure 5.13. Protein kinase B and cAMP-dependent protein kinase phosphorylate PDE3B in vitro.

Recombinant PDE3B was incubated with catalytically active PKB and [$\gamma^{32}\text{P}$]ATP for 20 minutes (see 2.2.5.1, a). The catalytic subunit of cAMP dependent protein kinase was also assayed as a control. The SDS polyacrylamide gel was dried and visualised with a phosphoimager. Phosphorylated PDE3B can be seen as a band at 135kDa.

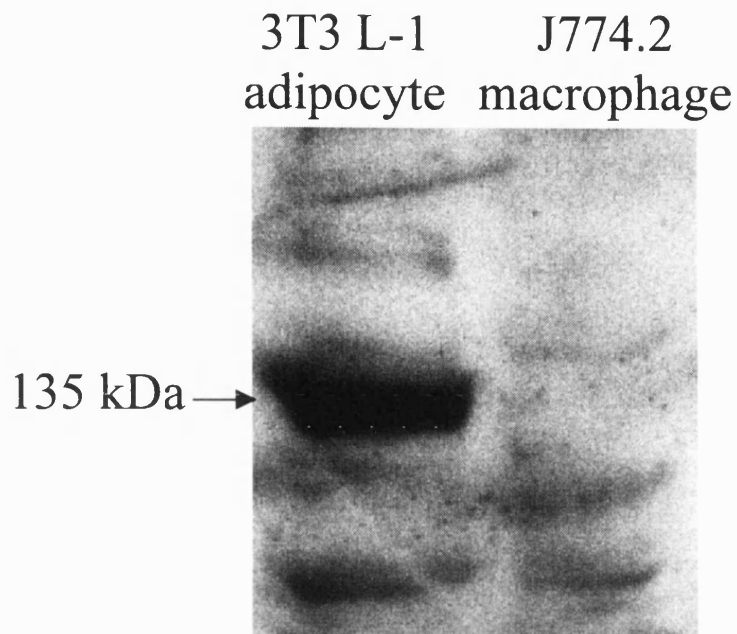


Figure 5.14. PDE3B is present in adipocytes but not macrophages

Unstimulated 3T3-L-1 adipocytes and J774.2 macrophages were lysed. Aliquots of lysates containing 250µg of protein were subject to SDS PAGE and western blotted with anti-phosphodiesterase 3B antibody. Proteins were visualised with a phosphoimager.

PKB consensus RxRxxS

Peptides:

Lo1 -IRPRRRAS²⁸⁰CV-
 Lo2 -IRPRRRS²⁷⁹ACV-
 Lo3 -KIFRRPS³⁰²LPC-

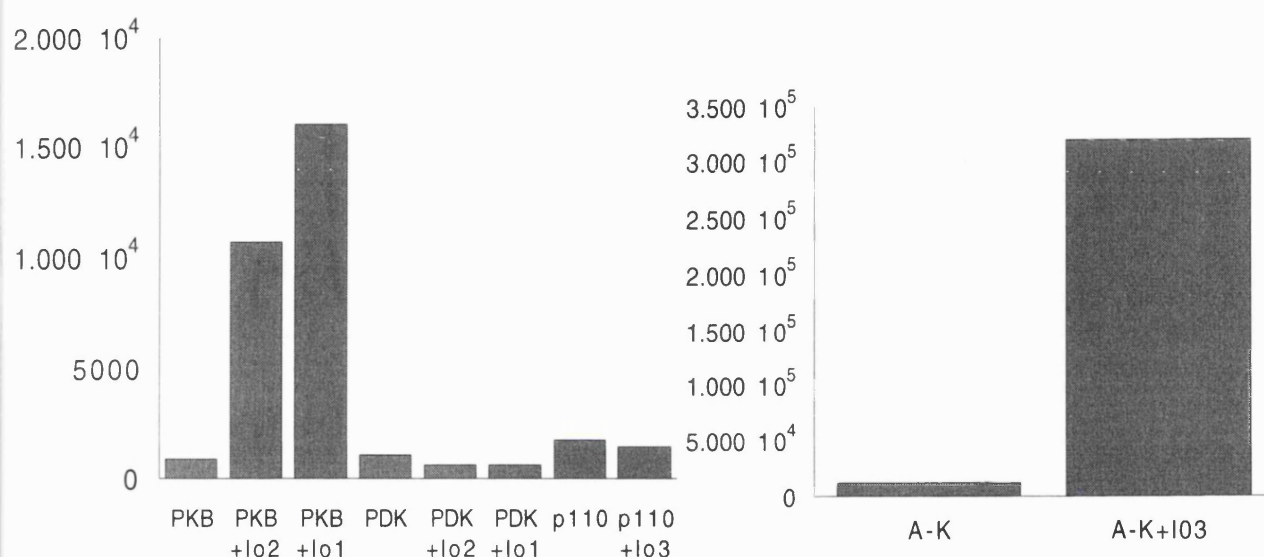


Figure 5.15. phosphorylation of synthetic peptides by PKB

Two synthetic peptides were synthesised corresponding to potential phosphorylation sites within (Lo2) or near (Lo1) a motif (RXRXXS) that is preferentially phosphorylated by PKB, in the rat PDE3B sequence. Also a peptide was synthesised corresponding to the formerly identified A-kinase consensus sequence. Recombinant catalytically active PKB, p110 α and PDK were used in a phosphocellulose cation-exchange paper protein kinase assay (see 2.2.5.1, b). Values are represented as CPM. Background values for the peptides alone have been subtracted from the above values. This experiment was carried out before this site was confirmed to be that which was phosphorylated by PKB.

5.4 Discussion

Chapters 3 and 4 provided evidence that the regulation of HSL by leptin and insulin in macrophages could at least in part explain why cholesterol ester deposition and foam cell formation is accelerated in insulin resistance and diabetes. Data presented in this chapter is a summary of experiments performed throughout the course of this work to characterise the signalling mechanism by which leptin and insulin might regulate HSL in macrophages.

5.4.1 *Leptin signalling in J774.2 macrophages*

Evidence indicates that leptin only signals inside cells where the long form of the leptin receptor is expressed (Baumann et al., 1996) and in many peripheral tissues OB-RL is absent or present in very low amounts compared to the short form (Ghilardi et al., 1996; Tartaglia, 1997). Despite this there are several reports that leptin has direct effects on several hematopoietic cell types and that these cells can express OB-R_L (Bennet et al., 1996; Gainsford et al., 1996; Lord et al., 1998; Martin-Romero et al., 2000; Santos-Alvarez et al., 1999). However this has not previously been demonstrated in cell culture models. In the current study we demonstrate that OB-R_L is expressed at levels clearly detectable by western blotting in J774 macrophages. Furthermore we demonstrate increased tyrosine phosphorylation of OB-R_L in response to low nM amounts of leptin, indicating that these receptors are active in the macrophages. It is well documented that the Janus kinases (JAKs) are associated with the leptin receptor and, upon leptin binding, autophosphorylate and tyrosine phosphorylate the receptor (Baumann et al., 1996). We were also able to demonstrate this tyrosine phosphorylation of

JAK-2 in leptin-stimulated J774.2 macrophages. The time frame of tyrosine phosphorylation of OB-R_L and JAK-2 matches well with the timecourse for the maximal effect of leptin on HSL activity (see figure 4.1). The J774.2 cell line is therefore a novel cell culture model for studying leptin signalling

