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The Role of Copper in Hypoxia Inducible Factor – 1 Expression

A Potential Mechanism of Chemical Hypoxia Preconditioning

For Ischaemia Reperfusion Injury

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Abbreviations

ARNT	Aryl Hydrocarbon Receptor Nuclear Translocator
BSA	Bovine Serum Albumin
CHO	Chinese Hamster Ovary
$CuCL_2$	Copper Chloride
DSF	Desferroxamine
EPO	Erythropoetin
Fe	Iron
G6P	Glucose-6-phosphatase
GK	Glucokinase
GSNO	S-nitrosoglutathione
HIF-1	Hypoxia Inducible Factor 1, two subunits HIF-1a & HIF-1b
HIF-1a	Hypoxia Inducible Factor 1 alpha
HIF-1b	Hypoxia Inducible Factor 1 beta, also called ARNT
HIF-2	Hypoxia Inducible Factor 2
HIF-3	Hypoxia Inducible Factor 3
HPC	Hypoxia Preconditioning
HBS	HIF-1 Binding Site
HRE	Hypoxic Responsive Element

IRI	Ischaemia Reperfusion Injury
iNOS.	Inducible Nitric Oxide Synthetase
MTT	Thiazolyl Blue; 3-[4, 5-Dimethylthiazol–2-yl]-2,5-diphenyltetrazolium bromide
NO	Nitric Oxide
NSS	Normal Sheep Serum
ODDD	Oxygen Dependent Degradation Domain
PHD (1-3).	Prolene HyDroxylase (1-3)
PBS	Phosphate Buffered Saline
PCK	Phosphophenolpyruvate
PK	Pyruvate kinase
RTPCR	Reverse Transcription Polymerised Chain Reaction
ROS	Reactive Oxygen Species
SOD	Super Oxide Dismutase
TBS	Tris Buffered Saline
TCM	Tissue Culture Media
Tet	N,N-Bis(2-aminoethyl)-1,3-propanedamine
Trien	Trithylenetetramine tetrahydrochloride
Bath	2,9-Diphenyl-1,10-phenanthrolinedisulfonic acid
VEGF	Vascular Endothelial Growth Factor
Zn	Zinc

Abstract

Introduction: Hypoxia Preconditioning (HPC) involves a short period of hypoxia prior to a more sustained ischaemia. This method reduces Ischaemia Reperfusion Injury (IRI) in vascular surgery. The molecular response to hypoxia and HPC is mediated by the transcription factor Hypoxia Inducible Factor 1 (HIF-1). HIF-1 activation involves cell signalling by Reactive Oxygen Species (ROS), which are normally buffered by metalloenzymes, in particular by the transition metal ion copper. We wished to assess how altering intracellular copper concentration would affect HIF-1 expression. This may provide a mechanism to artificially induce HPC and modify IRI in vivo.

Methods: The effect of altering copper levels on HIF-1 was assessed in cell culture, animal models and in human liver. HIF-1 activation was studied in HEP3B cell culture; HIF-1 nuclear protein by Western Blot, HIF-1 transcription activation by Northern Blot for VEGF mRNA and in a reporter construct. In vivo analysis for HIF-1 was performed in rodent models of copper excess and copper deficiency. In humans HIF-1 expression was assessed in patients with Wilson's disease and those with IRI following liver transplantation.

Results: Increased copper increased HIF-1 nuclear protein. Reduced copper reduced HIF-1 nuclear protein response to hypoxia. Increased copper increased VEGF mRNA. The reporter construct confirmed these findings. Animal models produced conflicting results that may have been due to confounding factors. Copper toxic biopsies from

patients with Wilson's disease showed no effect, this may reflect chronic copper exposure rather than an acute response. HIF-1 may be increased in IRI biopsies.

Conclusion: In cell culture intracellular copper concentration appears to affect the HIF-1 pathway of activation and response. In vivo analysis was less conclusive. Artificial control of HIF-1 may be feasible by altering intracellular copper balance

Chapter 1: Introduction

Summary of Chapter 1

This chapter reviews and summarises data in order to bring together key subjects that make this thesis; Ischaemia Reperfusion Injury (IRI), Hypoxia Preconditioning (HPC), Hypoxia Inducible Factor 1 (HIF-1), Reactive Oxygen Species (ROS) and Copper.

IRI is a common problem in vascular and transplant surgery. Cellular injury sustained during the hypoxic period of arterial occlusion or graft storage, is increased following reperfusion, with release of ROS and metabolic by-products in to the systemic circulation. This causes activation of the Systemic Inflammatory Response Syndrome (SIRS), with a negative impact on cardiac, respiratory and renal function. An established mechanism to reduce IRI is Hypoxic-preconditioning (HPC). HPC involves a short exposure of tissue to hypoxia, followed by reperfusion and recovery, before subsequent full exposure to hypoxic conditions. HPC has been proven experimentally and clinically to be effective. It reduces the effects of IRI in aortic surgery, and transplantation. HPC has two phases of response. First there is an initial response, characterised by release of reactive oxygen species (ROS), and later a molecular response characterised by increased gene transcription in response to the hypoxia. Whilst many aspects of the molecular response have been studied, and several pathways of gene activation identified, a key feature of HPC is the stimulus by hypoxia.

The molecular response of a cell to hypoxia is coordinated by the transcription factor HIF-1. HIF-1 is termed 'the master regulator of oxygen homeostasis'. In response to hypoxia HIF-1 initiates an array of genes that mediate a response that enable a cell to

adapt and survive in a hypoxic environment. Perhaps the most well known gene induced by HIF-1 is erythropoetin, which increases red blood cell production from the bone marrow. Other genes include VEGF, iNOS and control enzymes in glycolysis and gluconeogenisis that balance aerobic and anaerobic metabolism. HIF-1 has been identified as a key transcription factor involved in HPC and IRI.

In the last decade much research has concentrated on the control mechanisms of HIF-1, activation and identification, also oxygen sensing. Despite significant advances in the understanding of HIF-1 molecular biology, the exact method of how the cell senses oxygen concentrations, and communicates this to HIF-1 remains a subject of debate. ROS are proposed as being integrally involved in the oxygen sensing mechanism. ROS have traditionally been regarded as dangerous, their functions recognised as part of the mitochondria respiratory chain. Also they are involved in cellular defence associated with the neutrophil oxidative burst. However increasingly ROS are identified as signalling molecules in the cell and ROS have been shown to directly activate HIF-1 expression.

Under normal circumstances ROS are continually produced as a by-product of oxygen and cellular metabolism. They are tightly controlled in the cell by reduction to water or buffered by enzymes cabable of altering their own redox status. The key feature of theses redox enzymes is a transition metal ion core. This makes it possible for the enzymes to exist between two oxidative states and thus be able to buffer ROS. One of the main transition metal ions involved in cellular redox balance is copper. Copper has the ability to exist in two redox states, oxidized cupric Cu^{++} and reduced cuprous Cu^{+}.

Separate to the role of copper as a core ion involved in buffering ROS copper can independently create and catalyse the production of ROS by the Fenton reaction.

These features; the association of copper with ROS and the association of ROS with the oxygen-signalling pathway of HIF-1, suggest that copper may be involved in HIF-1 induction. If altering intracellular copper concentration affects HIF-1 induction, copper may have a role in mimicking HPC and thereby reducing IRI.

1.1.1. Ischaemia reperfusion & hypoxia preconditioning

IRI is a common problem in vascular and transplant surgery. Cellular injury sustained during a hypoxic period is increased following reperfusion, with release of ROS and metabolic by-products, in to the systemic circulation. Prolonged tissue hypoxia during surgery can lead to ischaemia, defined as a restriction in blood supply to a tissue with resultant damage or dysfunction. Tissue ischaemia causes a cascade of events, initially ATP depletion with dysfunction of membrane channels, and a switch to anaerobic metabolism, that leads to accumulation of lactic acid as an end product. Enzyme, mitochondrial dysfuntion and cell necrosis eventually occur. This cell damage and tissue necrosis further stimulates the inflammatory cascade with ROS release, cytokine activation and odema. Similar changes occour in the microcirculation, with endothelial membrane dysfunction, swelling and seperation of endothelial cells with luminal disruption leading to red cell clumping in the capillary beds (Blaisdell 2002).

Reperfusion compounds this damage, causing further injury (IRI), with both local and systemic consequences. The damage of IRI is due in part to inflammatory response of damaged tissues and also to the breakdown products released from cells. With restoration of normal blood flow these inflammatory and breakdown products are 'flushed' into the systemic circulation, hence the resultant SIRS. The inflammatory response in IRI is multifactorial, involving ROS, cytokines, the coagulation cascade and endotoxin (Norwood, Bown et al. 2004; Harkin, Arnold et al. 2007). Damaged endothelium shows increased expression of cell surface receptors, attracting white blood cells. In particular neutrophils carried to the area by the newly returning blood,

cause further activation and release of cytokines and ROS, (Chan, Ibrahim et al. 2003; Banga, Homer-Vanniasinkam et al. 2005). Breakdown products from damaged cells activate the coagulation cascade and in capillary beds red cells impact with rouleaux formation and thrombosis causing 'plugging' of vessels. Restored blood flow reintroduces oxygen to cells with damaged mitochondria and cell membranes. Consequently, increased ROS cannot be buffered or contained, leading to further damage.

IRI causes significant, local tissue swelling and inflammation. In vascular surgery this is characteristically seen in the lower limb following reperfusion of an occluded artery. IRI causes muscles in the calf to swell leading to compartment syndrome and the need for fasciotomies. This is particularly in the anterior compartment which contains mostly type I or red slow twitch fibres. These depend on aerobic metabolism and are therefore more sensitive to hypoxia. In contrast the gastrocnemius contains more type II or white fibres, using anaerobic metabolism of glycogen for energy that are more tolerant to hypoxia (Lindsay, Liauw et al. 1990).

The effect of IRI is to primarily compound established ischaemic damage. Secondary release of ROS, lactic acid, procoagulants and inflammatory mediators into systemic circulation occurs. The systemic effects of IRI and SIRS can lead to a cascade of acute lung injury and multiorgan failure. Clinicaly, this can been seen following vascular operations to repair ruptured AAA. Or lower limb reperfusion for acute limb ischaemia. Analysis showed that most mortality following these emergency operations was related to pulmonary complications (Blaisdell 2002), a situation that has remained unchanged over several decades (Groeneveld, Raijmakers et al. 1997; Norwood, Bown et al. 2004).

Hypoxia preconditioning (HPC) is an established mechanism to reduce IRI. HPC involves a short exposure of tissue to hypoxia, followed by reperfusion and recovery, before subsequent full exposure. HPC was first described by Murry in 1986 in a canine model. Murray demonstrated that brief periods of HPC before coronary occlusion reduced myocardial infarct size, despite no difference in collateral blood flow (Murry, Jennings et al. 1986). Cohen reinforced these findings in the rabbit and showed that brief coronary occlusion before a more prolonged occlusion results in not only reduced infarct size but, also significantly, improved recovery of systolic function (Cohen, Liu et al. 1991). This functional benefit of HPC was confirmed in the rat heart exposed to both acute and chronic hypoxia (Tajima, Katayose et al. 1994). These findings were reproduced in the rat brain, HPC was shown to prevent stroke following carotid ligation (Gidday, Fitzgibbons et al. 1994; Vannucci, Towfighi et al. 1998). Since these initial reports, HPC has proven effective in reducing IRI in all organs and experimental animals studied (Koti, Seifalian et al. 2003).

HPC is effectively a method of warning cells to prepare against further ischaemia. This prevents IRI damage and cell death. HPC also promotes earlier return to function. In Humans HPC has proven beneficial during coronary artery bypass grafting (Yellon, Alkhulaifi et al. 1993). More recently Clavien *et al* in the first randomised controlled trial in humans, showed that 10 minutes of HPC reduced hepatic injury following reperfusion in patients undergoing liver resection (Clavien, Selzner et al. 2003). In vascular surgery for abdominal aortic aneurysms, a technique of remote HPC significantly reduced complications and morbidity following operation. In a randomised controlled trial of 50 patients, the experimental group underwent ten

minutes of HPC by clamping the common iliac artery during operation for Abdominal Aortic Aneurysms (AAA). HPC improved postoperative respiratory function, reduced any rise in troponin levels, and protected renal function compared to the control group (Ali ZA, personal communication, submitted to Lancet).

Clinically HPC has proven effective in reducing IRI, preventing tissue death, and enhancing recovery. The mechanisms of how HPC mediates these effects, has been the subject of much research over the last decade. In order that pharmacological mimetics could be developed to ultimately artificially induce HPC in a clinical setting, for a review see (Jaeschke 2003; Koti, Seifalian et al. 2003; Selzner, Rudiger et al. 2003; Yellon and Downey 2003).

At a molecular level two phases of response from HPC can be seen. The early phase of HPC is related to free radical and ROS release. The delayed phase represents a molecular response with de-novo protein synthesis. The two phases are integrated, with ROS released in the acute phase, also possibly acting as signals to stimulate the delayed phase. The delayed phase of HPC was first termed 'Second Window of Protection' which is perhaps more meaningful name, reflecting the benefit of HPC (Yamashita, Hoshida et al. 1998). The implication for HIF-1 to be involved is seen in all stages of HPC. HIF-1 is induced both in response to the hypoxia and also by ROS seen during the initial phase of HPC. Further, the genes induced seen during the delayed phase of HPC are predominantly transcribed by HIF-1.

1.2.1. HIF-1 family of transcription factors

A well recognized response from exposure to hypoxia is increased blood red cell mass, characteristically seen in people exposed to low atmospheric oxygen concentration at altitude. Increased red cell production, is an effect mediated by Eythropoetin (EPO) and is the most studied and representative of the molecular responses to hypoxia. Two key breakthroughs on the molecular biology of EPO advanced knowledge this area of research and lead to the discovery of the transcription factor HIF-1. First the discovery of a hepatoma cell line (HEP3B) that on exposure to hypoxia increased EPO production. Experiments on HEP3B cells in culture demonstrated a correlation between hypoxia and increased production of EPO mRNA (Goldberg, Glass et al. 1987) and later that EPO transcription was regulated by hypoxia (Goldberg, Gaut et al. 1991; Tan and Ratcliffe 1991). The second breakthrough was cloning of the EPO gene. This enabled transfection experiments and development of reporter cell lines (Beck, Ramirez et al. 1991; Pugh, Tan et al. 1991). Analysis of the EPO gene highlighted a control sequence on the EPO promoter (Semenza, Nejfelt et al. 1991) and a DNA binding activity that bound specifically to this site (Semenza and Wang 1992). This factor was seen under hypoxia but not in normal conditions and named Hypoxia Inducible Factor 1 (HIF-1). HIF-1 was defined as the EPO transcription factor as it bound to the EPO gene enhancer sequence at a site required for transcriptional activation (Wang, Jiang et al. 1995).

1.2.2. HIF-1

Following identification HIF-1 was purified in a unique and large series of experiments, involving ion exchange and DNA-affinity chromatography. The transcription factor HIF-1 was found to be a dimer, comprising two subunits; alpha and beta (HIF-1a & HIF-1b) (Wang, Jiang et al. 1995). HIF-1a was a new protein but HIF-1b was previously known, called Aryl Hydrocarbon Receptor Nuclear Translocator (ARNT) (Hoffman, Reyes et al. 1991). Structurally both HIF-1 subunits were similar, with basic-helix-loop-helix (BHLH) motifs and PAS domains. The BHLH motif is found in many dimeric transcription factors. The helix-loop-helix domain mediates dimerisation and the basic domain contact with DNA. PAS is an eponym termed from the names of the first transcription factors discovered; Per and Sim proteins found in Drosophilae also mammalian (ARNT) and its dimerisation partner Aryl Hydrocarbon Receptor (Wang, Jiang et al. 1995; Wenger and Gassmann 1997; Semenza 2000; Semenza 2000). The PAS domain contains two internal homology units, A and B repeats, implicated in protein-protein interactions (Huang, Edery et al. 1993). Deletion analysis of HIF-1 confirmed these functions (Jiang, Rue et al. 1996). Residues 1-166 of HIF-1a were sufficient for heterodimerization with ARNT and residues 1-390 were shown to be required for DNA binding (figure 1.2.1).

Following the discovery of HIF-1 in the HEP3B cell line, HIF-1 was seen in all other cell lines studied; by transfection of the EPO 3' enhancer into reporter cell lines (Maxwell, Pugh et al. 1993), andusing electrophoretic mobility shift assay (EMSA) the EPO 3' enhancer sequences showed HIF-1 DNA binding activity in multiple cell lines (Beck, Weinmann et al. 1993; Wang and Semenza 1993). These experiments suggested

that HIF-1 was a ubiquitous transcription factor, present in all cells, that mediated the cellular response to hypoxia.

Early experiments found that HIF-1 mRNA was continually expressed and remained unchanged in response to hypoxiasuggesting that HIF-1 was regulated at protein level (Gradin, McGuire et al. 1996; Huang, Arany et al. 1996; Salceda, Beck et al. 1996; Wood, Gleadle et al. 1996; Wenger, Kvietikova et al. 1997). HIF-1 protein was not present in cells cultured under normal conditions, and levels rose with exposure to hypoxia. This HIF-1 activity was found to be dependent on the alpha subunit HIF-1a, as ARNT was continually present in the cell under normal conditions (Wang, Jiang et al. 1995; Huang, Arany et al. 1996; Jiang, Rue et al. 1996). Under hypoxic conditions HIF-1a accumulated in the nucleus. Early kinetic experiments showed that HIF-1a nuclear accumulation correlated with HIF-1 DNA binding activity (Wang and Semenza 1993) and subsequent hypoxic gene induction. Data that confirmed the control of HIF-1 activation was by the presence of HIF-1a protein in the cell.

In summary, research on EPO lead to the discovery of HIF. HIF is responsible for mediating the molecular response to hypoxia in all cells. The function of HIF is mediated by the alpha subunit of the protein (HIF-1a), which increases in response to hypoxia.

Figure 1.2.1: Structure of HIF-1a

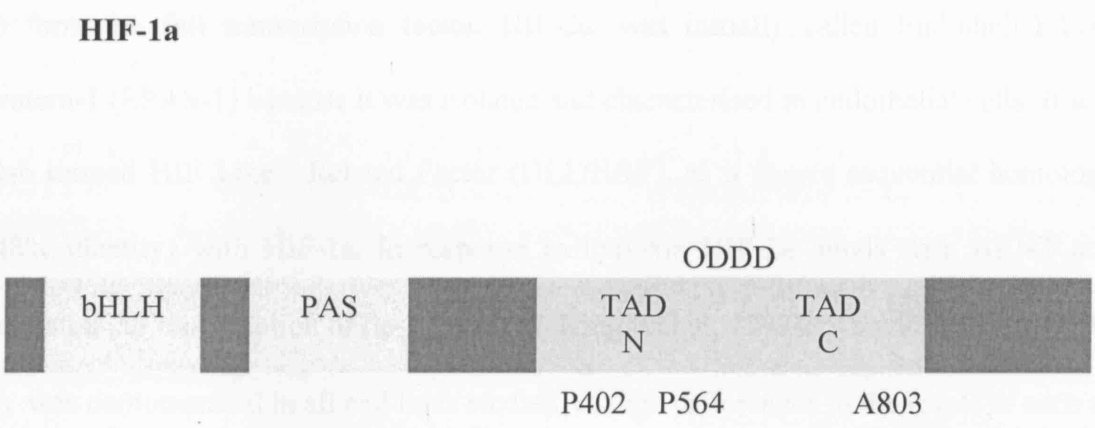

Figure 1.2.1: HIF-1a contains four important domains; the bHLH and PAS domains that mediate ARNT and DNA binding, also transactivation domains N and C (TAD-N & TAD-C), which are both located in the oxygen dependent degradation domain (ODDD). Key features of the ODDD are the location of specific prolene residues (P402 & P564) and asparganine (A803) that are involved in the control of HIF-1a. TAD-N contains specific prolene sites involved in targeting HIF-1 for destruction, and TAD-C interacts with transcriptional co-factors

1.2.3. HIF-2 and HIF-3

Following the identification of HIF-1, two further Hypoxia Inducible Factors were identified, HIF-2 & HIF-3. Both HIF-2 and HIF-3 were made up of two separate subunits, the functional subunits being HIF-2a and HIF-3a, which combine with ARNT to form the full transcription factor. HIF-2a was initially called Endothelial PAS protein-1 (EPAS-1) because it was isolated and characterised in endothelial cells. It was also termed HIF Like / Related Factor (HLF/HRF), as it shares sequential homology (48% identity) with HIF-1a. In response to hypoxia HIF-2a binds with ARNT and activated the transcription of tie-2 (Tian, McKnight et al. 1997). As with HIF-1a, HIF-2a was demonstrated in all cell lines studied, though differences in the levels of each of the transcription factors were apparent depending on the cell type evaluated. (Wiesener, Jurgensen et al. 2003). Regulation of HIF-2a was similar to HIF-1a, with induction by hypoxia and regulation at protein level (Wenger, Kvietikova et al. 1997; Wiesener, Turley et al. 1998). The third functional subunit, HIF-3a shared similar sequence identity with HIF-1a and HIF-2a (57% and 53% respectively) (Gu, Moran et al. 1998; Takahata, Sogawa et al. 1998) and expression of HIF-3a was seen in adult brain, thymus, lung, brain, heart and kidney (Hara, Hamada et al. 2001).

The three members of the HIF family are similar in structure and function, but differ in expression in tissues and response to hypoxia.

1.2.4. Function of HIF-1

HIF-1 is responsible for mediating the cellular response to hypoxia. The cellular response inducted by HIF-1 involves a plethora of genes involved in several pathways. These mediate adaptation to a reduced oxygen environment. These genes include those involved in; angiogenesis (VEGF), response to injury (HO-1, transferrin) and glucose metabolism (G6P, GK) (figure 1.2.3). In addition to this 'acute phase response' HIF-1 has a 'housekeeping' function, HIF-1 is essential for embryological development and maintaining normal cell physiology. Separate to these functions, HIF-1 can be over-expressed in disease and appears integral to several common disease processes.

1.2.5. HIF is essential for development

HIF-1 is essential for life demonstrated by the failure of HIF-1 -/- knockout mice to survive beyond embryonic day eleven (figure 1.2.2.). 'Knockouts' showed failure of development after day eight and death after day eleven. Developmental failure was characterised by massive mesenchymal cell death in cranial region and failure of neural tube closure. Cellular proliferation was also impaired. Levels of mRNA's encoding glucose transporters, glycolytic enzymes and VEGF were decreased. Vascularisation, initiated correctly, failed between D8.75 to D9.75. In comparison, HIF +/+ mice showed 7-8 fold increased HIF-1a expression at days 9.5 – 10 and further 12-18 fold increased expression from D11-12 (Iyer, Leung et al. 1998; Ryan, Lo et al. 1998). A similar situation was seen in ARNT -/- knockouts with stunted development and death by day ten (Maltepe, Schmidt et al. 1997). HIF-2a mutant mice similarly suffered embryonic lethality, although at a slightly later stage (Peng, Zhang et al. 2000).

Figure 1.2.2: HIF-1 is essential for normal foetal development

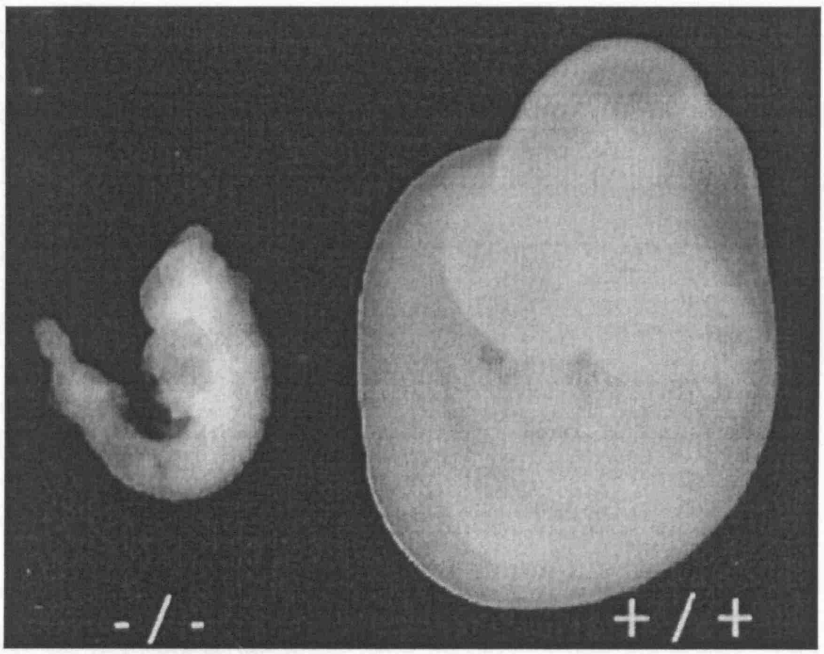

Figure 1.2.2: HIF-1(-/-) knockout mice at D10 of embryonic life compared to wild type controls (+/+). HIF-1 knockout mice show failure of development after day eight and death by day 11. (Courtesy of D. Stroka, University of Berne, Switzerland.)

1.2.6. HIF-1 is normally expressed in all tissues

The evidence for a 'housekeeping' function of HIF-1 is both spatial and temporal. HIF-1 was demonstrated in all cell lines studied and in vivo immunohistochemistry demonstrated baseline nuclear HIF-1a in all organs and tissues examined (Maxwell, Pugh et al. 1993; Stroka, Burkhardt et al. 2001). Variation did exist in the kinetics and level of HIF-1 induction between cell lines examined, which may reflect underlying cellular susceptibility of different tissue types to ischaemia. Increased HIF-1 response to hypoxia may be seen in cells from tissues most susceptible to ischaemia (Stroka, Burkhardt et al. 2001).

Surprisingly low baseline HIF-1 expression was seen in liver and renal tissue despite zones of significant hypoxia (Kietzmann, Cornesse et al. 2001; Zou and Cowley 2003). In the liver, anatomical structure and physiological function are well matched and in part controlled by the oxygen gradient from arterial to venous blood. The liver acinus is a segment of hepatocytes located between the hepatic arteriole and effluent venule. The acinus is essentially a functional microcirculation unit as blood flow creates an oxygenation gradient from well oxygenated hepatocytes around afferent arterioles (periportal zone) to low oxygenated hepatocytes around efferent venules (perivenous zone) (Ronald G. Thurman 1986). Studies elegantly demonstrated a zonal regulation of HIF-1 gene expression and function in the acinus. Glucose forming enzymes glucose-6-phosphatase (G6P) and phosphophenolpyruvate (PCK) were predominantly located in highly oxygenated periportal areas whereas glucose metabolising enzymes glucokinase (GK) and pyruvate kinase (PK) were located in perivenous zones (Jungermann and Kietzmann 1997). This situation could be reversed on reverse perfusion of the liver and the results were confirmed in cell culture under differing oxygen concentrations

(Jungermann and Kietzmann 2000). These findings demonstrated that zonal metabolism and gene expression in the liver was mediated by oxygen concentration. Interestingly despite this a zonal regulation of gene expression, HIF-1a expression was uniform without zonal variation (Kietzmann, Cornesse et al. 2001). On reverse perfusion experiments hypoxic zones and consequent gene expression were reversed but without increased HIF-1a expression. This switch between anaerobic and aerobic metabolism (termed the Pasteur effect) was mediated by HIF-1 (Seagroves, Ryan et al. 2001). These experiments show how HIF-1 expression can be uniform despite different oxygen tensions in same tissues. It also suggests that HIF-1 has both a 'housekeeping role' in control of gene expression under normal circumstances as well as an acute phase response to hypoxia. In relative oxygen abundance HIF-1 stimulates aerobic gene expression and in more hypoxic regions HIF-1 promotes anaerobic metabolism. In rats exposed to chronic hypoxia there was an initial increased HIF-1a expression that returned to normal with time. Reflecting adaptation of cellular function to the environment (Chavez, Agani et al. 2000). HIF-1 acts not only to respond to hypoxia but also to control cellular utilisation of available oxygen to prevent ischaemia.

1.2.7. HIF-1 mediates the cellular response to hypoxia

The acute role of HIF-1 in the cellular response to hypoxia enables the cell to survive in a reduced oxygen environment. The response to hypoxia is immediate and includes an ever-increasing array of hypoxia responsive genes (figure 1.2.3). The effect of HIF-1 induction is to reduce cellular oxygen utilization and increase oxygen delivery. HIF-1 induces increased cellular oxygen delivery in several ways: - increased respiration (tyrosine hydroxylase), increased vascularity (iNOS, VEGF) and, amongst other more sustained adaptations, increased red cell volume with increased EPO production. Hence

HIF-1 mediates cellular specific, local tissue and general systemic acute response to hypoxic stress.

1.2.8. HIF-1 can be pathologically elevated in disease

Pathological induction of HIF-1 is well illustrated in cancer. For malignant cells to grow they require energy, substrates and oxygen. Initially this can be achieved by simple diffusion from surrounding tissues. Beyond a certain size, suggested to be 2mm, the diffusion gradient is too great to maintain oxygen supply (Vaupel, Kelleher et al. 2001). To grow, a cancer must develop its own blood supply to enable adequate oxygen delivery. HIF-1 is involved in the angiogenesis of cancer, both directly by the hypoxia of developing cells and indirectly by cross talk from inflammatory and pathological signalling pathways found in carcinogenesis (Mabjeesh and Amir 2007).

Several lines of evidence support a role for HIF-1 in tumour pathophysiology. The range of genes inducted by HIF-1 activation in cell culture correlated with those seen in carcinogenesis. Key genes over-expressed in angiogenesis, such as iNOS and VEGF are hypoxia inducible genes. Inactivation of HIF-1a in embryonic stem cell tumours reduced VEGF expression, prevented large vessel formation and impaired vascular function (Carmeliet, Dor et al. 1998). A feature of cancer cells is a switch from aerobic to anaerobic metabolism, termed the Warburg effect. In normal physiology this is termed the Pasteur effect and mediated by HIF-1 (Semenza 2000). Immunohistochemistry of human tumour biopsies revealed increased HIF-1a and HIF-2a protein in most tumours studied (Talks, Turley et al. 2000). Increased HIF-1 was predominantly seen in tumours with poor prognosis, likely to metastasise, those with rapid growth with central necrosis. In brain tumours HIF-1a protein correlated with

tumour stage and grade (Zhong, De Marzo et al. 1999; Zagzag, Zhong et al. 2000). A similar finding seen in cervical (Birner, Schindl et al. 2000) and breast cancer (Laughner, Taghavi et al. 2001; Sowter, Raval et al. 2003; Yokoi, McCrudden et al. 2003).

Figure 1.2.3: Representative HIF-1 target genes

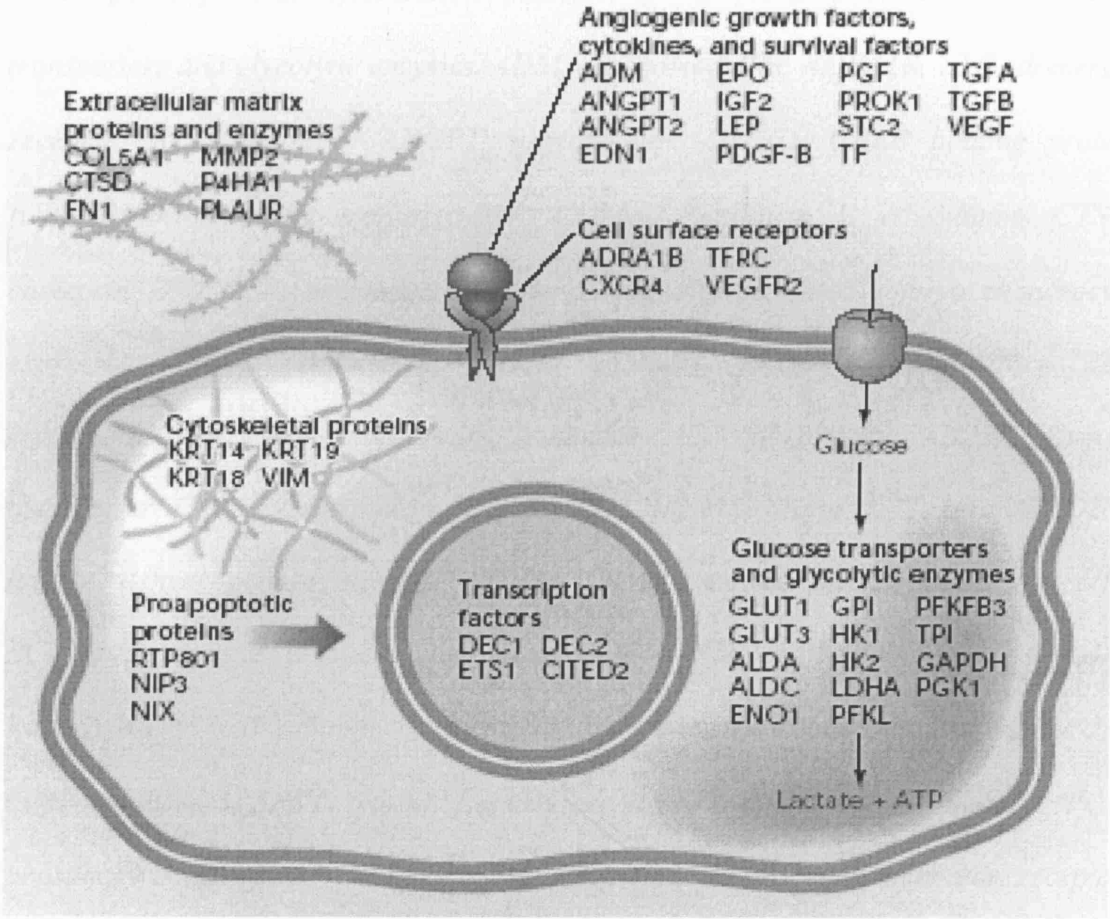

Figure 1.2.3. HIF-1 target genes (reproduced from Dr. D. Mole pHD).

Hypoxia inducible factor-1 (HIF-1) activates the transcription of genes encoding secreted signalling proteins, including angiogenic growth factors and survival factors, cell surface receptors, extra cellular matrix proteins and modifying enzymes, transcription factors, cytoskeleton proteins, proapoptotic proteins, and glucose transporters and glycolytic enzymes. ADM, adrenomedullin; ADRA1B, α1β-adrenergic receptor; ALD, aldolase; ANGPT, angiopoietin; CITED, CREB binding protein (CBP)/p300-interacting transactivator; COL5A1, collagen V α1-subunit; CTSD, cathepsin D; CXCR, chemokine receptor; DEC, differentiated embryo chondrocyte expressed; EDN, endothelin; ENO, enolase; EPO, erythropoietin; ETS, erythroblastosis virus transforming sequence; FN, fibronectin; GLUT, glucose transporter; GPI, glucose phosphate isomerase; HK, hexokinase; KRT, keratin; LDHA, lactate dehydrogenase A; LEP, leptin; MMP, matrix metalloproteinase; NIP, BCL2/adenovirus E1B 19-kDa-interacting protein; NIX, NIP3-like; P4HA1, prolyl-4-hydroxylase α1-subunit; PFKFB3, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase-3; PFKL, phosphofructokinase L; PGF, placental growth factor; PGK, phosphoglycerate kinase; PLAUR, urokinase-type plasminogen activator receptor; PROK, prokineticin (endocrine gland-derived VEGF); STC, stanniocalcin; TF, transferrin; TFRC, transferrin receptor; TGFA and TGFB, transforming growth factor-α and -β; TPI, triose phosphate isomerase; VEGFR, VEGF receptor; VIM, vimentin.

1.2.9. Activation, destruction and control of HIF-1

Early experiments found that HIF-1 mRNA was continually expressed and that levels remain unchanged with hypoxia (Gradin, McGuire et al. 1996; Huang, Arany et al. 1996; Salceda, Beck et al. 1996; Wood, Gleadle et al. 1996; Wenger, Kvietikova et al. 1997). HIF-1 activity was found to be dependent on expression of the alpha subunit, while HIF-1b (ARNT) was constitutively expressed (Wang, Jiang et al. 1995; Huang, Arany et al. 1996; Jiang, Rue et al. 1996). Under hypoxic conditions the protein, HIF-1a accumulated in the cell nucleus and this was associated HIF-1 DNA binding activity (Wang and Semenza 1993). These and subsequent studies correlated hypoxic gene induction, HIF-1a binding and HIF-1a nuclear accumulation, confirming that the rate-limiting step in HIF-1 induction was HIF-1a protein accumulation in cell nucleus.

1.2.10. Under normal conditions HIF-1a is destroyed

Under normal conditions although HIF-1 mRNA is continually expressed, the protein HIF-1a is rapidly destroyed. The mechanism for how HIF-1a was destroyed under normal conditions was discovered during studies on the hereditary cancer syndrome of von Hippel-Lindau disease (VHL). VHL is an autosomal dominant disease with a prevalence of 1/40,000 individuals, characterised by highly vascular haemangioblastomas of retina and central nervous system. Other tumours associated with VHL disease tend to be highly angiogenic; renal cell carcinomas, phaechromocytomas, endolymphatic sac tumours, pancreatic islet cell tumours, and papillary cystadenomas of the epididymis (males) or broad ligament (females) (Kondo and Kaelin 2001). A feature of the disease is that many VHL tumours express hypoxia inducible genes (VEGF, EPO). Germ line mutations that lead to inactivation or loss of

function of pVHL, can be demonstrated in patients (Stolle, Glenn et al. 1998) and hotspots for these mutations are in the α or β binding domains of VHL.

Analysis of the association between HIF-1 and VHL revealed that HIF-1a was destroyed in the cytoplasm by a process that involved the VHL tumour suppressor gene product (pVHL) (Maxwell, Wiesener et al. 1999). Analysis of this interaction revealed the β domain of pVHL bound to a highly conserved region in the ODDD of HIF-1a (Cockman, Masson et al. 2000). This was confirmed by analysis of interaction in COS7 cells, that over express HIF-1a in normoxia (Tanimoto, Makino et al. 2000) and fusion proteins containing specific regions of HIF-1a and pVHL (Ohh, Park et al. 2000). It was also investigated by studying the interaction of HIF-1a with recombinant pVHL (Kamura, Sato et al. 2000). Following binding of HIF-1a by pVHL, HIF-1a is destroyed by a process of poly-ubiquitination and proteosomal degradation. Ubiquitination of the HIF-1a/pVHL complex required all 5 subunits of pVHL coupled via the α domain of pVHL to elongins B & C (Ohh, Park et al. 2000). Recruitment of Cul2 with Rbx1 formed a ubiquitin ligase complex that induced polyubiquitination via E1 ubiquitin activating and E2 ubiquitin conjugating enzymes. This enabled degradation by 26S proteosome (Kamura, Sato et al. 2000) (figure 1.2.4.)

The HIF-1a pVHL binding is facilitated by hydroxylation of specific prolene residues in the TAD-C (transactivating domain C) region of the ODDD. Kinetic experiments (as detailed later), revealed that hydroxylation of HIF-1a is instantaneous. The fast nature of hydroxylation is best illustrated by the fact that the experiments had to be performed inside a hypoxic chamber. Initially one target amino acid on the ODDD was identified that underwent hydroxylation, prolene 564 (Ivan, Kondo et al. 2001;

Jaakkola, Mole et al. 2001) This process was dependent on oxygen, iron and oxalogluterate as substrates. A further prolene 402 on the ODDD was also identified (Masson, Willam et al. 2001). Subsequently three prolene hydroxylases (PHD1-3) were identified from a family of propyl–4-hydroxylases enzymes (Bruick and McKnight 2001) that can hydroxylate HIF-1a □at one of the two prolene sites on ODDD. Analogous prolene residues were later found on HIF-2a and HIF-3a (Safran and Kaelin 2003).