The acute stimulation of HSL activity by leptin was completely blocked by 100nM wortmannin, suggesting this effect of leptin requires PI 3-kinase. We investigated PI 3-kinase activation by leptin in macrophages and found that there was increased recruitment to anti-phosphotyrosine IPs and increased PI 3-kinase lipid kinase activity in vitro. Therefore this data is consistent with the stimulation of PI 3-kinase being involved in the pathway by which leptin regulates HSL. The mechanism by which leptin activates PI 3-kinase in J774 cells was not determined. However, leptin-induced activation of PI 3-kinase has been shown previously in C₂C₁₂ myotubes, where it was established that JAK-2 phosphorylates IRS-2 which then recruits PI 3-kinase (Kellerer et al., 1997). It is possible that this same signalling mechanism is being used in macrophages. In other cell types PKC (Berti et al., 1997), ERK-1 /2 (Berti and Gammeltoft, 1999; Bouloumie et al., 1999) and the JNK-SAPK pathway (Berti and Gammeltoft, 1999; Bouloumie et al., 1999) have been shown to be activated by leptin. It will be of interest to determine whether these are also activated by leptin in macrophages.

5.4.2 Role of PI 3-kinase and PDE3B in the regulation of HSL by insulin and leptin

In contrast to these studies with leptin the insulin-induced downregulation of HSL activity was not blocked by wortmannin. This was surprising considering

that it is well established that the same effect of insulin on HSL activity in adipocytes is PI 3-kinase dependent. We have verified that insulin is able to activate PI 3-kinase in these cells, albeit weakly. However the data suggests that this is not the mechanism used in macrophages to exert acute effects on HSL activity.

Studies in adipocytes show that insulin reduces phosphorylation of HSL via activation of PDE3B which hydrolyses cAMP, and thus reduces the activity of A-kinase. Leptin has also been shown to activate PDE3B in pancreatic β cells (Zhao et al., 1998). Therefore, as both leptin and insulin activate PI 3-kinase in macrophages, we might expect insulin and leptin to stimulate PDE3B and inhibit HSL activity in a wortmannin-sensitive manner, which is not the case. The reason for this could lie in our observation that no PDE3B was detected in J774.2 macrophage lysates, but was clearly detected in 3T3 L-1 adipocytes. This could mean that insulin is regulating HSL in J774.2 cells by mechanisms which do not involve PDE3B, or in fact changes in cAMP levels. Such mechanisms have been reported in adipocytes (Londos et al., 1985), where insulin causes A-kinase-independent inhibition of lipolysis. Londos et al suggest that insulin either activates an endogenous inhibitor of the lipase or activates phosphatases to reduce HSL phosphorylation and activation .

Due to the lack of PDE 3B in macrophages, it is tempting to speculate that other PDE sub-types are responsible for the insulin-induced reduction in HSL activity. Indeed other phosphodiesterase sub-types PDE 4 and PDE 1 have previously been identified in human monocyte-derived macrophages (Gantner et al., 1997). In FDCP2 myeloid cells IL4 activates PDE 4 by mechanisms using IRS, PI 3-

kinase and MAP kinase (Ahmed et al., 1999). This suggests that insulin could also regulate this isoform in macrophages, although this remains to be established. While insulins' effect on HSL activity is not blocked by wortmannin, ruling out PI 3-kinase involvement, it could potentially still act via the MAP kinase pathway. Interestingly PDE3 and PDE4 have both been shown to be involved in inhibition of FFA release from fat cells (Harmelen et al., 1999). However, the signalling pathway preceding PDE3, but not PDE4, involves PI 3-kinase. The fact that different PDEs have been shown to be regulated via different mechanisms to carry out similar functions could help explain why we are observing the same effect on HSL activity in macrophages and adipocytes, but that PI 3-kinase is not involved in the macrophages.

It is also not clear whether the leptin-induced increase in HSL activity in macrophages is due to a rise in cAMP levels. Previous reports on the effect of leptin on cellular cAMP levels differ depending on cell type. In chromaffin cells leptin causes an increase in cAMP levels, but the mechanism for this was not investigated (Takekoshi et al., 1999). In contrast, leptin has been shown to decrease cAMP levels in islets by activating PDE3B (Zhao et al., 1998). A possible explanation for our observed effects on HSL is that in the absence of PDE3B, such as in J774 cells, leptin will act to increase cAMP resulting in increased HSL phosphorylation and activation.