Figure 1.2.4: Pathway of HIF-1a degradation

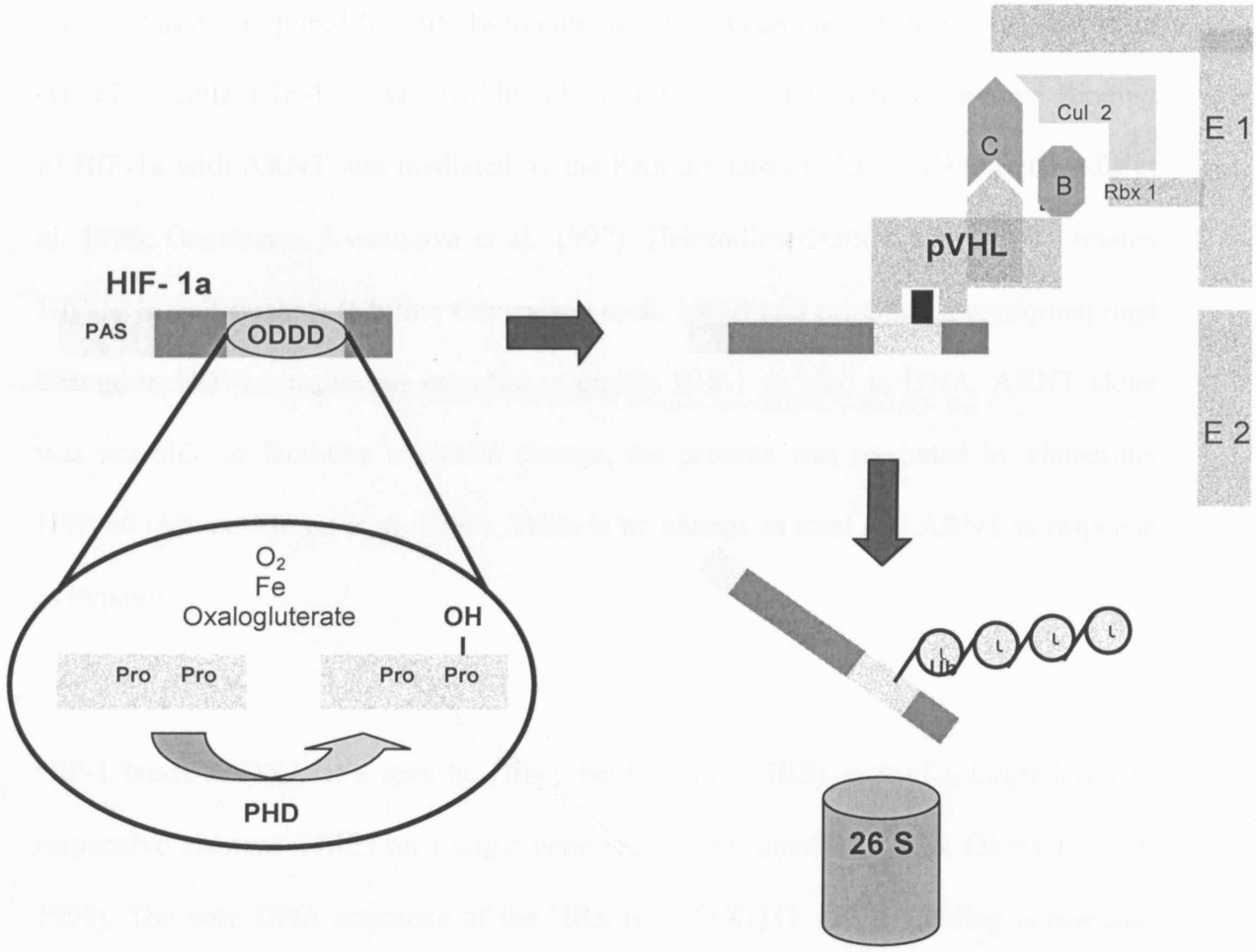

Figure 1.2.4: In the presence of oxygen, iron and oxalogluterate, HIF-1a is hydroxylated at one of two specific prolene sites on the ODDD by one of three specific PHD's. This facilitates binding by the β domain of pVHL, the α domain forms a ubiquitin ligase complex with Elongins B & C (B, C), Cul2 and Rbx 1 proteins. Polyubiquitination (Ub) is initiated by the E1 activating and E2 conjuqating enzymes (EI, E2) and degradation by the 26S proteosome (26S).

1.2.11. Activation of hypoxic gene induction requires co-factors

Under hypoxic conditions HIF-1a is not hydroxylated or destroyed by the pVHL pathway. HIF-1a is able to move □into the cell nucleus, a process mediated by unmasking of a nuclear localization signal (Kallio, Okamoto et al. 1998). Although ARNT was not required for HIF-1a to enter nucleus (Gassmann, Chilov et al. 2000), in ARNT -/- cells, HIF-1a □was unable to bind to DNA or induce transcription. Binding of HIF-1a with ARNT was mediated by the PAS domain of HIF-1a (Wood, Gleadle et al. 1996; Gassmann, Kvietikova et al. 1997). Heterodimerisation with ARNT retains HIF-1a in cell nucleus (Chilov, Camenisch et al. 1999) and produces a conformational change in HIF-1a, a process essential to enable HIF-1 to bind to DNA. ARNT alone was not able to facilitate allosteric change, the process was mediated by chaperone HSP-90 (Minet, Mottet et al. 1999). There is no change in total cell ARNT in response to hypoxia.

HIF-1 binds to DNA at a specific HIF-1 binding site (HBS), part of a larger hypoxic responsive element (HRE) on a target gene promoter sequence (Kallio, Okamoto et al. 1998). The core DNA sequence of the HBS is G/ACGTG. HIF-1 binding is essential but not adequate alone for hypoxic gene transcription. Further co-factor binding to HBS is necessary to form the complete transcriptional complex with Polymerase II and initiate hypoxic gene transcription. HIF-1a binds CBP/p300, modular transcription co activators, that provide scaffold for assembly of further transcriptional activation complexes (figure 1.2.5) (Arany, Huang et al. 1996; Kallio, Okamoto et al. 1998; Dames, Martinez-Yamout et al. 2002). These additional factors for HIF-1 are specific to the target gene to be transcribed. Hepatocyte nuclear factor 4 (HNF-4) binds to ARNT for EPO transcriptional induction (Gallon, Tsuchiya et al. 1995) activating

transcription factor-1 (ATF-1) for the lactate dehydrogenase A gene (Kvietikova, Wenger et al. 1995), and activator protein 1 (AP-1) for VEGF (Shih and Cliffy 2001).

Figure 1.2.5: Activation pathway of HIF-1

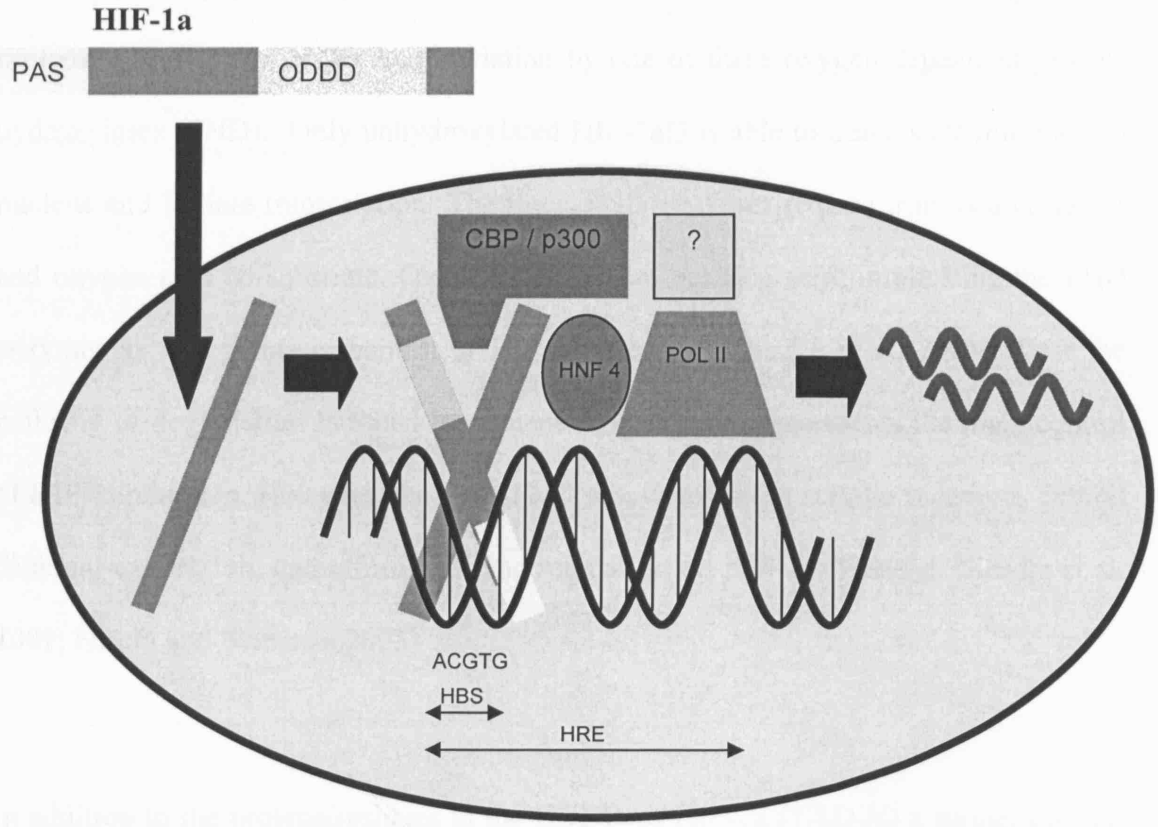

Figure 1.2.5: In response to hypoxia HIF-1a moves from cytoplasm of cell into the nucleus. In nucleus HIF-1a heterodimerised to ARNT, which enables binding to HIF-1 binding site (HBS) on consensus sequence ACGTG. The HBS is part of a larger hypoxia responsive element (HRE). Binding of structural complex protein CBP/p300 to HIF-1 facilitates binding of cofactor transcription factors. Cofactor binding (?) e.g. hepatocyte nuclear factor 4, is specific to gene transcribed. A transcriptional activation complex with Polymerase II is formed to induce hypoxic gene transcription.

1.2.12. Control of HIF-1

The key step in HIF-1 mediated gene expression in response to hypoxia is stabilisation of the HIF-1a protein. Under normal conditions the HIF-1a subunit is targeted for proteosomal degradation by hydroxylation by one of three oxygen-dependent prolene hydroxylases (PHD). Only unhydroxylated HIF-1a□ is able to translocate into the cell nucleus and initiate transcription. The three PHD enzymes require iron as a co-factor and oxygen as a co-substrate. Oxygen acts as rate limiting step, implicating the PHD enzymes as key points in control of HIF-1a (Epstein, Gleadle et al. 2001). Thus the pathway of degradation initiated by prolene hydroxylation represents the main control of HIF-1 induction. However, the three PHD's have differing cellular locations, exhibit different expression, and afford differing regulation on HIF-1a (Epstein, Gleadle et al. 2001; Hirota and Semenza 2005)

In addition to the prolene residues in the ODDD of HIF-1a (TAD-N) a further domain (TAD-C) where co-factors bind to form the HIF-1 transcriptional complexcontains a conserved asparagine residue (Asn 803). Hydroxylation of this residue by an asparganine hydroxylase termed FIH-1 ("factor inhibiting HIF-1"), under normal conditions inhibits the HIF-1 transcriptional complex (Mahon, Hirota et al. 2001). FIH-1 prevents recruitment of the p300/CBP family of transcriptional co-activators, so prevents formation of the HIF transcriptional complex(Lando, Peet et al. 2002).

Separate to these hydroxylation controls of HIF a further control mechanism exists at the transcriptional level. HIF-1 induction of the p35srj gene has been described (Bhattacharya, Michels et al. 1999). The p35srj gene product interacts directly with the

HIF-1 binding domain of p300/CBP. Thus, in chronically hypoxic cells, one can envisage that p35srj down regulates HIF-1-mediated transcription by controlling the access of HIF-1a to p300/CBP. P35srj therefore creates a negative feedback loop that is linked to HIF-1 transcriptional activity.

In summary the PHD's and FIH-1 are involved in the oxygen-sensing pathway and directly affect HIF-1 activation. Furthermore negative feedback mechanisms may exist. However, location and expression of these enzymes vary. Stimulation and inhibition by these enzymes, and their control, remains the target for future research.

1.2.13. HIF-1 induction is instantaneous

Activation of HIF-1a expression and HIF-1 DNA binding occurs within physiologically relevant oxygen tensions (Jiang, Semenza et al. 1996). In cell culture HIF-1a expression and DNA binding increased two-fold as oxygen concentration decreased from 20 to 6% (approximately 140-42 mmHg) and ten-fold between 6 and 0.5% oxygen (approximately 42-4 mmHg). HIF-1a expression increased exponentially with increased hypoxia and a maximal response was seen when cells were exposed to anoxia and severe hypoxia (Jewell, Kvietikova et al. 2001). HIF-1a protein was detected in the nucleus at two minutes, the same time taken for culture media to equalize oxygen tension and become hypoxic. HIF-1a □nuclear protein accumulated rapidly for 30 min at all oxygen concentrations then slowed until maximum level was reached at 60min. HIF-1 DNA binding by EMSA mirrored these kinetics. Re-oxygenation rapidly reduced HIF-1a nuclear protein after 4 min, with complete absence after 32 min. HIF-1a nuclear protein reduction was prolonged following exposure to anoxia for 1 hour. The rate of HIF-1 degradation in response to re–oxygenation was inversely related to duration and severity of hypoxic stress, so the longer and more hypoxic the exposure the longer HIF-1 activation persisted (Berra, Richard et al. 2001).

In normal tissues, cells are exposed to partial pressure of oxygen at 40-50 mmHg (Semenza 2001). Reduction in tissue oxygenation would therefore correspond to the steep portion of HIF-1 response curve, seen in cell culture. Transcriptional response to hypoxia may therefore respond to physiologically relevant conditions. In vivo experiments demonstrated baseline level of HIF-1a nuclear protein in all tissues. Exposure of mice to 6% O_2, equivalent to 9100m above sea level, demonstrated HIF-1a nuclear protein increased in all tissue studies. Kinetics varied between organ tissues

studied. Brain tissue was the most sensitive and produced increased HIF-1a expression response at all ranges of hypoxia, maximal at 6% oxygen. HIF-1a induction correlated with EPO expression in serum. HIF-1a mRNA levels remained unchanged. Reperfusion kinetics were similar to in vitro with $t_{1/2}$ at 15 min (Stroka, Burkhardt et al. 2001).

In Summary HIF-1a induction is immediate and the degree of expression is related to the intensity of the hypoxic stimulus. Following removal of the hypoxic stimulus HIF-1a similarly returns to normal levels of expression.

1.2.14. HIF-1 and hypoxia preconditioning

In the last decade research has mostly focused on HPC in the myocardium or brain, although all tissues have been studied. Following a natural history study showing HIF-1 induction in the prenumbra of the brain following middle cerebral artery ligation (Bergeron, Yu et al. 1999), studies were performed to elucidate the effect of HPC on HIF-1 expression. In a rodent model rats were exposed to hypoxia (8% O_2) or chemically pre-treated, either with desferroxamine (DSF) or cobalt for three hours before ligation of the common carotid artery. The brains were analysed seven days post procedure. HPC and to a lesser extent pre-treatment with cobalt and DSF, increased HIF-1 expression and reduced infarct size in response to carotid ligation. The level of HIF-1 expression correlated with the degree of brain protection afforded by HPC (Bergeron, Gidday et al. 2000). Studies on the kinetics of HPC found that a window of two or three days was required for HPC to mediate a protective effect (Miller, Perez et al. 2001). Further experiments confirmed this and found a greater protective effect to be associated with a greater initial HPC stimulus and that more than three hours of hypoxic exposure was necessary to significantly reduce infarct size. HPC results also correlated with HIF-1 DNA binding and EPO expression (Prass, Scharff et al. 2003). Further in studies on the mouse brain the effect of HPC was associated with increased HIF-1, VEGF and EPO protein levels (Bernaudin, Nedelec et al. 2002).

In the kidney of a rodent model IRI was assessed following 45 minutes of ischaemia; an experimental group of rats were pre-treated with cobalt and compared with controls. The cobalt group showed reduced rise in serum creatinine and on histological analysis reduced tubular damage. IHC showed increased HIF-1 protein and increased expression

of several HIF-1 genes (HO-1, EPO, Glut-1 and VEGF) were seen on RT-PCR (Matsumoto, Makino et al. 2003).

Studies looking at HPC in the heart found exposure of mice to intermittent hypoxia protected the heart from subsequent ischaemic insult 24 hours later but not at 30 minutes. Cardiac protection induced by HPC was lost in HIF-1a +/- mice. The level of EPO expression strongly correlated with these results (Cai, Manalo et al. 2003). Similar results were achieved by pre-treatment with cobalt, with reduction of infarct size seen in the murine heart, which correlated with increase HIF-1 DNA binding activity. The effect was blocked by the antioxidant DDTC, suggesting a role for ROS in the HPC - HIF-1 pathway. Furthermore the effect of HPC was lost in iNOS knockout mice (Xi, Taher et al. 2004).

The role for HIF-1 in cardiac protection to IRI was strengthened by transfection experiments. Adenoviral transfer to continuously express HIF-1 in cardiomyocytes mimicked the protective effect of HPC with expression of HIF-1a protein and several target genes including iNOS, VEGF and Glut 1 (Date, Mochizuki et al. 2005).

Another mechanism to implicate HIF-1 as a key factor in HPC and IRI was the use of PHD specific inhibitor (DMOG). The increased HIF-1a provided cardio protection and reduced infarct size following after IRI with reduced IL8 and neutrophil activation and increased HO-1 expression (Ockaili, Natarajan et al. 2005). The same group confirmed this effect by using RNA interference to silence PHD2, thereby stabilising HIF-1. Consequent over-expression of HIF-1 in transfected mice mimicked the effect of HPC

and resulted in significantly reduced infarct size following IRI (Natarajan, Salloum et al. 2006).

In summary the results of these experiments suggest a role for HIF-1 in HPC to provide protection against IRI. Activation of hypoxia inducible genes and increased EPO production was associated with reduced tissue ischaemia. Artificial or over expression of HIF-1a appeared to be effective in inducing HPC and reducing IRI.

1.3.1. Proposals for the oxygen sensor

How does a cell actually sense oxygen? In a comprehensive review Bunn and Poyton examined possible theories and models (Bunn and Poyton 1996). Oxygen sensing ability varies between cells types and includes specialised oxygen sensing pathways and a more general hypoxia response system involving HIF-1, which is endogenous to all cell types.

The specialised oxygen responsive cells are responsible for acute physiological responses to changing atmospheric oxygen and the body's oxygen demand. Chemoreceptors in carotid body sense arterial oxygen partial pressure and directly affect brainstem respiratory stimulus function. These chemoreceptors comprise Type I glomus cells with oxygen responsive potassium channels that generate calcium action potentials with subsequent neurotransmitter release.

Separate to these the more general HIF-1 system is endogenous to all cell types. Two of the most popular models proposed for the oxygen sensor include the involvement of reactive oxygen species (ROS) or a heme containing protein sensor (figure 1.3.1). In the last decade, with dissection of the HIF-1 pathway, evidence has swung between the different possible models. Following identification of the PHD's it was thought that these proteins were themselves the direct oxygen sensors. However, increasingly more layers appear to be implicated in the oxygen-sensing pathway and to date no precise mechanism has been identified.

Figure 1.3.1: Possible mechanisms of oxygen sensing

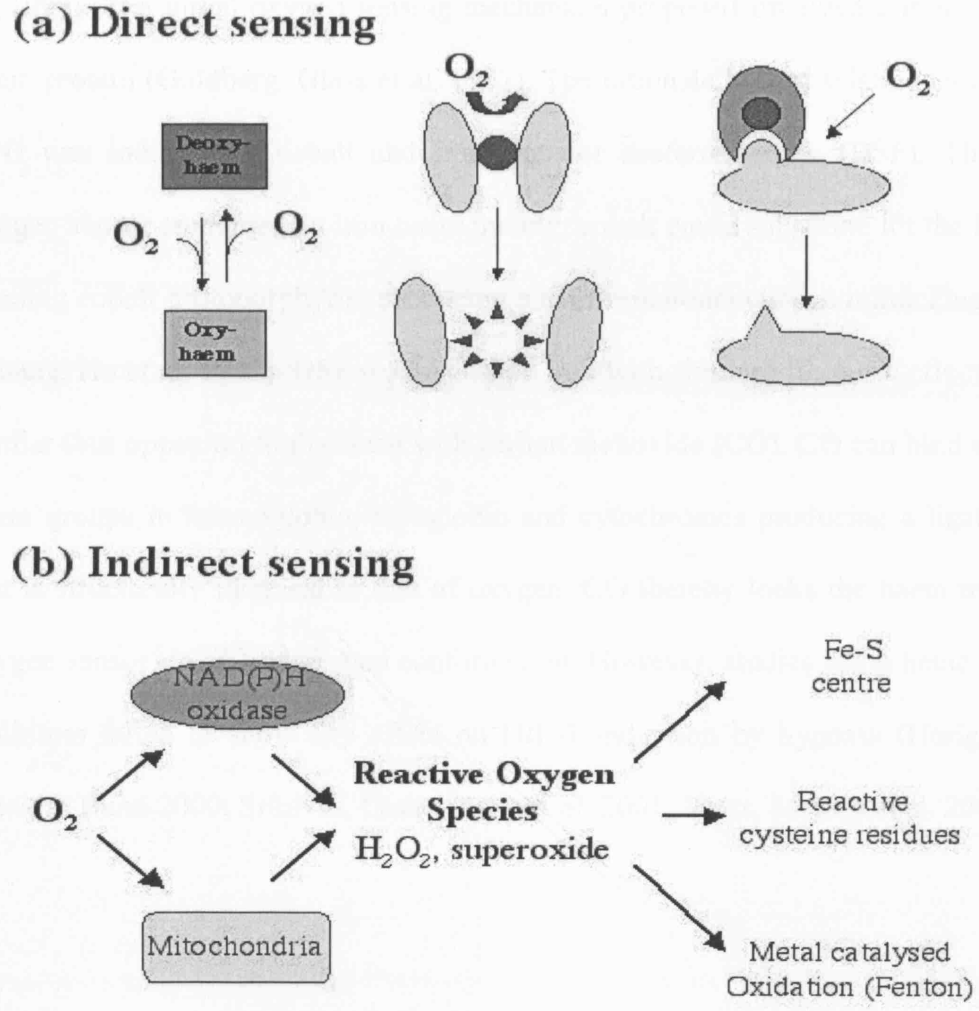

Figure 1.3.1: Direct sensing of oxygen may involve binding to haem, modification at an Fe-S centre or enzymatic modification. Indirect sensing may involve reactive oxygen species (either increased or decreased) by an NAD(P)H oxidase, or mitochondrial processes, which attack an Fe-S centre, reactive cysteine residues, or effect metal catalysed oxidation, through Fenton reaction (kindly reproduced from D. Mole MRCP).

1.3.2. The oxygen sensor may be a haem protein

EPO induction in response to hypoxia has been the most studied pathway of HIF-1 activation. EPO was an ideal model, and could be studied using HEP3B cell cultures, which expressed EPO in response to hypoxia (Ratcliffe, Ebert et al. 1997; Bunn, Gu et al. 1998). The initial oxygen sensing mechanism proposed involved a iron containing haem protein (Goldberg, Glass et al. 1987). The rationale behind this model being that EPO was induced by cobalt and iron chelator desferroxamine (DSF). Thus if the oxygen sensor contained an iron haem moiety, cobalt could substitute for the iron atom forming cobalt protoporphyrin, producing a conformational change mimicking hypoxia (Huang, Ho et al. 1997). DSF would chelate iron with similar effect. An effect that was similar (but opposite) to that seen with carbon monoxide (CO), CO can bind to ferrous haem groups in haemoglobin, myoglobin and cytochromes producing a ligation state that is structurally identical to that of oxygen. CO thereby locks the haem moiety (or oxygen sensor) in an oxygenated conformation. However, studies using heme synthesis inhibitors failed to show any effect on HIF-1 induction by hypoxia (Horiguchi and Franklin Bunn 2000; Srinivas, Leshchinsky et al. 2001; Vaux, Metzen et al. 2001).

1.3.3. Reactive Oxygen Species

Reactive Oxygen Species (ROS) have been of interest in cell biology for many years. ROS have been studied in two main functional areas, the neutrophil respiratory burst and in the mitochondrial electron transport chain. ROS have traditionally been regarded as harmful. ROS are formed by the sequential reduction of molecular oxygen, with hydroxyl radicals as the end product of ROS reduction. ROS are extremely reactive, with short half-life, and short diffusion distance. They are frequently indiscriminate and react with the first molecule encountered. These characteristics provide neutrophils with an effective and potent mechanism of destruction, and ROS in this role serve to facilitate cellular defence. Apart from being deliberately generated for a functional role in the neutrophil, ROS are also produced by all cell types as a by-product of oxygen metabolism in the mitochondria. In this latter pathway ROS present an instant, temporary response mechanism dependent on oxygen balance and cellular redox status.

In addition to the neutrophil and mitochondria, several cellular pathwaysalso generate, all involve the sequential reduction of molecular oxygen (figure 1.3.2). The first step, a one-electron reduction, is catalysed by NADPH oxidase and produces superoxide. This is the principle reaction seen in neutrophil respiratory burst. NADPH itself is a relatively complex molecule comprising five subunits. Two membrane bound subunits (p22-phox and gp91-phox) form a heme containing catalytic core of the enzyme flavocytochrome b558. Translocation of three cytoplasmic components (p47-phox, p67-phox and p40-phox) to the inner surface of cell membrane is assisted by GTPase rac2, to form the fully active enzyme complex. Flavocytochrome b558 enables electron transfer across the cell membrane to facilitate reduction of molecular oxygen and rapid

accumulation of superoxide. Cytochrome gp-91 subunit acts as a H+ channel to enable charge compensation (Hancock, Desikan et al. 2001).

The second step utilises specific superoxide dismutase (SOD) enzymes that reduce superoxide to hydrogen peroxide. The functional component of SOD incorporates one of three transition metal complexes containing; manganese, copper or zinc. The key feature of these enzymes is the use of a transitional metal ion that can alter its redox status, between oxidized and reduced forms, thereby acting as an electron donor.

The final step for hydrogen peroxide can be along several pathways. Hydrogen peroxide can be subsequently reduced to water by catalase, or decomposed by glutathione and thioredoxin dependent peroxidases. In parallel to these pathways other reducing agents, including metallothionen and haemoxygenase act as buffers for ROS. Alternatively, hydrogen peroxide can be reduced further to form hydroxyl radicals or 'free radicals'. It is these end products of oxygen reduction that represent most reactive and potent form of ROS. Generation of hydroxyl radicals can be facilitated by the Fenton and Haber-Weiss reactions; both reactions are reliant on ability of free metal ions to act as redox catalysts. Although Iron is the predominant catalyst, other transition metals, notably copper, provide a means for hydroxyl radical generation by Fenton chemistry.

Figure 1.3.2: Pathways of ROS generation

NADPH oxidase

(1) $O_2 + e^- \rightarrow O_2^{\cdot -}$

$2O_2 + NADPH \rightarrow 2O_2^{\cdot -} + NADPH^+ + H^+$

Superoxide dismutase

(2) $2O_2^{\cdot -} + 2\,H^+ \rightarrow H_2O_2 + O_2$

Glutathione / Catalase

(3) $H_2O_2 \rightarrow H_2O$

Fenton Reaction

(4) $H_2O_2 \rightarrow OH^{\cdot} + OH^-$

(4i) $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\cdot} + OH^-$

(4ii) $Fe^{3+} + O_2^{\cdot -} \rightarrow Fe^{2+} + O_2$

Haber-Weiss reaction

(5) $O_2^{\cdot -} + H_2O_2 \rightarrow OH^{\cdot} + OH^- + O_2$

Figure 1.3.2:

(1) NADPH catalyses reduction of molecular oxygen (O_2) *to superoxide* ($O_2^{\cdot -}$).

(2) Further reduction of superoxide produces hydrogen peroxide. The reaction can occur spontaneously especially at low pH. However, under physiological conditions a family of superoxide dismutase enzymes act as catalysts.

(3) Glutathione is oxidized by Glutathione peroxidase thereby reducing and removing hydrogen peroxide. The redox cycle is completed by reduction of glutathione by NADPH, which is itself liked to the Krebs cycle by conversion of glucose.

(4) Further reduction leads to formation of highly reactive hydroxyl radicals. In equation 4i, the Fenton reaction, ferrous iron is oxidized to the ferric state with generation of hydroxyl radicals. Equation 4ii, reduction of ferric iron to ferrous state occurs by oxidation of superoxide.

(5) Haber-Weiss reaction. Net reaction of equations 4i, the Fenton reaction, & 4ii. Hydroxyl radical is generated by interaction of superoxide and hydrogen peroxide. It provides a means to generate highly reactive free radicals from less radical and more controlled species. A reactive transition metal such as copper can mediate the reaction.

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1.3.4. ROS are involved in cell signalling

ROS have traditionally been regarded as dangerous and therefore tightly controlled in oxygen-utilising enzymatic reactions involved in cellular defence (oxidases, peroxidases, lipoxygenases) or in the mitochondrial cytochromes. The tight control of ROS is reflected by the spatial location of NADPH oxidase, found in neutrophils and mitochondria, and of superoxide dismutases in the mitochondria and cytoplasm.

Evidence for ROS as signalling molecules developed as it was seen that many proteins triggered ROS production as secondary messengers; PDGF, EGF, AgII and a host of cytokines (Finkel 2000). The discovery of a NADPH oxidase capable of generating ROS in non-phagocytic cells suggested a second role for ROS, separate to cellular defence. Similarly the rac family of proteins, essential for assembly of NADPH oxidase and a family of NADPH-dependent superoxide generating enzymes (NOX) (similar to the gp91-phox subunit of NADPH oxidase capable of generating superoxide) were also described in non phagocytic cells (Suh, Arnold et al. 1999; Cheng, Cao et al. 2001).

The question was whether the ROS generated a toxic or non-toxic signalling response? ROS are markers of cellular redox and hence cellular oxygenation status. Rapidly generated with nanosecond half-lives, ROS would provide ideal triggers for intra-cellular signal transduction. Experiments affecting ROS, addition of hydrogen peroxide or inhibition of production by catalyse, affected several protein signal transduction mechanisms (Finkel 2000), as well as calcium signalling (Suzuki, Forman et al. 1997). ROS have since been shown to have an important role in activation of various kinases; mitogen activated protein kinase (MAP kinase), extracellular regulated kinase (ERK

kinase) and c-jun N terminal kinase (JNK). ROS act as secondary messengers for a variety of genes in response to oxidative stress (Morel and Barouki 1999). Also a feedback pathway is possible as ROS activate antioxidant enzymes involved in protection from oxidative stress (e.g. glutathione peroxidase, metallothionen and thioredoxin) (Allen and Tresini 2000).

1.3.5. ROS signalling are involved in the HIF-1 pathway

ROS are a by-product of aerobic metabolism and are rapidly generated with short half-life and direct local effects. Therefore ROS provide an ideal response mechanism to alteration in cellular oxygenation. Also, other oxygenation responsive transcription factors are stimulated by ROS, such as the hyperoxia responsive transcription factor NFκβ. In the last decade the concept that HIF was redox sensitive derived from various sources. However, much disagreement exists about the exact nature and pathway of ROS involved in HIF-1 induction, also the source of these ROS. Two models are presented here looking at ROS and HIF-1.

A large group from Germany that includes W. Jenkleman, J. Fandrey, T. Keitzmann, K. Jungerman, and H. Acker have built a wealth of experimental evidence consistently supporting a role for ROS in oxygen sensing (Kietzmann and Gorlach 2005). An alternative theory proposed by Professor Chandel's group in America supports the role of the mitochondria in ROS generation, suggesting a functioning cytochrome chain to be essential for HIF-1 induction (Bell, Emerling et al. 2005).

1.3.6. Cytoplasmic ROS mediate HIF-1 signalling

The role for ROS in oxygen sensing and signalling was supported by the finding that hydrogen peroxide in cell culture mimicked effect of oxygen by inhibition of EPO induction in HEPG2 cells (Fandrey, Frede et al. 1994; Fandrey, Frede et al. 1997). Hydrogen peroxide also mimicked oxygen dependent gene expression with increased PCK and reduced GK mRNA expression (Jungermann and Kietzmann 1997). Further support was given by use of the hydroxyl radical chelator DMTU, which reversed the effect of hydrogen peroxide on EPO induction (Canbolat, Fandrey et al. 1998). It also reduced PCK and increased GK mRNA expression. (Kietzmann, Porwol et al. 1998). Direct evidence for ROS in the control of HIF-1 came from experiments showing that hydrogen peroxide affected HIF-1a nuclear accumulation (Huang, Arany et al. 1996; Wang, Jiang et al. 1995). The effect of ROS in mimicking oxygenation was further supported by finding that hypoxia reduced ROS in cell culture and production of ROS increased proportionally with increased oxygen tension (Kietzmann, Fandrey et al. 2000).

These data supported the role that hydrogen peroxide generated hydroxyl radicals were involved in oxygen sensing. The German group suggested that in vivo ROS could be produced by the Fenton reaction. Further experiments supported this theory; iron and a Fenton reaction were colocatised in the same perinuclear cytoplasmic space, as well as oxygen dependent generation of hydroxyl radicals associated with GK and PCK control (Kietzmann, Porwol et al. 1998). Developing these experiments they co-localised the Fenton reaction and hydroxyl radicals to the perinuclear endoplasmic reticulum; the same place they identified HIF-1a under normoxia. Introducing a ROS scavenger led to

reduced PHD activity and promoted HIF-1 induction with involvement of Ref-I and recruitment of p300 (Liu, Berchner-Pfannschmidt et al. 2004)

They suggested that the oxygen sensor itself was similar to known ROS generators such as a cytosolic non-mitochondrial cytochrome similar to NADPH oxidase (Gorlach, Holtermann et al. 1993; Ehleben, Bolling et al. 1998; Zhu and Bunn 1999). Cell lines deficient of two key subunits of the neutrophil NADPH oxidase, gp91 and p22 had reduced hypoxic gene induction (Wenger, Marti et al. 1996). Inhibition of NADPH-P450 inhibited hypoxic EPO induction and over expression increased HIF-1a DNA binding (Osada, Imaoka et al. 2002). Mice lacking gp91 showed deficient oxygen sensing (Fu, Wang et al. 2000). Over expression of p22 in transgenic mouse resulted in increased hydrogen peroxide production and increased HIF-1a and VEGF (Khatri, Johnson et al. 2004).

In Summary these data suggested ROS were directly involved in the pathway of HIF-1 activation and that a cytochrome similar to NADPH oxidase, located in the cytosol, containing a metal core capable of generating ROS, may act as an oxygen sensor (Kietzmann and Gorlach 2005) (figure 1.3.3).

Figure 1.3.3: Possible pathway of HIF-1 induction by ROS

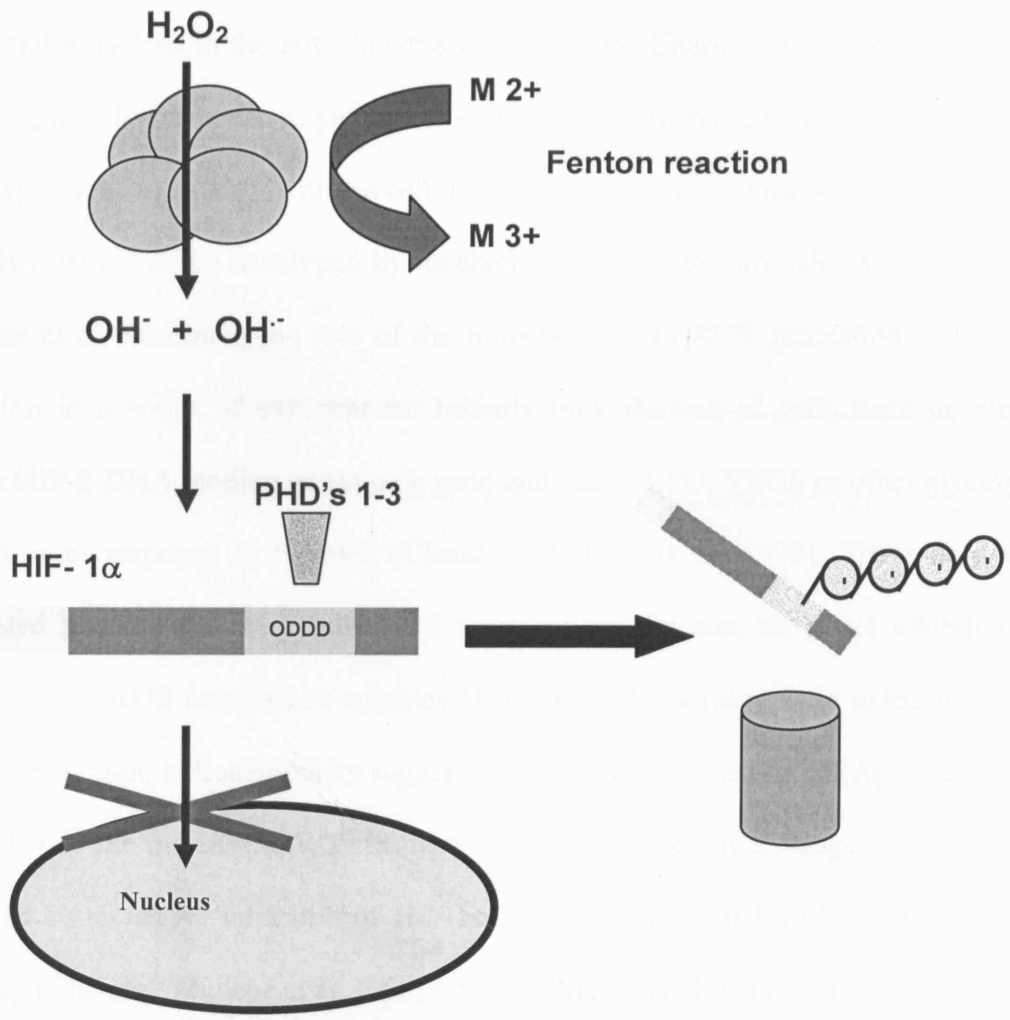


Figure 1.3.3. A proposed mechanism of oxygen sensing. Sequential reduction of molecular oxygen produces ROS as a normal by product. A cytochrome similar to NADPH oxidase, containing a metal core (M) capable of generating ROS, may act generate hydroxyl radicals, which directly activate the prolene hydroxylases and promote HIF-1a destruction.

1.3.7. Mitochondrial ROS mediate HIF-1 signalling

Separate proposals for the origin of ROS responsible for HIF-1 signalling have centred on the role of the mitochondria. It is suggested that 2% of all oxygen that undergoes sequential reduction in the mitochondria ends as ROS (Chance, Oshino et al. 1973). It seems logical that the main organelle responsible for oxygen use would be connected to the oxygen sensing system within a cell. Data were developed in mitochondria deficient HEP3B cells (p^0 cells) developed by incubation in ethidium bromide over 2-3 weeks. Chandel et al confirmed the role of the mitochondria in ROS generation and HIF-1 induction in a series of experiments. Initially they showed p^0 cells were unable to induce HIF-1 DNA binding or hypoxic gene induction (EPO, VEGF or other glycolytic mRNA's) in response to hypoxia (Chandel, Maltepe et al. 1998). These data also suggested that in wild type cells ROS increased in hypoxia, an effect inhibited by antioxidants. ROS localised to complex III of the mitochondria were associated with HIF-1a induction, although others suggested complex I to be essential (Agani, Pichiule et al. 2000). The increase in ROS in hypoxia was mimicked by hydrogen peroxide and inhibited by catalase. Induction of HIF-1a was also associated with the PI-3 kinase pathway (Chandel, Maltepe et al. 1998; Chandel, McClintock et al. 2000).