5.4.3 HSL translocation

Phosphorylation of HSL clearly plays an important role in the regulation of its catalytic activity, but the magnitude of this activation is small compared to the total increase in lipolysis, suggesting that other mechanisms may be involved in

regulating HSL function. At least part of this could be explained by the interaction of the lipase with its substrate and associated intracellular membranes. Redistribution of triglyceride lipase activity is observed upon lipolytic stimulation in adipocytes (Egan et al., 1992; Hirsch and Rosen, 1984). Proteins such as perilipin (Greenberg et al., 1991; Mooney and Bordwell, 1991) and lipotransin (Syu and Saltiel, 1999) have been identified at the surface of the lipid droplet and are thought to have a role in regulating adipocyte lipolysis. Lipotransin is thought to act as a docking protein which transports HSL to the droplet then dissociates allowing direct association of HSL with the droplet surface (Syu and Saltiel, 1999). Perilipin, on the other hand is a protein which is thought to compete with HSL and must be removed for stimulation of lipolysis (Blanchette-Mackie et al., 1995; Greenberg et al., 1991; Souza et al., 1998). Perilipin is not detected in loaded J774 macrophages, but similar processes may occur in macrophages and prove to be an important feature of regulation of nCE activity. Understanding these processes could be vital for determining why there is excessive abnormal lipid deposition in cells, such as foam cells in atherosclerotic plaques.

5.4.4 Effect of thiazolidinediones in macrophages and adipocytes

The thiazolidinedione (TZD) class of insulin-sensitising drugs decrease plasma glucose, insulin and TAG levels in insulin-resistant subjects. The effect of TZDs on insulin signalling remains unclear. Several reports show increases in the tyrosine phosphorylation state of IRS-1 and the insulin receptor (Maegawa et al., 1999; Young et al., 1995), whilst other groups have reported no change (Hulin et al., 1996; Zhang et al., 1994). In adipocytes we found that several TZDs increased basal IRS-1 phosphorylation and p85 recruitment but the insulin-induced

increase in phosphorylation was not further increased by TZD treatment. The same treatment led to a reduction in basal HSL activity. These results could explain why experiments carried out in animal models show a reduction in circulating TAG and FFA levels even in insulin-deficient streptozotocin diabetic rats, suggesting that this is not due to sensitisation to insulin. Rosiglitazone was the only TZD tested that appeared to have an effect on PI 3-kinase recruitment in macrophages and cause a reduction in HSL activity. In these studies drug treatment was for four hours, therefore any effects seen could be due to altered expression of certain genes. In macrophages we would not expect the reduction in HSL activity to be due to the increased PI 3-kinase recruitment, as insulin-induced reduction in HSL activity is not blocked by wortmannin. We have shown previously that there is differential regulation of HSL expression in macrophages and adipocytes (see chapter 3), so it will be important to determine if these drugs are altering expression levels of hormone-sensitive lipase in these two cell types. Whatever the mechanism is, the reduction in HSL activity due to TZD treatment in macrophages would result in reduced CE hydrolysis, which could potentially contribute to foam cell formation.

PPAR γ has recently been identified as the major functional receptor for the thiazolidinediones (Spiegelman, 1998) and is expressed in macrophages (Vamecq and Latruffe, 2000). The idea of TZD treatment contributing to atherosclerosis has arisen in a number of other studies which have shown PPAR γ activation to cause effects which would have an unfavourable impact on atherosclerosis. For example, it has been shown that PPAR γ promotes monocyte/macrophage differentiation and uptake of oxidised LDL (Nagy et al.,

1998; Ricote et al., 1998; Tontonoz et al., 1998), as well as increasing adhesion molecule expression of human vascular endothelium (Chen et al., 1999). It should be noted that macrophage uptake of oxLDL is designed to eliminate harmful extracellular debris and only turns pathological when too much lipid accumulates and the cell becomes trapped in the lesion. Therefore future studies should address whether PPAR inhibition in foam cells will limit oxLDL accumulation. However to consider the influence of PPAR γ ligands on the risk for atherosclerosis the multiple effects on systemic lipid and glucose metabolism must be taken into account, in addition to their effects on vascular cells and macrophages. PPAR γ may be proatherogenic in some contexts and protective in others. For example the PPAR γ dependent regulation of the scavenger receptor, CD36, is not restricted to macrophages. Expression of CD36 is also increased in adipose tissue and skeletal muscle (Tontonoz and Nagy, 1999). In contrast to the pro-atherogenic effect in macrophages in the other tissues it functions as a fatty acid transporter and may help control blood lipid levels. Also studies presented in chapters 3 and 4 suggest that high glucose levels could be a major factor influencing CE accumulation in macrophages. Therefore the TZD-induced improvement in glucose homeostasis in addition to lowering lipids may give a net anti-atherogenic effect.

5.5 Conclusion

The mechanism by which insulin stimulates a reduction in HSL activity is different in macrophages and adipocytes. Although PI 3-kinase is activated by insulin in J774.2 macrophages, the signalling pathway for HSL regulation does not depend on PI 3-kinase or PDE3B activity. In contrast, the increase in HSL

activity caused by leptin, does require PI 3-kinase activity. OB-R_L is easily detectable and activated in J774.2 macrophages. Therefore a novel cell culture model for leptin signalling has been identified.

6. General Discussion

Atherosclerosis-related disorders are the principal cause of death in the western world. Insulin resistance and type 2 diabetes are well recognised risk factors for coronary heart disease and type 2 diabetics have a 2-3 fold increased risk of developing macrovascular heart disease (Steiner, 1994; Stout, 1993). In hyperinsulinemic individuals who have not yet developed type 2 diabetes there is an association between insulin resistance, obesity and high circulating levels of triglycerides and glucose (Steiner, 1994). This cluster of abnormalities predispose to atherosclerosis and cardiovascular disease. However, the molecular mechanisms affected by these factors which could account for accelerated plaque formation have not been established. Understanding why these macrovascular complications occur in diabetes will lead to better methods of preventing premature morbidity and mortality in diabetes.

The earliest lesion of atherosclerosis is characterised by an accumulation of lipid laden macrophages, known as foam cells, in the sub-endothelium (Ross and Glomset, 1976). The bulk of the lipid in macrophages is in the form of cholesterol esters (CE) (Ross, 1993; Stary et al., 1994). In the cytosol of macrophages a cycle exists between free and esterified cholesterol and the balance of this cycle is a major determinant of the level of intracellular CEs in foam cells (Brown et al., 1980). CEs must be broken down to cholesterol before they can be excreted from the cell.

The role of HSL as the principle nCE in most cell types is highlighted by the very recent observation that nCE activity cannot be detected in most cells derived from mice in which both copies of the HSL gene have been deleted (Osuga et al., 2000). One surprising finding of these studies is that nCE activity was still found in peritoneal macrophages from HSL knockout mice (Osuga et al., 2000). This contrasts with the strong evidence that HSL is the major enzyme responsible for the neutral cholesterol esterase activity in macrophages, required for hydrolysis of CE to liberate free cholesterol (Khoo et al., 1981; Reue et al., 1997; Small et al., 1989; Hart et al., 2000). On balance the fact that HSL clearly is able to act as a nCE and is present in macrophages, means that it is highly likely that proper regulation of this enzyme could be vital for maintaining cholesterol homeostasis and the prevention of foam cell formation. For this reason, the work presented in this thesis has investigated how factors associated with insulin resistance and diabetes affect the regulation of HSL and lipid homeostasis in macrophages.

A hallmark of insulin resistance and diabetes is high levels of plasma insulin and glucose. In adipocytes insulin is known to exert an antilipolytic function by downregulating HSL activity, but at the commencement of this work it was not known if insulin also affected HSL activity in macrophages. Obesity and high circulating levels of leptin are also commonly associated with diabetes. Leptin is known to alter lipid homeostasis in other cell types, such as B cells and adipocytes, but again its role in macrophages had not been determined previously. Therefore the experiments presented here have focussed on the effects of these two agents in macrophages under different glucose conditions.