Despite these findings other researchers demonstrated a reduction of ROS in response to hypoxia and ability for HIF-1 induction in p^0 cells, although different results were found for the role of hydrogen peroxide. (Srinivas, Leshchinsky et al. 2001; Vaux, Metzen et al. 2001).

More recently three groups provided complimentary evidence supporting the mitochondria as an integral part of the oxygen-sensing pathway. Using a redox sensitive probe, they confirmed previous studies using dye conversion assays to show ROS increased in hypoxia. RNA interference techniques to inhibit complex III prevented this ROS increase and prevented HIF-1 induction, an effect also seen by disruption of cytochrome C (Brunelle, Bell et al. 2005; Guzy, Hoyos et al. 2005; Mansfield, Guzy et al. 2005). Data that directly conflicts that presented earlier from the German group.

1.4.1 Copper

Copper is essential for life, fulfilling an important role in cellular biology. It is the ability of copper to cycle between two oxidative states, oxidised Cu^{++} and reduced Cu^{+} that enables copper to play a key role in regulation of cellular redox chemistry (Camakaris, Voskoboinik et al. 1999). By forming an active redox component of several metal core enzymes, copper plays an integral role in a number of biological processes including mitochondrial oxidative phosphorylation, free radical chemistry, neurotransmitter production, and iron metabolism. Essential for cellular function, copper is paradoxically highly toxic. Uncontrolled, copper can rapidly generate ROS with consequent damage on lipids, proteins and DNA. Consequently copper homeostasis is tightly regulated, manifest by an apparent lack of free copper to exist intracellularly as all intracellular is copper tightly bound with high specificity to target proteins (Rae, Schmidt et al. 1999). Failure of copper homeostasis is dramatically illustrated by genetic conditions resulting in copper excess in Wilson's disease and copper deficiency in Menkes syndrome. The original description by Wilson referred to copper as a "morbid toxin".

1.4.2. Copper homoeostasis is tightly regulated

Copper homeostasis is balanced between absorption from the gastrointestinal tract and excretion in the bile; urinary excretion is negligible under normal circumstances. At all times copper is bound to proteins (ceruloplasmin, albumin and metallothioneins). Ceruloplasmin harbours over 95% of plasma copper and delivers copper to extra hepatic tissues via ceruloplasmin receptors. Albumin and transcuprein are thought to play an important role in delivering copper to hepatocytes. Metallothioneins sequester copper into an extremely stable heptacopper thiolate cluster and provide a buffer mechanism for these other proteins (Camakaris, Voskoboinik et al. 1999). Interestingly ceruloplasmin knockout mice showed no defect in copper absorption from intestine, uptake to liver or bilary excretion, suggesting significant flexibility and reserve to control copper homeostasis. (Meyer, Durley et al. 2001).

At a cellular level copper haemostasis is tightly controlled. Free intracellular copper is postulated not to exist and copper is continually bound to transport proteins called metallochaperones, which escort copper to destination cuproenzymes (Valentine and Gralla 1997). Copper needs to be in its reduced state (Cu^{+}) for intracellular copper trafficking (Rosenzweig and O'Halloran 2000). Thus copper uptake is initiated by a metalloreductase reduction of Cu^{++} to Cu^{+}. The copper transport protein Ctr1 can then transport reduced copper across the plasma membrane (Lee, Prohaska et al. 2001; Lee, Pena et al. 2002). Strongly expressed in liver and kidney Ctr1, is essential for embryonic development, although exact function of copper in embryological development remains unknown.

Different specific metallochaperones then escort copper to the target cuproenzymes. These include; COX 17 transports copper to SCO1 in the mitochondrial intermembrane space and subsequently cytochrome C oxidase in the mitochondria, and CCS transports copper to SOD1 in the cytosol (Rae, Schmidt et al. 1999; Schmidt, Kunst et al. 2000). The secretory pathway is mediated by HAH 1 (Atx1/Atox1 in yeast) via the ATPase 7(A&B) transport proteins (Hamza, Faisst et al. 2001) to ceruloplasmin which acts as a multicopper oxidase (Fet3 in yeast). The secretory pathway is dependent on ATPase 7, which is located in trans-golgi network (Suzuki and Gitlin 1999) and near the canillicular membrane of hepatocytes (Schaefer, Hopkins et al. 1999). The inherited disorders of copper homeostasis are manifest by genetic defects in the ATPase transport proteins, which prevent copper uptake from the gut in Menkes syndrome leading to systemic copper insufficiency, or prevent copper excretion leading to liver toxicity in Wilson's disease.

At cellular level, the gene defect in ATPase 7B is found predominantly in the liver. Located on transgolgi network, ATPase 7B mediates incorporation of copper into apo-ceruloplasmin. Under normal conditions elevated copper results in re-distribution of both ATPase 7A & 7B. ATPase 7A is predominantly expressed in extra-hepatic tissues and in response to increased copper moves to plasma membrane presumably to efflux copper from the cell. ATPase 7B is predominantly expressed in the liver and in response to increased copper moves to intracellular vesicles promoting excretion of copper in to bile, on cannilicular side of hepatocytes. The defect in ATPase 7B results in a failure of excretion of copper and abnormal intracellular localisation of the protein, noticeably within mitochondria, which coincides with the oxidative damage seen.

1.4.3. Copper controls cellular redox balance

Copper has two main functions in the regulation of redox chemistry in the cell. The first is mediated by cuproenzymes, which include; cytochrome oxidase in the electron transport chain of the mitochondria and the cytosolic form of superoxide dismutase (CuSOD), which catalyses hydrogen peroxide formation from superoxide. Although predominantly cytosolic a small proportion of CuSOD is found in the intermembrane space of mitochondria and may be an important first line of defence against superoxide generation by incomplete reduction of oxygen in the mitochondria. Secondly copper can directly mediate free radical production by the Fenton and Haber Weiss reactions (figure 1.4.3).

Figure 1.4.3: Pathway of copper mediated ROS generation

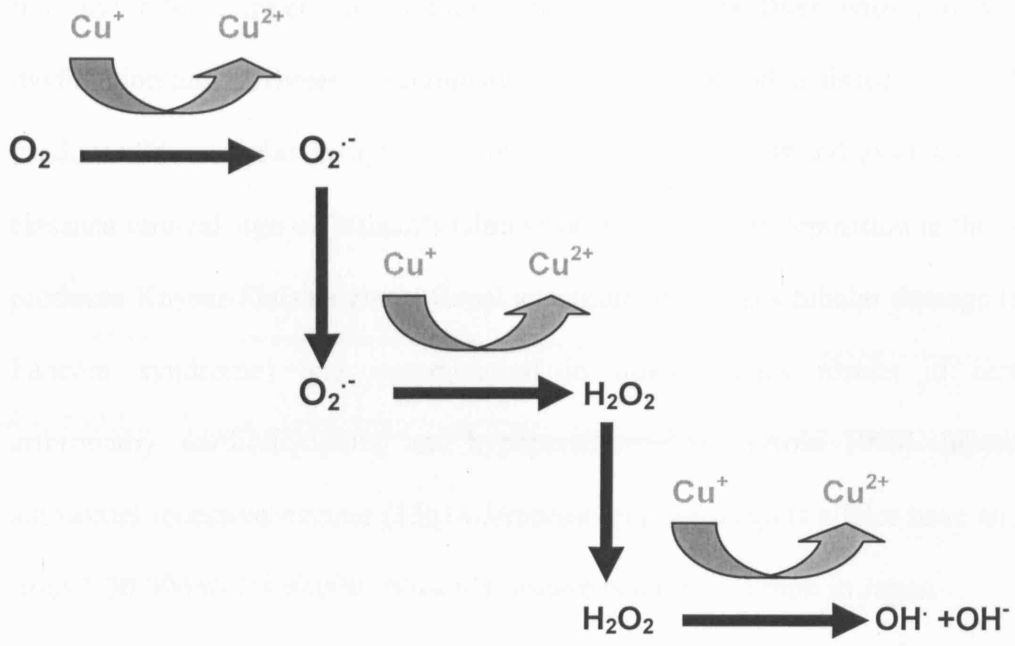


Figure 1.4.3: Copper mediated ROS generation. Copper has the ability to exist in two redox states, oxidized cupric Cu^{++} and reduced cuprous Cu^{+}. Copper is integrally involved in ROS balance in the cell and is ideally positioned to be involved in ROS generation by the Fenton reaction as demonstrated here.

1.4.4. Wilson's Disease

Described in 1912 by S. A. Kinnier Wilson and attributed to copper toxicity by Cumings in 1948, Wilson's disease results from failure to excrete copper from the liver into the bile. Copper concentration increases in the liver with progressive liver dysfunction and cirrhosis. Accumulation also occurs in other tissues. In the brain, this produces Parkinsonian symptoms of involuntary movements and dysdidacokinesis. The classical clinical sign of Wilson's Disease is excess copper deposition in the cornea that produces Kayser-Fleisher rings. Renal accumulation causes tubular damage (secondary Fanconi syndrome) and accumulation in other tissues results in osteoporosis, arthropathy, cardiomyopathy and hypoparathyroidism. (Aoki 1999). Inherited in an autosomal recessive manner (13q14.3/recessive) homozygous alleles have an incidence from 1:30,000 to 1:100,000. Wilson's disease is more common in Japan.

Biochemical serum changes include; serum ceruloplasmin levels decrease and non-ceruloplasmin copper levels increase. Urinary copper excretion increases greatly as, does hepatic copper content. Treatment includes low copper diet, chelation by D-penicillamine or Trienthine Tetramine and blockage of intestinal absorption by zinc or potassium. Zinc works by induction of metallothionein (Shimizu, Yamaguchi et al. 1999).

In Wilson's disease the increased copper in the liver leads to lysosomal uptake and degradation of copper-loaded metallothionein that then promotes lipid peroxidation and hepatocellular damage. Sub cellular localisation demonstrates copper accumulation in the mitochondria of patients with Wilson's disease. This is concomitant with a defect in

mitochondrial respiratory-chain activity, suggesting a possible further mechanism of ATPase 7B in mitochondria copper transport. Excess copper is released in the serum where copper is loosely bound to albumin and other ligands and can readily diffuse out of the vascular compartment and deposit in tissues (Sternleib and Scheinberg 1993).

Models for Wilson's disease include Beddlington terrier, Long-Evan Cinnamon rat and toxic milk mouse (Brewer 1998; Terada and Sugiyama 1999). Several other clinical conditions associated with copper excess; diabetic patients with vascular complications of diabetic retinopathy, hypertension and arteriopathy (Beshgetoor and Hambidge 1998), primary bilary cirrhosis (PBC), primary sclerosing cholangitis (PSC) and hepatocellular carcinoma (HCC). Additionally copper excess has been indicated in neurodegenerative diseases of Alzheimer's, familial amyotrohic lateral sclerosis (ALS) and prion diseases. In Alzheimer's disease, increased copper concentration found in grey matter was postulated to interact with amyloid precursor protein to induce ROS generation and consequent cellular damage (Strausak, Mercer et al. 2001).

1.4.5. Menkes Syndrome

Described by J. H. Menkes in 1962, and associated with copper deficiency in 1972 by Danks, Menkes syndrome is a failure of intestinal copper absorption and a low serum copper that leads to reduced activity of copper containing enzymes. Inherited in an X-linked recessive (Xq13.3/recessive) manner it is a rare disease with, reported incidence of 1:300,000 live births. Uniformly lethal by three years, copper deficiency results in connective tissue and vascular defects, skin laxity, hernias, macrovascularisation and arterial rupture. Cerebral and cerebellar degeneration causes severe mental and physical

retardation and a peculiar 'steely' hair. Milder (allelic) forms have subsequently been described, the mildest being occipital horn syndrome. (Sternleib and Scheinberg 1993; Aoki 1999).

The defect is in a transporting P-type ATPase (ATP7A) similar to that seen in Wilson's Disease. Under normal conditions increased copper induces ATPase 7A to move from the trans-Golgi network to plasma membrane, which enables copper to move out of the intestinal cell to blood proteins. In Menkes Disease, despite normal uptake by intestinal cells the defect in ATPase 7A function prevents transport out from the cell and hence absorption from the gut. The result is low serum copper, ceruloplasmin, liver copper and consequent loss of function of cuproenzymes (Kodama and Murata 1999; Kodama, Murata et al. 1999). Management remains parenteral copper.

Clinical manifestations of the disease can in part be explained by loss of function of associated cuproenzymes. Dopamine β-hydroxylase is a critical enzyme in catecholamine synthesis. Superoxide dismutase is responsible for processing of free radical superoxide to hydrogen peroxide. Defective SOD function is seen in an inherited disease Amyotrophic Lateral Sclerosis (ALS), a motor neurone disease (Rowland and Shneider 2001). Classical motor neurone disease is also associated with loss of cytochrome C oxidase function (Strausak, Mercer et al. 2001).

Models for Menkes disease include Mottled or Brindled mouse, Macular mouse and Blotchy mouse (occipital horn syndrome). Several clinical conditions can also give rise to copper deficiency. These include inadequate copper intake in premature babies,

babies fed only on cows' milk, malabsorption syndromes or inadequate supplementation in patients on long term TPN. Excessive loss can be seen in nephritic syndrome due to caeruloplasmin excretion or patients undergoing CAPD (Beshgetoor and Hambidge 1998).

In summary, copper is important in cellular redox balance and the control of ROS. Copper deficiency may have direct impact on cupproenzymes, in particular SOD. Copper toxicity may have direct effect on ROS, facilitating production of hydroxyl free radical generation by the Fenton reaction. Furthermore copper may independently act to speed up iron dependent Fenton reaction generation of ROS. Consequently intracellular copper balance may affect cellular ROS and redox balance.

Hypothesis

'Copper is involved in the HIF-1 induction pathway and a change in copper concentration will affect HIF-1 expression'

Chapter 2: Methods

Cell culture

Cell lines

Hep3B cells were obtained from European Collection of Animal Cell Cultures (Biologics, Salisbury, UK). Chinese Hamster Ovary cells (CHO) were a gift from Dr D. Stroka PHD (Department of Visceral Surgery, University of Berne, Switzerland) and primary hepatocytes a gift from Melanie Baker (Liver labs, University of Birmingham, UK).

Culture conditions

Maintenance: Cells were maintained in Tissue Culture Medium (TCM), composed of Dulbecco's Modified Eagle's Medium (DMEM; Sigma D5671, Sigma-Aldrich Co. Irvine UK) supplemented with 10% heat inactivated Foetal Calf Serum (FCS) and 1% Penicillin-Streptomycin-Glutamine (Gibco BRL Life Technologies LTD, Paisley Scotland). Cells were grown with 10 or 20ml of TCM on 80 or 150cm^2 plates (Nalge Nunc International) at 37^{o}C in a 5% CO_2 water-jacketed incubator until confluent and ready for passaging.

Confluence was defined, when cells have exhausted all surface area and growth was stopped due to contact inhibition. To passage cells, TCM was removed, cells washed with PBS and detached by incubation for 3-5min with Trypsin–EDTA (Gibco BRL Life Technologies LTD, Paisley Scotland). The cell suspension was then centrifuged at 640rpm for 5 min, the supernatant discarded and the pellet re-suspended in an

appropriate amount of TCM. Cell suspension was re-plated at a ratio of 1:3 for HEP 3B and 1:4 for CHO to allow for growth.

Freezing: For long-term storage, pre-confluent cell cultures were harvested and centrifuged as per protocol. Aliquots of $5x10^6$ cells were re-suspended in 1ml TCM, containing 50% FCS and 10% dimethyl sulfoxide (DMSO) (Sigma-Aldrich Co. Irvine UK). Cell aliquots were frozen at $^{-}80^{o}C$, wrapped in tissue paper to ensure a steady reduction in temperature. For long-term storage cells were kept in the gaseous phase of liquid nitrogen.

Thawing: Cells were recovered by quickly thawing in a $37^{o}C$ water bath. Cell suspension was immediately transferred to 10ml TCM, mixed and centrifuged to remove all DMSO. Cells then re-suspended in appropriate amount of TCM and plated.

Experimental conditions

Cells were used for experiments in 80cm^2 plates when 50-80% confluent.

Hypoxia: Cells were incubated under hypoxic conditions of 2% O_2, 5% CO_2 and the remainder N_2 at 37°C using a dual gas water-jacketed incubator (Model 3131 Forma Scientific Inc. Ohio USA).

Chemicals: Chemicals were obtained from Sigma-Aldrich Co. Irvine UK unless otherwise described. All chemical used are listed in appendix. All chemical solutions were freshly made in sterile water at 0.1M solutions before being added directly to TCM. For copper chelation, chemicals were added four days before the cells were used for experimentation.

Cell viability

MTT cell viability assay: The MTT (Thiazolyl Blue; 3-[4, 5-Dimethylthiazol–2-yl]-2,5-diphenyltetrazolium bromide) assay (Sigma-Aldrich Co. Irvine UK) quantifies cell proliferation by activity of mitochondrial dehydrogenases. MTT a water-soluble tetrazolium salt produces a yellowish solution. This is converted to an insoluble purple formazan by dehydrogenase enzymes. Stock solution was prepared with 5mg/ml MTT in RPMI-1640 without phenol red and passed through a 0.2μm filter.

Cells were counted on a hemocytometer, seeded onto a 96 well plate at a concentration of 10,000 per well in 125μl TCM. After settling overnight 12.5μl of MTT stock solution was added to each well and incubated for 4 hours. The formazan produced was then dissolved with 100μl of 0.1M HCL in isopropanol. The absorbency of converted dye was measured at a wavelength of 570nm with background subtraction of 630-690nm.

Trypan blue: Cell viability and cell number was also determined by Trypan blue (Sigma-Aldrich Co. Irvine UK) exclusion using a standard hemocytometer chamber. Trypan blue is excluded from viable cell membranes and taken up by dead cells.

Intracellular copper concentration

Whole cell copper concentration was measured by atomic absorption spectrophotometer at department of Clinical Pharmacology, University of Berne, Switzerland. The assay works by argon plasma (4000°K) breaking all molecules to atoms and measuring a specific band, characteristic for element of interest (Copper).

Western blot

Preparation of nuclear extracts: Cells were washed twice with Phosphate Buffered Saline (PBS) (4°C) on ice and incubated for 10min in 500µl cell lysis buffer (appendix) with a protease inhibitor cocktail added (appendix). Cells were scraped from plate and cytoplasmic fraction separated by centrifugation at 5,550rpm (4°C) for 5min. The remaining pellet was suspended in 30µl nuclear extraction buffer (appendix) with a protease inhibitor cocktail (appendix) and incubated on ice for 15min, vortexing every 5min. The nuclear extract was then separated by centrifugation at 14,000rpm (4°C) for 5min.

Protein concentration: The concentration of nuclear protein extracted was determined by coumassie method (Bio-Rad). Values were compared to a standard curve generated from serial dilutions of Bovine Serum Albumin (BSA), analysed by spectrophotometer at 620nm (Labsystems Multiskan Biochromatic, Finland).

Gel electrophoresis: Equal concentrations of the nuclear proteins (20µg) were mixed with lamelli buffer (4µl) and water (total 16µl) then denatured by incubating at 95°C for 5 min. Protein separation was performed using a 7.5% SDS-polyacrylamide denaturing gel (appendix) run at 100mV for 60-90 min. Pre-stained molecular weight markers (Rainbow marker, Amersham, Buckinghamshire, UK) were used to monitor protein migration.

Transfer: Proteins were transferred onto a nitrocellulose membrane (HYBOND ECL, Amersham) by semi-dry transfer at 300mA for 60min (Trans-Blot SD, Bio-Rad). Transfer and equal protein loading was confirmed by staining the membrane with ponceau red. The membrane then washed in PBS for 30 min prior to overnight incubation in 4% milk/PBS at 4°C.

Immunoblotting: The primary antibody was mouse monoclonal anti-HIF-1a (Novus Biologicals Inc. Littleton, CO, USA) diluted 1:500 in 4% milk / PBS, this was applied to membrane for 4 hours at room temperature with gentle rotation. The membrane was then washed for 15 min twice in PBS and incubated with horseradish peroxidase conjugated goat anti-mouse antibody (Pierce, Rockford IL. USA), diluted 1:5000 in 4% milk/PBS for 1 hour at room temperature with gentle rotation.

To confirm equal protein loading per lane immunoblotting for Sp1 was performed after completion of Western Blot for HIF-1a. Primary antibody was rabbit polyclonal IgG anti-Sp1 (Santa Cruz Biotechnology, Santa Cruz, CA. USA) diluted 1:5000 in 4% milk/PBS applied to membrane for 1 hour at room temperature with gentle rotation. The second antibody was horseradish peroxidase conjugated goat anti-rabbit antibody (Dako, Denmark), diluted 1:5000 in 4% milk/PBS for 1 hour at room temperature with gentle rotation. Washes were the same as for HIF-1a protocol.

Detection: Membrane was washed three times in PBS for 15 min then incubated with enhanced chemi-luminescence assay solution for 60 seconds (appendix). Signal was detected by exposing membrane to film (X-omat AR, Kodak) for 3-5min and developing.

Northern blot

RNA extraction: RNA was isolated from cells using RNAzol B (Biogenesis, Poole, England) following a modified manufacturer protocol. Cells were washed in PBS (4°C) and incubated with RNAzol B (1ml per 80cm plate). Cell lysate then shaken vigorously with 100µl chloroform for 15s, incubated on ice for 5min and centrifuged at 12,000rpm (4°C) for 15min. The aqueous phase was removed and mixed with an equal volume of isopropanol overnight at -20°C. The RNA was pelleted by centrifugation for 15min at 12,000rpm (4°C) and washed with 800µl 70% ethanol before further centrifugation at 7,500rpm (4°C) for 8min. The remaining RNA pellet was air dried, re-suspended in 10-20µl DEPC-treated water and dissolved at 56°C for 5-10min.

RNA quantification: Quantification was by spectrometer analysis at 260nm (Helio, Unicam, Cambridge UK) using DEPC-treated water as a zero marker.

Gel electrophoresis: Equal amounts of total cellular RNA (25µg) were made up to equal volumes with DEPC-treated water mixed with 3 volumes of loading buffer, then denatured by incubating at 68°C for 5 min. RNA was separated on formaldehyde gel, run at 70mV for 4-5 hours in MOPS (1x) running buffer (appendix). Ribosomal RNA was visualised by UV transillumination to monitor separation and loading.

Transfer: The formaldehyde gel was washed in DEPC-treated water 3x for 5min. RNA was transferred to a nylon membrane (Hybond-N+, Amersham,

Buckinghamshire, UK) by overnight capillary blotting in 10x SSC buffer. The membrane was then baked at 80°C for 2 hours.

Hybridization: Probes for mouse full length mouse HIF-1a or VEGF cDNA fragments were labelled with [α-^{32}P] dCTP using a standard random primed Klenow polymerase reaction. The membrane was initially incubated at 42^{0}C for 2 hours with 10ml of pre-hybridisation mix, before overnight incubation at 42^{0}C with hybridisation mix. The membrane was washed twice with 2x SSC and 0.1% SDS for 5 min at 42^{0}C and again with 0.2x SSC and 0.1 % SDS for 1Hr at 42^{0}C.

Detection: Autoradiograph performed by exposing membrane to film (X-omat AR, Kodak), with an intensifying screen for 24-48 hours at ˉ70^{0}C. The film was then developed.

Reporter gene assay

Reporter construct: A HIF-1 responsive luciferase reporter was constructed (figure 2.1). The firefly luciferase reporter gene plasmid (pH3SVL) containing a total of 6 HBS's derived from transferrin HRE was constructed by inserting 2 copies of the oligonucleotide TfHBSww into *Sma*I site of the plasmid pGLTfHBSww.

Stable transfection: pH3SVL was linearized with *Xmn*I, mixed with *Eco*R1-linearized neomycin expression vector pSV2neo at a molar ratio of 100:1, and coelectroorated into CHO cells. Following limited dilution and selection in 2mg/ml G418 (Alexis, Laufelfingen, Switzerland), a hypoxia reporter cell line (termed HRCHO5) was chosen on efficiency of the hypoxia reporter gene induction.

Transfected CHO cells were seeded at a density of $1x10^6$ cells with 3ml of TCM onto 6 well plates (Nalge, Nunc International) and allowed to settle for 4hr. Exposure to hypoxia or chemical stimulus was for 24hr.

Preparation of Cell lysates: All TCM was removed, cells were washed twice with PBS (4°C) and Reporter Lysis Buffer (appendix) added. Cells were removed by scraping and a single freeze thaw performed to ensure cell lysis. Cell lysate then separated by centrifugation at 12,000rpm (4°C) for 2min. Protein concentration was determined by coumassie method (Bio-Rad) as described before

Luciferase Assay: Quantification of luciferase activity was determined by Promega Luciferase Assay System (Promega, Madison USA) as per manufacturers instructions. 100µl of Luciferase Assay Reagent was added to 20µl of each cell lysate on a 96 well plate. Samples were read in a Buthold luminometer using 100µl injection mix. Light emission was measured after a 2.05s delay for 10s and a second measurement was taken after a 10s delay for a further 10s.

Immunohistochemistry

Tissue preparation: Tissue blocks were immediately frozen in isopentene pre-chilled by immersion in liquid nitrogen, before storage at ¯80°C. For animals this was immediately after euthanasia and for human specimens immediately after resection of the specimen.

Frozen sections (6 microns) were mounted on Superfrost Plus slides (Menzel-Glaser, Germany) and dried on a 50°C hotplate for 2 min. Then fixed in 4% formaldehyde in PBS (pH 7.4) for 10 min and washed in PBS (3x over 10 min).

Antibody incubation: At the time experiments were performed, two antibodies against HIF-1a were available. The first was a commercially available Novus E2 monoclonal antibody. The second was a gift from Dr. R. Wenger, Zurich and was a polyclonal chicken antibody (Camenisch, Tini et al. 1999). The antibody protocols used are listed below, and further details on the protocols used are given in the relevant chapters.

All antibodies were diluted in 0.05mol/l TBS containing 0.1% tween-20 and 10% NSS. Slides were incubated with the anti-HIF-1a antibody or 10% NSS (negative control) overnight at 4°C. The secondary horseradish peroxidase (HRP)-conjugated antibody was used at 1:100 concentration in TBS + 0.1% tween with 10% NSS for 45min at RT, and a tertiary (HRP)-conjugated antibody was used at 1:100 concentration in TBS +

0.1% tween with 10% NSS for 45min at RT. Slides were washed between antibody incubations in TBS for 10min

Detection: Peroxidase reaction was detected by incubation with diaminobenzidine (DAB) for 10min at RT, prepared as per manufacturers instructions with 45mg of sodium azide and filtered 2x before use. Sequential sections were counterstained with hematoxylin, dehydrated and mounted in DPX medium.

Polyclonal Protocol:

	Antibody	Manufacturer
Primary	Polyclonal chicken IgY anti-HIF-1a	Wenger (Switzerland)
Secondary	G134A Rabbit anti-chicken IgY - (HRP)	Pierce, Rockford, IL. USA
Tertiary	Goat anti-rabbit - (HRP)	DAKO, Carpenteria, CA. USA

Monoclonal Protocol:

	Antibody	Manufacturer
Primary	Mouse monoclonal anti-HIF-1a	Novus Biologicals Inc. Littleton, CO, USA
Secondary	Goat anti-mouse antibody - (HRP)	Pierce, Rockford, IL. USA
Tertiary	Goat anti-rabitt - (HRP)	DAKO, Carpenteria, CA. USA

(HRP)- horseradish peroxidase

HIF-1a quantification in human tissue

Isolation of nuclear proteins from tissue: Frozen tissue (500mg) (-80°C) was crushed in liquid nitrogen using a pre-chilled pestle and mortar. The powder was homogenized in 1ml of chilled C1 buffer (appendix) with a protease inhibitor cocktail added (appendix). Dunce manual type tissue grinder (Wheaton, USA) was used (ten strokes). After addition of NP-40 (10ul of 10%), the solution was incubated on ice for ten minutes. A further five strokes to homogenize tissue was performed and solution transferred to eppindorf tubes.

Non-cellular material and cytoplasmic fraction were separated by centrifugation for 5min at 1,000rpm (4°C) and discarded. The pellet, containing nuclei and cellular debris, was washed with 1 ml of C1 buffer and re-centrifuged at 1,000rpm (4°C) three times or until clean. Final spin was at 1,500rpm for 5min to ensure all C1 buffer was removed.

The pellet of nuclei was re-suspended in 50-100µl of C2 buffer (appendix) with 5-10µl 4M KCL (to avoid dilution of salt concentration) and incubated on ice for 30min, vortexing every 10min. Nuclear protein was separated by centrifugation at 12,000rpm (4°C) for 5min. Supernatant was separated into aliquots and stored at -80°C.

HIF-1a ELIZA: The TransAM HIF-1 Kit (Activ Motif, Rixensart, Belgium) was used according to manufacturers' protocol.
(www.activemotif.com/download/manual/transam-hif.pdf)

Statistics

Where appropriate data were analysed using the 'Instat 3' statistics software (www.graphpad.com). For comparison of normally distributed continuous data T-tests were used as appropriate, non-parametric data were compared by Mann Whitney test. For comparisons of multiple variables a Kruskall Wallis non-parametric ANOVA was used with a Dunn's multiple comparisons test. Where different tests were used specific details are given in the text.

Chapter 3: Effects of Increased and Reduced Copper on Cells in Culture

Summary of Chapter

Introduction: Cell culture was established using the HEP3B cell line, as HEP3B cells are the most studied cell line in the published literature on HIF-1a. The experimental protocols for HIF-1a are established and well validated. We wished to produce conditions of increased copper and reduced copper in cell culture. Copper in the form of copper chloride, $CuCl_2$ (μM) was added to the culture medium or in order to reduce copper, copper specific chemical chelators were used. The experiments performed here assess the affect of increased and reduced copper on intracellular copper concentration and the effect of the chemicals used on cell viability.

Method: HEP3B cells in culture were incubated either with addition of $CuCl_2$ or specific copper chelators. Intracellular copper concentration was measured by atomic absorption spectrophotometer. Cell viability was measured by cell count, Trypan Blue exclusion and MTT assay.

Results: Increased copper produced a 25% increase in intracellular copper concentration. High doses were toxic to the cells. All copper chelators studied produced a similar significant reduction (50%) in intracellular copper concentration, without evidence of cell toxicity.

Conclusion: Increased copper did produce a rise in intracellular copper concentration but this was not dose dependent. Copper depletion reduced intracellular copper concentration.

Introduction

Cell culture was established using the HEP3B cell line and experiments were performed with cells cultured under normal conditions in standard tissue culture media (TCM) as described. The experimental protocols for HIF-1a were essentially unchanged since the original papers by Semenza and Wang over a decade ago; they are therefore established and well validated.

Copper excess

For copper excess experiments cells were established in culture and the TCM was changed, fresh TCM was added supplemented with increased copper ($CuCL_2$) at concentrations indicated, for time periods indicated before harvesting for analysis.

The effect of copper excess on cells in culture has been looked at in several previous studies. In human glioma and neuroblastoma cell lines no increase in intracellular copper concentration was initially seen in response to additional copper in TCM up to 64uM. However, increased intracellular copper was seen at a concentration of 250uM in TCM. The increased copper promoted cell growth and increased intracellular free radical production. Cells were more suseptible to oxidative stress when exposed to the pro-oxidant ascorbic acid. (Watt and Hooper 2001). The effects of increased exogenous copper were seen in several cells lines. Low levels of exogenous copper increased activity of the inflammatory PI3K/AKT pathway whereas high concentrations (500uM) increased intracellular ROS (Ostrakhovitch, Lordnejad et al. 2002). In neuronal cell cultures increased exogenous copper increased hydrogen peroxide production and altered oxidative balance. In these experiments amyloid peptides were proposed to

directly bind and reduce copper extracellularly. Copper in a reduced form (Cu^{+}) mediated hydrogen peroxide production by Fenton chemistry. Hydrogen peroxide formed, was able to cross the cell membrane, alter intracellular redox balance and increase susceptibility of the cell to oxidative stress (Huang, Cuajungco et al. 1999). This proposed mechanism suggested that increased extracellular copper could alter intracellular redox balance without affecting intracellular copper concentration.

Studies in Keratinocyte lines, for models in wound healing, showed direct induction of VEGF in response to exogenous copper. Further, copper reduction in normal TCM inhibited the normal hydrogen peroxide mediated induction of VEGF. Copper mediated VEGF induction was inhibited by addition of wormannin, a specific inhibitor of PI3K. Suggesting that copper induced VEGF by affecting cellular redox status, involved the PI3K pathway associated with intracellular signalling. In vivo evidence in BalbC mice showed reduced time to wound healing in wounds treated with topical copper solution, associated with increased VEGF expression at wound edges (Sen, Khanna et al. 2002).

Taken together, data suggest that copper excess may exert different effects on cells; by induction of gene transcription or stimulation of cell signalling pathways. Copper may further indirectly affect cells by generation of ROS extracellularly, which mediate their effects on cell membrane or permeate through to exert an effect intracellularly. Considerable variability exists in cell toxicity to exogenous copper. Increased cell proliferation was reported in several cell lines and the concentration of exogenous copper in experiments varied from 2 - 500uM. Of note many studies were performed in different conditions; serum free media, use of non-specific chelators. Also different types of cell culture were studied including; embryonic undifferentiated, differentiated

specific neuronal, primary and immortalised cell lines. In part these many variations may explain the variation in results and findings.

Copper reduction

Three different copper specific chelating agents were used to produce copper reduction. The chemicals were N,N-Bis(2-aminoethyl)-1,3-propanedamine (Tet also termed 2,3,2-tetraamine or TETA), Trithylenetetramine tetrahydrochloride (Trien) and 2,9-Diphenyl-1,10-phenanthrolinedisulfonic acid (Bath).

Tet: Tet is the most studied of the three chelators used. Hopkins & Failla studied copper depletion in Jurkat human T Lymphocytes by incubation with 20μM Tet. Their results demonstrated selective reduction in cellular copper concentration and activity of the copper enzyme Superoxide Dismutase 1 (SOD1). Addition of 22μM $CuCL_2$ reversed the effect of Tet. Their results demonstrated role for copper in IL-2 mRNA expression. The activity of IL-2 decreased with increased incubation with Tet (24 to 48 hours), further exposure had no influence on IL-2 expression. Intracellular copper levels were decreased by 30-35% compared with controls and the enzyme activity of SOD-1 decreased by 30-40%. Treatment with Tet had no effect on cell replication or function, determined by cell count and trypan blue exclusion. Mitochondrial function, assessed by almar blue dye reduction, was reduced by 5%. They concluded that Tet selectively reduced intracellular copper concentration and SOD-1 activity without adverse effect on general cellular activities (Hopkins and Failla 1997; Hopkins and Failla 1999).

Similar results were seen using Tet in U937 human promonocyte cell line with significant reduction in intracellular copper concentration without effect on cellular or mitochondrial function. However, no effect was seen on SOD-1 function in U937 cells. In separate experiments copper depletion did result in reduced SOD-1 function in the murine macrophage cell line RAW264.7 (Huang and Failla 2000; Huang, Failla et al. 2001).

Use of Tet, again in Jurkat cells, showed no effect on cell viability assessed by WST-1 reagent. Comet assay showed no effect on DNA in response to copper deficiency, but on exposure to hydrogen peroxide, increased DNA damage was seen in cells pre-treated with Tet. An effect reversed by simultaneous pre-treatment with Tet and additional copper. Suggesting copper deficiency increased Jurkat susceptibility to oxidative DNA damage (Pan and Loo 2000).

Use of Tet in the hepatoma cell line HEPG2 demonstrated similar reduction in intracellular copper concentration (Zhang, Wang et al. 1995; Wu, Zhang et al. 1997). Two passages in 20μM Tet reduced intracellular copper by 45-77%. In these experiments Hep G2 cells showed increased Apolipoprotein A-1 (ApoA-1) expression. ApoA-1 is a major component of HDL, expressed in the liver (Wu, Zhang et al. 1997). One of the few in-vivo experiments showed the Tet increased lipoproteins in copper deficient animals (Hing and Lei 1991).

Trien: Use of the copper chelator Trien in the human neurobalstoma cell line SH-SY5Y showed a similar effect to the experiments with Tet. Trien was non-toxic with no effect on cell morphology or proliferation. Cells incubated for three days with

Trien produced a dose dependent depletion of intracellular copper (33% or 75% with 25 or 125μM Trien). A decrease in function of the copper containing enzymes cytochrome C oxidase and SOD-1 was seen. No effect was seen on non-copper cytosolic or mitochondrial enzymes (LDH and succinate-ubiquinone reductase). Other anti-oxidant enzymes, catalase and glutathione peroxidase were unaffected. To assess the response to oxidative stress cells were treated with paraquat (a superoxide generator). Increased apoptosis was seen in copper depleted cells, with increased caspase 3 and activated p53 expression. The authors concluded that reduced copper reduced intracellular defence to oxidative stress (Rossi, Marchese et al. 2001).

In-vivo, the role of copper in angiogenisis and development of HCC was assessed by addition of Trien and the clinically used drug Penicillamine to drinking water of a murine Xenograft model for HCC. Both chemicals reduced tumour volume. The effect was also seen in animals fed a copper deficient diet. In vitro analysis demonstrated no cytotoxic effect of Trien but reduced proliferation of endothelial cells. The HCC tumour cells demonstrated increased apoptosis in response to Trien (Yoshii, Yoshiji et al. 2001). Further in-vivo analysis in rodents fed a copper deficient diet showed reduced liver copper levels and reduced ceruloplasmin. Also reduced cytochrome C oxidase and SOD-1 activity (Prohaska 1991).

Bath: Bath is reported to be a selective Cu^{+} chelator. Studies looking at a role of copper in p53 induction suggested different effects mediated by Cu^{+} compared with Cu^{++} as the use of different copper chelators produced different results. PDTC a metal compound, reported to increase intracellular copper, down regulated p53 activity. This effect was prevented by Bath (Verhaegh, Richard et al. 1997). PDTC increased

intracellular copper, presumably by affecting transmembrane transport as copper is transported across the cell membrane as Cu^{+} (Iseki, Kambe et al. 2000). The use of Bath in our experiments would make interesting comparison as the balance between oxidized and reduce copper is reported to directly affect cellular ROS generation and Fenton Chemistry (Ivanov, Parkinson et al. 2000).

Taken together these results on the use of copper chelators suggest that they reduce intracellular copper concentration. This is by reducing copper bound to proteins in the cell. As no free copper exists intracellularly the reduced cell copper concentration would correspondingly be associated with reduced SOD-1 and cytochrome oxidase function. The effect of this alone may affect intracellular gene expression. Copper deficiency also appears to reduce the ability of a cell to sustain oxidative stress.

Experiment 3.1.1: Intracellular copper concentration

Introduction: HEP3B cells were established in culture under routine conditions and TCM. To produce conditions of copper excess or copper depletion cell cultures were incubated with either with the addition of $CuCl_2$ (4 hours) or specific copper chelators (4 days). Whole cell copper concentration was measured by atomic absorption spectrophotometer. Controls used were taken from three different batches of HEP 3B cells, grown and harvested separately.

Results: Addition of copper to TCM produced a rise in whole cell copper concentration. This was not dose dependent. Addition of 50μM copper produced a 25% increase in whole cell copper concentration (figure 3.1.1a). Further increased copper concentration of TCM did not cause a further increase in whole cell copper concentration.

All the chemicals used for copper chelating produced a 50% reduction in intracellular copper (figure 3.1.1b). No dose dependent effect was seen. No difference in whole cell copper concentration reduction was seen between the three chemicals used.