Almost all previous studies on HSL regulation have been carried out in adipocytes. Therefore, many of the experiments with insulin were carried out in parallel in 3T3 L-1 adipocytes, where we generally know what effect to expect. In adipocytes HSL is activated by agents that raise cAMP levels and insulin counteracts this by causing a reduction in cAMP. Experiments presented in chapter 3 demonstrated that insulin also counters maximally stimulated HSL activity, stimulated by cAMP analogues, in macrophages. We have also demonstrated that insulin acutely causes a reduction in basal HSL activity similar to that in adipocytes, but is much more transient. The magnitude of the reduction in maximally stimulated HSL activity in macrophages is approximately the same as the reduction of basal HSL activity, caused by insulin. Therefore it is unclear if insulin is counteracting the effects of cAMP, by reducing cAMP levels, or if this is the same mechanism employed by insulin to reduce basal HSL activity. In an effort to establish this it would be useful to determine how intracellular cAMP levels change upon insulin stimulation in macrophages. Further studies presented in chapter 5 revealed that insulin reduces HSL activity in macrophages by a completely different mechanism to the well-characterised signalling pathway in adipocytes (see chapter 1). This is probably due to the fact that these two cell types have different functions and are therefore likely to express different proteins. An example of this can be seen in chapter 5, where western blotting for PDE3B reveals that this protein is completely absent from the macrophages. In adipocytes, HSL is activated by A-kinase phosphorylation at several sites and insulin causes net dephosphorylation of HSL primarily by lowering cAMP levels and A-kinase activity. In macrophages it has also been shown that increased nCE activity

corresponds with increased phosphorylation of HSL. However our data suggests another mechanism is employed by insulin for HSL dephosphorylation in macrophages, which does not involve PI 3-kinase or PDE3B. Activation of phosphatases is a possible explanation, but further studies would be required to address this issue.

In addition to the acute effects of insulin, differential effects on HSL expression and activity were observed upon chronic incubation with insulin. Incubation with high glucose and insulin caused a significant increase in HSL activity/expression in adipocytes and decrease in activity/expression in macrophages. It would be fascinating to determine the different mechanisms of HSL gene regulation in the two cell types, which unfortunately was beyond the scope of this investigation. Both increased serum FFA levels and atherosclerosis are observed in insulin resistance and diabetes. We can hypothesise that these abnormalities observed in diabetes may be partly due to effects on HSL, as the predicted physiological effects due to these changes in HSL expression/activity would be increased FFA in the circulation, due to increased HSL activity in adipose tissue, and increased atherogenesis due to increased lipid accumulation in macrophages. This is further supported by our finding that when J774 macrophages are incubated with modified LDL and labelled oleate more cholesterol oleate accumulates in the cells maintained in media containing high glucose and insulin. It would be worth extending these studies into human macrophages to determine if the effects observed in this murine cell culture line occurs in human cells. Ultimately comparisons of HSL activity and expression

and lipid loading could be made in human diabetic versus non-diabetic macrophages.

Recent studies have shown that leptin can have profound effects on macrophage function and it is emerging that leptin itself is an immune modulator. Leptin upregulates the inflammatory immune response by increasing macrophage phagocytosis and expression of pro-inflammatory cytokines. This is an essential response for protection against infection, however it is vital that this regulation of the inflammatory response is tightly controlled. As mentioned in chapter one, atherosclerosis is now thought to be a chronic inflammatory condition, and inappropriate upregulation of the immune response would exacerbate this process. This is particularly relevant in obesity where serum leptin levels are elevated and there is an increased susceptibility to vascular disease. Previously, cultured macrophages from C57BL/6 mice and GM-CSF transgenic mice have shown high levels of labelling with ^{125}I -leptin, indicating the presence of leptin receptors. However, this binding represents both long and short forms of the leptin receptor. It is thought that only the long form of the leptin receptor (OB-R_L) is the functional form capable of intracellular signalling and previous studies have not determined whether this was expressed in macrophages. Indeed no cultured cell lines have previously been described which express high levels of OB-R_L. However, OB-R_L was expressed to high levels in the J774.2 macrophage-like cell culture line and is clearly tyrosine phosphorylated in response to leptin stimulation in these cells. The J774.2 cell line will therefore prove to be extremely useful for future studies to investigate leptin signalling. Surprisingly OB-R_L is highly tyrosine phosphorylated in unstimulated, serum

starved J774.2 cells. A possible explanation for this is that these macrophages are capable of producing leptin. There is no evidence to date to suggest that this is the case, but as leptin clearly plays a role in the immune response the possibility that immune cells may be able to express this protein is intriguing.

Work presented in chapter 4 demonstrates a novel function of leptin for HSL regulation in macrophages. Leptin acutely increases HSL activity. This would be expected to increase CE breakdown in foam cells which would be beneficial for delaying the atherosclerotic process. However, when J774.2 macrophages were incubated with modified LDL and labelled oleate for 24 hours CE accumulation was similar to that observed with insulin. Leptin and low glucose media reduced intracellular CE levels, but leptin in combination with 20mM glucose significantly increased CE accumulation. The increase in CE accumulation reflects exactly the reduction in HSL activity observed under these conditions, so it is possible that these two effects of leptin and glucose may be associated. The fact that the change in HSL activity/expression and CE accumulation was the same upon chronic incubation with insulin and leptin suggests that these two agents could be utilising the same signalling machinery within the macrophage to chronically regulate CE homeostasis. Both leptin and insulin show beneficial effects on CE accumulation in low glucose conditions. Therefore it seems that glucose, while it does not cause increased lipid accumulation alone, is a prominent factor in the pathological effects of insulin and leptin on CE accumulation. Although the effects on lipid accumulation are relatively small, cells were only incubated for 24 hours. Insulin resistance, on the other hand can be manifesting itself undetected for years prior to the development of full blown

type 2 diabetes, therefore our observations could be physiologically significant. The change from the lipid lowering anti-atherogenic effects of low glucose to the lipid accumulating pro-atherogenic effects of insulin and leptin with high glucose, could have important implications for insulin-resistance and obesity related atherosclerosis. Therefore it is essential to determine the underlying mechanism for regulating HSL expression and how this is altered under conditions of high glucose. The HSL promoter contains cis acting elements for peroxisome proliferator-activated receptors (PPARs) and sterol regulatory element binding proteins (SREBPs) , so it is possible that these factors are involved (as discussed in chapter 3).

As mentioned in chapter 4, peroxisome proliferator-activated receptor- γ (PPAR- γ) is abundant in macrophage foam cells. PPAR- γ belongs to a subfamily of nuclear receptors involved in the control of various aspects of lipid metabolism. The ligands for PPAR- γ are diverse and include oxidised LDL particles such as 9- and 13-HODE and the synthetic thiazolidinediones. Because TZDs improve insulin sensitivity, glucose tolerance and the lipidemic profile in type 2 diabetic patients, it is generally thought that activation of PPAR- γ is beneficial . However it is known that PPAR- γ in macrophages promotes scavenger receptor expression and oxLDL uptake . Despite this the drugs are used widely in the treatment of type 2 diabetes and do not cause vascular complications. A possible explanation for this is that the normalisation of glucose and lipid levels compensates for these effects on foam cell formation. Interestingly, it has recently been reported that there is improved insulin sensitivity in heterozygous PPAR- γ deficient mice . Therefore it would seem that contrary to enhancing

insulin sensitivity, PPAR- γ , along with its natural ligands, might serve to reduce insulin action. If this were the case, the decreased expression of PPAR- γ receptors in these heterozygous mice would heighten insulin sensitivity. We tend to think of TZDs as pure activators of PPAR- γ , when in reality most have been shown to be partial agonists, which also makes them partial antagonists, which can compete with the natural full agonists. Thus it is possible TZDs could act to delay foam cell formation by preventing PPAR- γ -induced increase in scavenger receptor expression. It would be interesting to determine how TZD incubation would affect our lipid loading experiments to give an insight into PPAR- γ involvement in the increased accumulation.