Conclusion: Increased copper produced a 25% increase in intracellular copper concentration. Copper chelation resulted in a 50% reduction in intracellular copper concentration.

Figure 3.1.1a: Intracellular copper concentration – copper excess

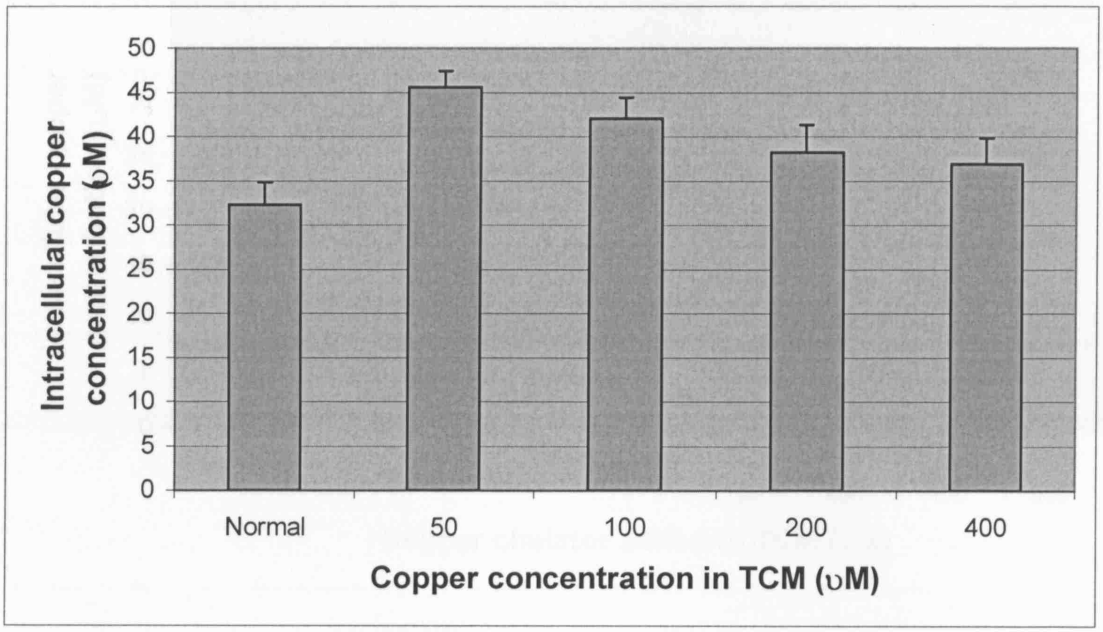


Figure 3.1.1a: Effect of increased copper in TCM on intracellular copper concentration. HEP3B were cells grown in TCM with different concentrations copper (μM). Copper ($CuCL_2$) was added to TCM for 4 hours. Cells were harvested and Intracellular copper concentration measured by atomic absorption spectrophotometer. Copper excess produced a 25% increase in intracellular copper concentration, which was not dose dependent (ANOVA p=0.02). The experiment was undertaken with each concentration of copper increased incrementally, double the previous, no results were seen for 800uM copper in TCM confirming the cell viability assays (see below) that copper was toxic at these levels.

Figure 3.1.1b: Intracellular copper concentration – copper depletion

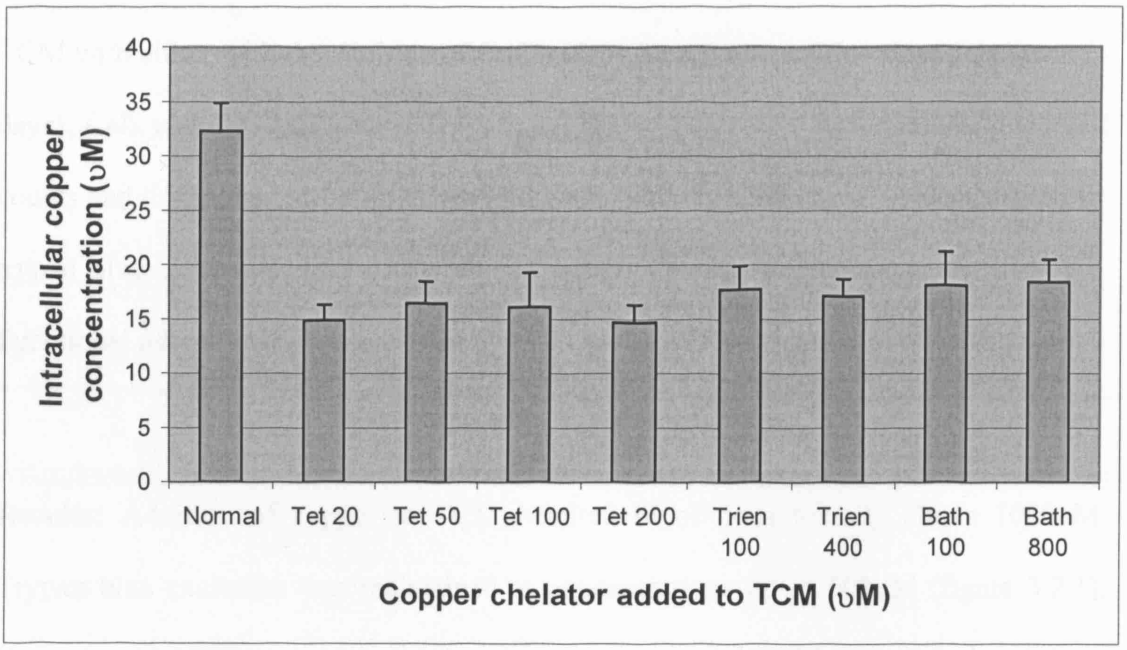


Figure 3.01b: Effect of excess depletion in TCM on intracellular copper concentration. HEP3B cells were grown in TCM with copper chelators at different concentrations (□M) for 4 days. N,N-Bis(2-aminoethyl)-1,3-propanedamine (Tet), Trithylenetetramine tetrahydrochloride (Trien) and 2,9-Diphenyl-1,10-phenanthrolinedisulfonic acid (Bath). Cells were harvested and intracellular copper concentration was measured by atomic absorption spectrophotometer. Copper chelation produced a 50% reduction in intracellular copper concentration (ANOVA $p<0.001$). Cells were incubated with Tet 20uM for a further 14 days and no difference in intracellular copper concentration was seen.

Experiments 3.2.1-2: Cell viability

Introduction: HEP3B cells were established in culture under routine conditions and TCM with either with the addition of $CuCl_2$ (4-24 hours) or specific copper chelators (4 days). Cell viability was assessed in several ways. Subjective measures included cell counts and time required between cell splits. Objectively cell survival was measured by trypan blue exclusion, loss of membrane integrity being a final stage of cell death. Functional assessment was measured by MTT assay reflecting mitochondrial function.

Results: Addition of copper to TCM resulted in obvious toxicity above 1000uM. Trypan blue exclusion was maintained at concentrations up to 600uM (figure 3.2.1). However, on MTT assay reduced function was noted at 400uM, although this did not alter with time (figures 3.2.2 & 3.2.3). HEP3B cells were grown with 400uM $CuCl_2$ in TCM for 48 hours without effect on cell count.

The chemicals used for copper depletion had no effect on cell count, Trypan Blue exclusion or MTT assay. HEP3B cells grown in TCM with 20uM Tet were grown for 14 days without effect on cell count.

Conclusion: Copper excess produced cell toxicity at high doses. Copper depletion had no effect on cell viability.

Figure 3.2.1: Trypan blue exclusion in HEP3B cells with increased copper

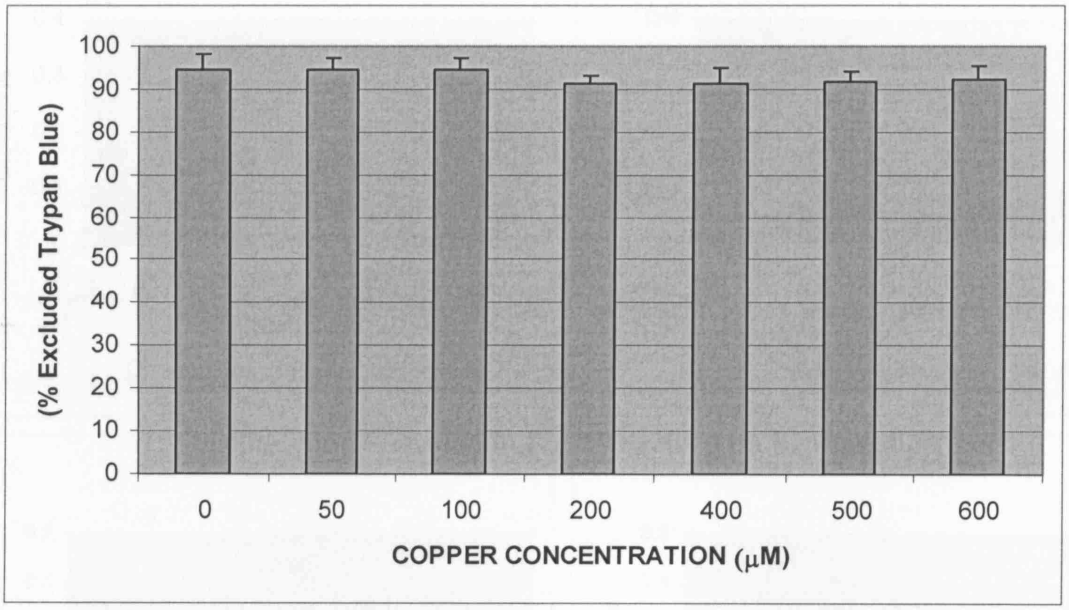


Figure 3.2.1: Cell viability assessed by Trypan blue exclusion in HEP3B cells incubated for four hours with increased copper in TCM. No difference was seen in Trypan Blue exclusion at the concentrations shown. High concentrations of copper (>1000uM) resulted in cell death.

Figures 3.2.2a-d: MTT assay in HEP3B cells with increased copper

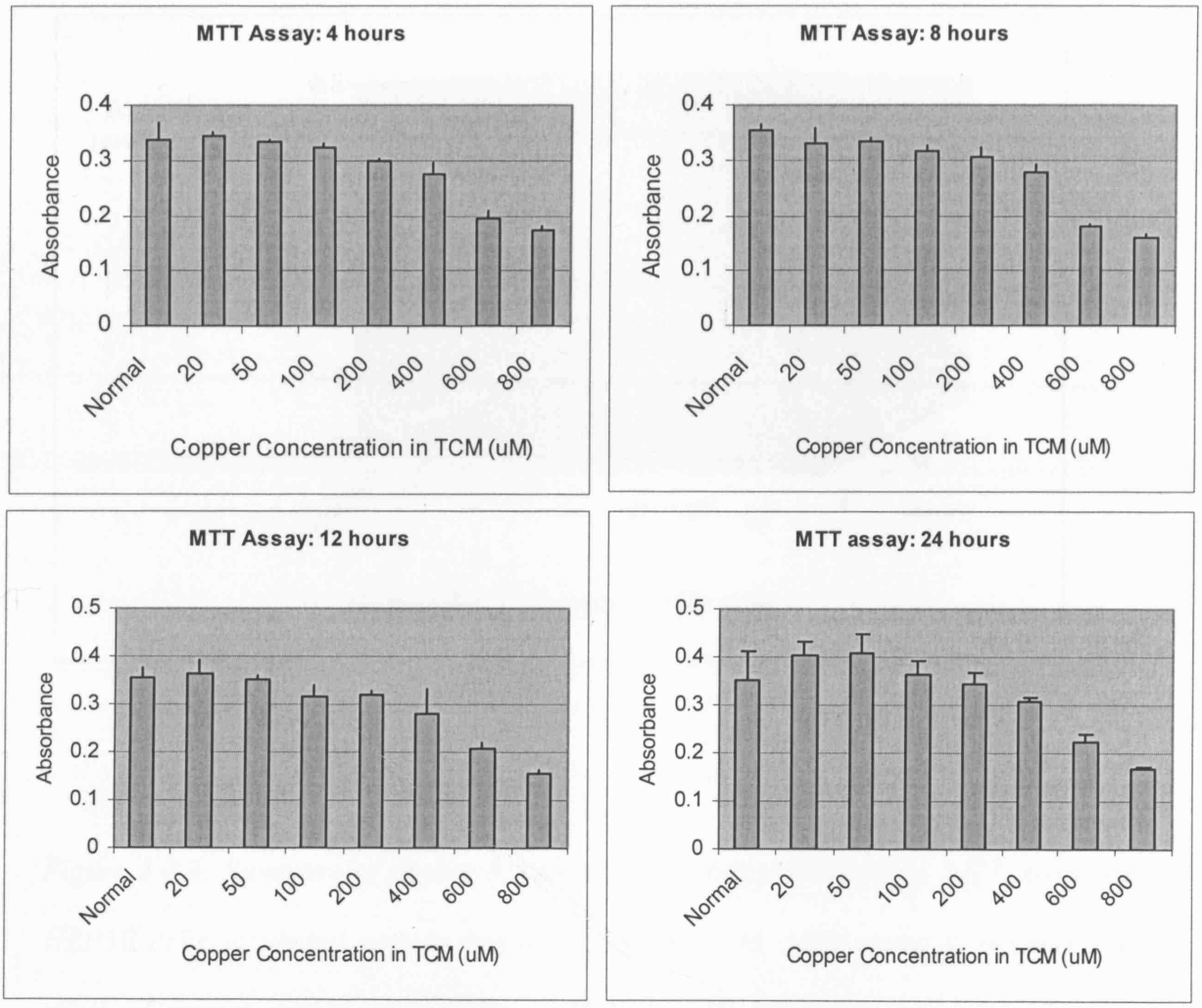


Figure3.2.2a-d: Cell viability assessed by MTT assay for HEP3B cells incubated with increased copper in TCM. Results are presented for the different time periods assessed (a-d). Analysis of results by ANOVA showed a decrease in cell viability at 400uM copper ($p<0.05$). The mean reduction in cell viability was 20% at 400uM $CuCL_2$, this did not alter from 4 to 24 hours.

Figure 3.2.3: MTT assay in HEP3B cells with increased copper over time

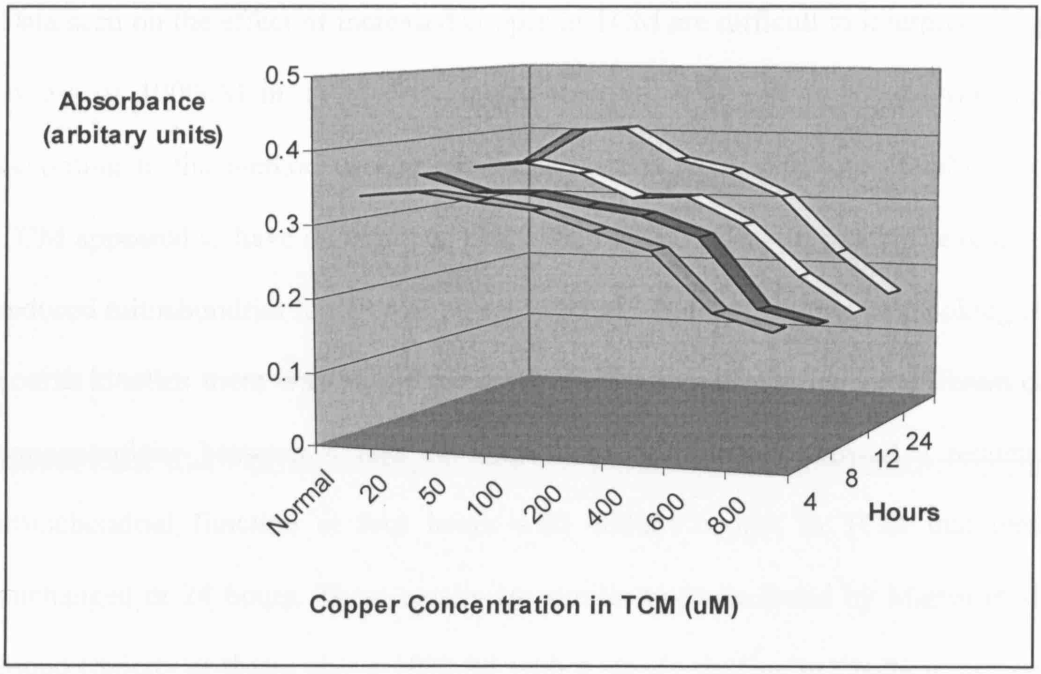


Figure 3.2.3: Summary of figures 3.2.2a-d, cell viability assessed by MTT assay for HEP3B cells incubated with increased copper in TCM. MTT assay to measure the effect of copper excess on viability of HEP3B cells in culture over time (hours). Analysis of results by ANOVA revealed decrease in cell viability at 400uM copper ($p<0.05$). The mean reduction in cell viability was 20% at 400uM $CuCL_2$, this did not alter from 4 to 24 hours.

Discussion

Data seen on the effect of increased copper in TCM are difficult to interpret. Copper in excess of 1000uM in TCM was clearly toxic to cells, above 600uM was variable according to the method used to assess cell viability. Addition of 400uM copper to TCM appeared to have no effect on cell toxicity assessed by trypan blue exclusion but reduced mitochondrial function as assessed by MTT assay. Despite this looking at time course kinetics there was no difference in MTT assay results for the different copper concentrations between 4 and 24 hours. The MTT assay showed a reduction in mitochondrial function at four hours with 400uM copper in TCM that remained unchanged at 24 hours. These results are similar to those found by Martin et al who found toxicity at doses above 1000uM with a steady decline in function, assessed by MTT assay up to that concentration. At 500uM, the optimal concentration used in their experiments on the same cell lines (HEP3B and CHO) there was a 60% reduction in MTT assay compared to the 25% reduction seen with our results (Martin, Linden et al. 2005).

Data on intracellular copper concentration are similarly confusing. Increased exogenous excess copper was reported to significantly increase intracellular copper concentration (Verhaegh, Richard et al. 1997; Narayanan, Fitch et al. 2001) whereas others have not reported such response (Watt and Hooper 2001). The results seen here showed a 25% increase in intracellular copper with low dose copper (50uM) in TCM. Intracellular copper concentration reduced towards control levels with increased exogenous copper (400uM) in TCM. The experiment was undertaken with each concentration of copper

increased incrementally, double the previous, no results were seen for 800uM copper in TCM confirming the cell viability assays that copper was toxic at these levels.

The expected response to increased exogenous copper was to see a parallel increase in intracellular copper concentration. In Wilson's Disease intracellular copper concentrations in end stage liver disease have been reported at several thousand times higher than normal (Sternleib and Scheinberg 1993). Under normal circumstances intracellular copper is tightly regulated. All copper is protein bound. The normal response of cells to increased intracellular copper concentration is mediated by expression of relevant cell surface receptors with reduced expression of ctr1 and therefore reduced movement of copper across the cell membrane (Lee, Prohaska et al. 2001). Further intracellular movement of ATPase7a & 7b would increase intracellular transport and promote export of any excess copper from the cell (Schaefer, Hopkins et al. 1999). The fact that copper did not increase intracellularly with increased copper in TCM may reflect these cellular responses (reduced ctr1 expression and altered ATPase 7 location) to maintain homeostasis.

Trypan blue exclusion showed preservation of the cell membrane up to 600uM copper whereas the MTT assay showed reduced mitochondrial function. This may reflect different effects of copper toxicity on cellular function; with an effect on the mitochondria at lower concentrations and disruption of the cell membrane at higher concentrations. Free copper is highly toxic due to the ability of copper to generate ROS production by Fenton chemistry. A possible mechanism of action may be by the generation of extracellular ROS that are able to traverse the cell membrane. Increased intracellular ROS could then alter cellular redox balance and disrupt mitochondrial

function. Alternatively the reduction in MTT assay may reflect the first stages of increased apoptosis in response to copper toxicity (Joseph R. Prohaska, personal communication). Thus excess copper, although not exerting direct effect on cell itself may mediate toxicity by increased extracellular ROS production, which are able to traverse the cell membrane and directly affect the cell. Further experiments to confirm the effect of copper toxicity on apoptosis verses necrosis could involve measurements of capsase 3 or COMET scores.