In addition to the A-kinase-induced phosphorylation sites on HSL a 'basal' phosphorylation site (Ser-565) is phosphorylated by several enzymes including, AMP-activated protein kinase, Ca²⁺ / calmodulin-dependent kinase II and glycogen synthase kinase-4, but not by A-kinase (Garton et al., 1989; Yeaman et al., 1994). The identity of the protein kinase that phosphorylates HSL in intact cells at Ser565 has not been determined. Ser565 and Ser563 are mutually exclusive, thus, AMP kinase, or another kinase, might act as an inhibitor of HSL activity by blocking A-kinase phosphorylation (Garton and Yeaman, 1990). This hypothesis is supported by the finding that aminomidazole carboxamide ribonucleotide (AICAR), which leads to activation of AMP kinase, partially inhibits lipolysis in adipocytes (Sullivan et al., 1994). However, the effects of AICAR on HSL phosphorylation have not been determined. The importance of Ser-565 in the acute insulin and leptin response in macrophages may merit further investigation. Regulation of AMP kinase may also be of interest in the

chronic effect of these agents. AMP kinase is activated, under conditions of elevated AMP, due to phosphorylation by AMP kinase kinase and is downregulated by phosphatases. Evidence suggests that AMP kinase has a role in inactivating fatty acid synthesis and cholesterol synthesis and stimulating glucose uptake and oxidation (Hardie and Carling, 1997). AMP kinase can respond to the level of glucose in the medium and act as a cellular fuel gauge detecting changes in the energy charge of the cell (Hardie and Carling, 1997). Activation of fatty acid synthase gene expression by high glucose levels has been shown to be markedly reduced by incubation of cells with okadaic acid, an inhibitor of protein phosphatases. A similar reduction was obtained by incubation with AICAR. This indicates that AMP kinase can inhibit glucose activated gene expression, therefore any change in activation of this enzyme in our studies could be important. It will be of interest to determine the effect of AICAR on acute and chronic HSL activity.

In summary, cardiovascular disease is the most common complication of non-insulin-dependent diabetes mellitus (NIDDM) and is the principal cause of death in NIDDM patients. Therefore it is critical that we understand the molecular mechanisms underlying the cause of accelerated atherosclerosis in diabetes. The work presented here has identified a novel role of insulin and leptin for HSL regulation and lipid homeostasis in macrophages. This could have important implications for foam cell formation and while it is unlikely to be the sole basis for increased atherogenesis, these findings will provide some useful insights towards mechanisms by which factors associated with insulin resistance could be contributing to atherogenesis.

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Abbreviations

ACAT	acylCoA cholesteryl acyltransferase
AGE	advanced glycation endproduct
A-kinase	cAMP dependent protein kinase
Apo B	apolipoprotein B
CAD	coronary artery disease
cAMP	cyclic adenosine monophosphate
CE	cholesterol ester
ERK-2	extracellular signal regulated kinase
FFA	free fatty acid
HDL	high density lipoprotein
IGT	impaired glucose tolerance
IRS	insulin receptor substrate
JAK-2	janus activated kinase-2
LDL	low density lipoprotein
LPL	lipoprotein lipase
MAP kinase	mitogen-activated protein kinase
MCP-1	monocyte chemotactic protein-1
nCE	neutral cholesterol esterase
OB-R	leptin receptor
OLDL	modified/oxidised LDL
PDE	phosphodiesterase
PKC	protein kinase C
PI 3-kinase	phosphatidylinositol 3-kinase
PIP ₃	phosphatidylinositol 3,4,5 trisphosphate
PPAR	peroxisome-proliferator activated receptor
SH2	Src homology 2 domain
STAT	signal transducers and activators of transcription
TAG	triglyceride
TNF α	tumour necrosis factor α
TZD	thiazolidinedione
VLDL	very low density lipoprotein