The effect of copper chelation on intracellular copper concentration was as expected and in line with previous reports. No significant difference was seen between chemicals used and the intracellular reduction in copper concentration was not dose dependent. All chemicals irrespective of concentration in TCM reduced intracellular copper concentration (mean 50%, range 46% - 54 %). No one has previously made comparison between the different chemicals. Interesting no difference was seen in intracellular copper reduction between Bath (a specific Cu^{+} chelator) and Tet or Trien. Data may be interpreted to suggest that of total intracellular copper half is essential for cell survival and proliferation. Thus under situations of reduced copper concentration a cell will only relinquish half of its bound copper. Copper reduction would be at expense of copper bound in metalloenzymes; SOD-1 and mitochondrial cytochromes (Professors Mark Failla & Joseph R. Prohaska, personal communications).

Alternate methods to attain copper deficient cells would be to culture cells in serum free media, PBS, or the use of Chelex (a non specific metal ion resin www.bio-rad.com). In studies on HIF no group has used serum free media or PBS for cell growth or experimentation. Cell culture techniques provide invaluable information in

physiological and molecular research. However, they are not truly physiological states. Cell culture utilises immortalised cell lines, designed to grow and proliferate without genetic alteration from a single cell, clones. Hence by definition immortalised cell lines or clones are different from heterogeneous tissue that they model. Conditions of cell growth, use of DMEM, addition of serum and antibiotics are all well described and established for the study of HIF-1 in HEP3B cells. Although reasonably ubiquitous each cell line has ideal conditions for proliferation to maintain 'healthy' growth. Use of serum free media would be a method to obtain copper depleted cells. Without copper in the TCM intracellular copper would have to be divided between proliferating cells. However, serum free media would be non-specific for copper and other transition and trace elements need to be added. An alternative method would be use of 'Chelex'. Chelex is a general metal chelator, a gel that acts by removing all metals from a solution. The resultant solution or TCM would then be supplemented with trace and transition metals, with exclusion of copper. Both methods increase complexity of experiments hence margin for error. Alteration of TCM, addition of chemicals etc may directly affect cells themselves. For experiments on HIF-1 all groups have used well-described conditions of cell culture with HEP3B cells and conditions unchanged in a decade. Variation from these conditions has not been studied. Use of non-physiological serum free media or PBS may have direct effect of cell physiology, gene expression and ability to respond to stimuli. Incubation in serum free media may have direct effect on HIF pathway itself. Alone alterations in TCM may produce false positive or false negative results. For these experiments we used cell culture conditions identical to those initially described in experiments by Wang & Semenza, which have been copied throughout the last ten years by all groups. Different groups described use of specific copper chelators. For each chemical reduction in intracellular copper concentration of

40-70% was reported. These data support these findings and represent an effective method for specifically reducing intracellular copper concentration without affecting cell physiology, proliferation or survival.

Chapter 4: Effect of Increased and Reduced Copper on HIF-1a in Cell Culture

Summary of Chapter

Introduction: We wished to assess how the conditions of increased and reduced copper in cell culture seen in Chapter 3 affected HIF-1a expression in HEP3B cells.

Method: HEP3B cells in culture were incubated with the addition of different concentrations of exogenous copper and also for different time periods. For copper depletion experiments HEP3B cells in culture were incubated with copper specific chemical chelators for four days. Cells were then exposed to hypoxia, cobalt, DSF or GSNO (a NO donor), all stimuli known to induce HIF1a.

Results: Increased copper produced a significant increase in HIF-1a expression; this was maximal at 400uM $CuCl_2$ in the TCM. The induction of HIF-1a by exogenous copper was rapid within hours and comparable to the effect produced by hypoxia.

All copper chelators studied produced a similar significant reduction in the normal levels of HIF-1a expression in response to hypoxia and nitric oxide. No response was seen with DSF or cobalt.

Conclusion: Increased copper increased HIF-1a expression. Reduced copper reduced the normal HIF-1a expression in response to hypoxia or nitric oxide.

Experiment 4.1.1: Effect of copper excess on HIF-1a induction

Introduction: HEP3B cells were exposed to different concentrations of copper in the form of copper chloride, $CuCl_2$ (μM) in TCM for a period of four hours. This time period was used initially as the HIF-1 response to hypoxia is maximal in four hours. Cells were harvested on ice and nuclear protein extracted. Western blot analysis for HIF-1a was performed on nuclear protein extracts as described in Chapter 3. Positive and negative controls (HEP3B cells under hypoxia or normoxia) were run in parallel during each experiment and protein loading per lane was quantified by subsequent immunoblotting of the membrane for SP1 as an internal control. SP-1 is a ubiquitous cell protein and is constitutively expressed at steady state.

Results: HIF-1a protein was detected in nuclear extracts of HEP3B cells exposed to copper at varying concentrations for a period of four hours (figure 4.1.1). A small amount of HIF-1a nuclear protein was seen at a concentration of 20μM $CuCl_2$. Increased HIF-1a nuclear protein was seen corresponding to increased in copper concentration in TCM. Maximal HIF-1a nuclear protein was seen at a concentration of 400μM $CuCl_2$ in TCM.

Conclusion: Copper excess induced HIF-1a in cell culture.

Figure 4.1.1: HIF-1a induction by copper in HEP3B cells

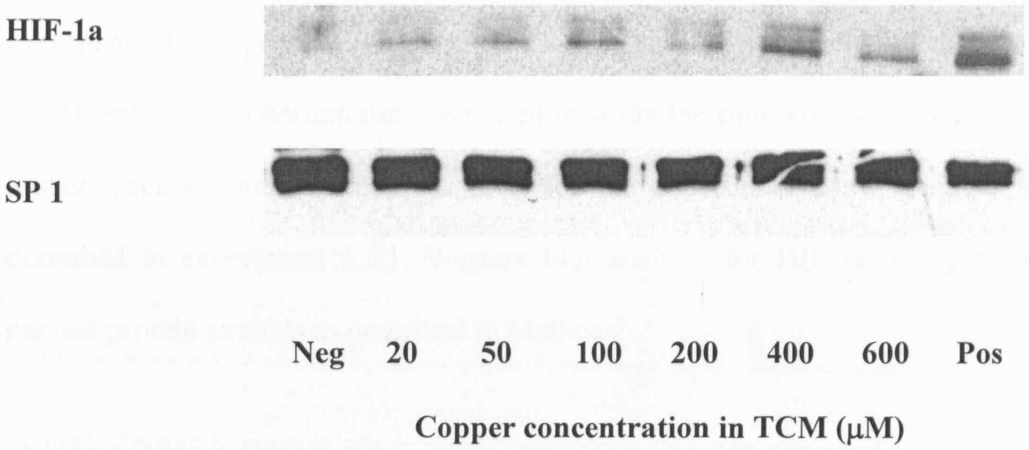


Figure 4.1.1: Western blot analysis was performed on nuclear protein extracts prepared from HEP3B cells exposed to different concentrations of $CuCl_2$ (μM) for a period of 4 hours. Immunostaining was performed for HIF-1a. HEP3B cells were exposed to hypoxia as positive (Pos) controls or normoxia as negative (Neg) controls. Protein loading per lane was quantified by subsequent immunoblotting of the membrane for SP1 as an internal control.

Experiment 4.1.2: Duration of copper excess on HIF-1a induction

Introduction: A concentration of 400μM $CuCl_2$ in TCM (from the results of experiment 4.1.1) produced maximal and consistently reproducible increase in HIF-1a. □□Therefore this concentration was used to study the time kinetics of HIF-1a nuclear protein accumulation. Techniques used for the experiment were identical to those described in experiment 4.1.1. Western blot analysis for HIF-1a was performed on nuclear protein extracts as described in Methods.

Results: Time kinetics for HIF-1a nuclear protein accumulation in response to 400μM $CuCl_2$ is shown in figure 4.1.2. HIF-1a nuclear protein was seen in HEP3B cells at two hours of exposure to copper excess. This response was sustained for 12 hours. Lower concentrations did produce same response but as in figure 4.1.1 in a dose dependent manner (data not shown).

Conclusion: Copper excess produced a rapid and sustained induction of HIF-1a in cell culture.

Figure 4.1.2: Time kinetics of HIF-1a induction by copper in HEP3B cells

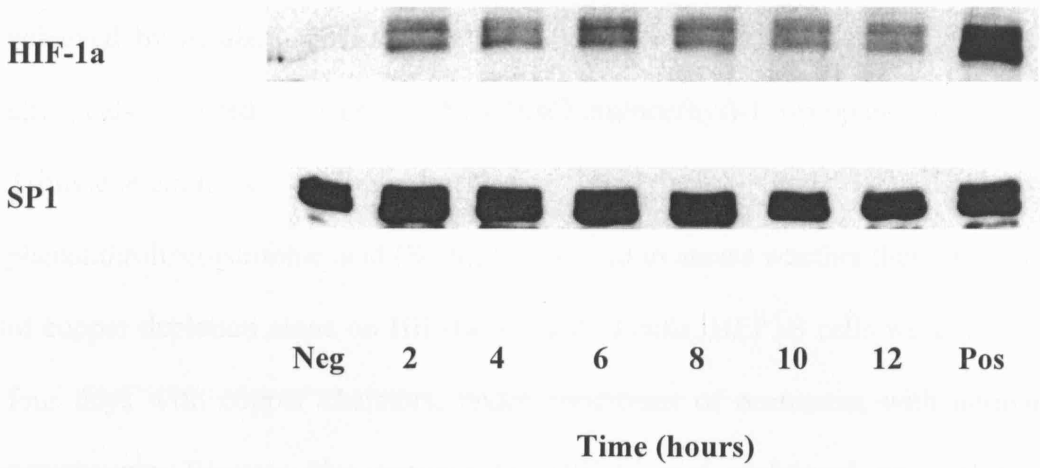


Figure 4.1.2: Western blot analysis performed on nuclear protein extracts prepared from HEP3B cells exposed to 400μM $CuCl_2$ for different time periods (hours). Immunostaining was performed for HIF-1a. HEP3B cells were exposed to hypoxia as positive (Pos) controls or normoxia as negative (Neg) controls. Protein loading per lane was quantified by subsequent immunoblotting of the membrane for SP1 as an internal control.

Experiment 4.2.1: Effect of copper depletion on HIF-1a induction

Introduction: In Chapter 3 we demonstrated that intracellular copper depletion was achieved by incubating HEP3B cells in culture with different copper chelators. The chemicals used were N,N-Bis(2-aminoethyl)-1,3-propanedamine (Tet), Trithylenetetramine tetrahydrochloride (Trien) and 2,9-Diphenyl-1,10-phenanthrolinedisulfonic acid (Bath). We wished to assess whether there was any effect of copper depletion alone on HIF-1a in HEP3B cells. HEP3B cells were incubated for four days with copper chelators, under conditions of normoxia, with normal TCM constituents. Western blot analysis for HIF-1a was performed on nuclear protein extracts as described in Methods.

Results: Under normal cell culture conditions copper depletion had no effect on HIF-1a nuclear protein accumulation in HEP3B cells. This was regardless of chemical or concentration of chemical used in TCM (figure 4.2.1). Similarly duration of incubation with the different copper chelators (4 hours – 4 days) had no effect on HIF-1a nuclear protein accumulation in HEP3B cells (data not shown).

Conclusion: Copper depletion had no effect on HIF-1a under normal conditions of cell culture.

Figure 4.2.1: Effect of copper depletion on HIF-1a induction under normoxia

HIF-1a

Chemical	**0**	**Tet**	**Tet**	**Tet**	**Trien**	**Trien**	**Bath**	**Bath**	**0**
Concentration (υM)	**Neg**	**20**	**50**	**100**	**100**	**400**	**100**	**800**	**Pos**

Figure 4.2.1: Western blot performed on nuclear protein extracts prepared from HEP3B cells pre-treated with copper depletion for 4 days. Immunostaining was performed for HIF-1a. Chemicals used were N,N-Bis(2-aminoethyl)-1,3-propanedamine (Tet), Trithylenetetramine tetrahydrochloride (Trien) and 2,9-Diphenyl-1,10-phenanthrolinedisulfonic acid (Bath). HEP3B cells, without any pre-treatment were exposed to hypoxia as positive (Pos) controls or normoxia as negative (Neg) controls.

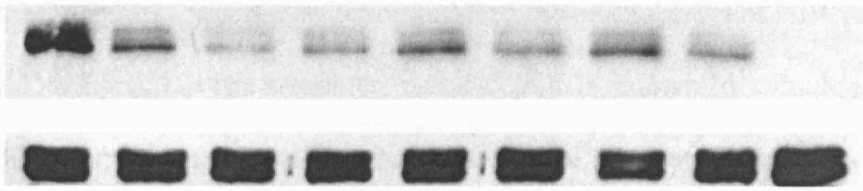
Experiment 4.2.2: Effect of copper depletion on HIF-1a induction by hypoxia.

Introduction: In experiment 4.2.1 we demonstrated that intracellular copper depletion had no effect on HIF-1a induction under normal conditions in cell culture. We now wished to assess whether copper depletion affected the normal response of HIF-1a induction by hypoxia in HEP3B cells. HEP3B cells were incubated for four days with copper chelators under conditions of normoxia with normal TCM constituents. The cells were then exposed to hypoxia for a period of four hours. Western blot analysis for HIF-1a was performed on nuclear protein extracts.

Results: The use of copper chelators to produce intracellular copper depletion resulted in reduced HIF-1a nuclear protein accumulation in response to hypoxia (figure 4.2.2). The reduction in HIF-1a induction was significant, however complete abolition of normal response was not seen. The effect of copper depletion on HIF-1a induction by hypoxia paralleled the results seen in Chapter 3, where the copper chelators produced a reduction but not complete abolition in total intracellular copper concentration.

Conclusion: Copper depletion reduced the normal HIF-1a response to hypoxia.

Figure 4.2.2: Effect of copper depletion on HIF-1a induction by hypoxia



Chemical	0	Tet	Tet	Tet	Trien	Trien	Bath	Bath	0
Concentration (υM)	Pos	20	50	100	100	400	100	800	Neg

Figure 4.2.2: Western blot analysis performed on nuclear protein extracts prepared from HEP3B cells pre-treated with copper chelators for 4 days and exposed to hypoxia for 4 hours. Immunostaining performed for HIF-1a. Chemicals used were N,N-Bis(2-aminoethyl)-1,3-propanedamine (Tet), Trithylenetetramine tetrahydrochloride (Trien) and 2,9-Diphenyl-1,10-phenanthrolinedisulfonic acid (Bath). HEP3B cells, without any pre-treatment were exposed to hypoxia as positive (Pos) controls or normoxia as negative (Neg) controls. Protein loading per lane was quantified by subsequent immunoblotting of membrane for SP1 as an internal control.

Experiment 4.3.1: Effect of copper depletion on HIF-1a induction by cobalt.

Introduction: In experiment 4.2.2 copper depletion reduced the normal level of HIF-1a induction in response to hypoxia. The transition metal cobalt is known to stimulate HIF-1a induction under normal conditions. We therefore wished to assess whether the effect of copper depletion in HEP3B cells affected HIF-1a induction in response to cobalt. Initially the copper chelator, Tet was used. HEP3B cells were pre-treated with different concentrations of Tet for four days and exposed to cobalt 100μM for 6 hours. Western blot analysis for HIF-1a was performed on nuclear protein extracts.

Results: HIF-1a nuclear protein accumulation in response to cobalt exposure was reduced in HEP3B cells pre-treated with Tet at a concentration of 100μM for four days. No HIF-1a nuclear protein was seen in HEP3B cells pre-treated with 200μM Tet, or at concentrations above that (figure 4.3.1).

Conclusion: Copper depletion inhibited HIF-1a induction by cobalt.

Figure 4.3.1: Effect of copper depletion on HIF-1a induction by cobalt

HIF-1a

Concentration (υM)	0	100	200	300	400	500	Negative

Figure 4.3.1: Western blot for HIF-1a *on nuclear protein extracts prepared from HEP3B cells pre-treated with different concentrations (μM) of N,N-Bis(2-aminoethyl)-1,3-propanedamine (Tet), over a period 4 days and then exposed to Cobalt 100μM for 6 hours. HEP3B cells, without any pre-treatment were exposed to cobalt as positive (Pos) controls. HEP3B cells, without any pre-treatment and no exposure to cobalt were used as negative (Neg) controls.*

Experiment 4.3.2: Duration of copper depletion on HIF-1a induction by cobalt.

Introduction: In experiment 4.3.1 copper depletion inhibited HIF-1a induction in response to cobalt. This result of inhibition of HIF-1a induction appeared to contradict the result seen in response to hypoxia where copper depletion only reduced the level of HIF-1a induction (experiment 4.2.2). In order to further explore the effect of copper depletion on HIF-1a induction in response to cobalt we assessed the time kinetics for this response. HEP3B cells were pre-treated with 200μM of the copper chelator, Tet for different time periods and then exposed to cobalt 100μM for six hours. Western blot analysis for HIF-1a was performed on nuclear protein extracts.

Results: Tet (200μM) abolished HIF-1a induction in response to cobalt. The effect was seen at all time periods studied and following the simultaneous addition of Tet with cobalt, without need for pre-treatment (figure 4.3.2).

Conclusion: The copper chelator, Tet may have a direct effect on cobalt.

Figure 4.3.2: Time kinetics of copper depletion on HIF-1a induction by cobalt

Figure 4.3.2: Western blot for HIF-1a on nuclear protein extracts prepared from HEP3B cells pre-treated with 200μM tet (N,N-Bis(2-aminoethyl)-1,3-propanedamine) for different time periods (hours) prior to exposure to Cobalt 100μM for 6 hours. HEP3B cells, without any pre-treatment were exposed to hypoxia as positive (Pos) controls. HEP3B cells, without any pre-treatment and no exposure to cobalt were used as negative (Neg) controls.

Experiment 4.3.3: Effect of Tet on HIF-1a induction by cobalt.

Introduction: The results from experiments 4.3.1 & 4.3.2 showed that copper depletion using Tet inhibited HIF-1a induction by cobalt. However, several factors suggest this to be a false positive result instead Tet may have a direct action on cobalt itself rather than represent an effect of copper depletion on HIF-1a induction by cobalt. The reasons to suggest this are; the dose dependent and high concentration of Tet required, complete abolition of HIF-1a induction by cobalt and that the effect of Tet was immediate. To test whether Tet was acting as a chelator of cobalt HEP3B cells pre-treated with 200μM Tet for 4 days and then exposed to increasing concentrations of Cobalt (μM) for six hours. Western blot analysis for HIF-1a was performed on nuclear protein extracts as described

Results: As before HIF-1a nuclear protein accumulation was abolished at concentrations of 100μM cobalt in TCM. When concentration of cobalt was increased to 400μM HIF-1a nuclear protein accumulation was seen (figure 4.3.3)..

Conclusion: The copper chelator, Tet effect on HIF-1a induction by cobalt was a false positive result as Tet appears to be an effective chelator of cobalt.

Figure 4.3.3: Effect of different concentrations of cobalt

Figure 4.3.3: Western blot for HIF-1a on nuclear protein extracts prepared from HEP3B cells pre-treated with 200μM Tet (N,N-Bis(2-aminoethyl)-1,3-propanedamine) for 4 days and exposed to increasing concentrations of Cobalt (μM) for 6 hours. HEP3B cells, without any pre-treatment were exposed to hypoxia as positive (Pos) controls. HEP3B cells, without any pre-treatment and no exposure to cobalt were used as negative (Neg) controls.

Experiment 4.3.4: Effect of copper depletion on HIF-1a induction by cobalt.

Introduction: The results from experiments 4.3.1 - 3 suggested that Tet had a direct effect on cobalt, that it was an effective cobalt chelator. Therefore these results did not represent and effect of copper depletion. To assess the effect of copper depletion in HEP3B cells on HIF-1a nuclear protein accumulation in response to cobalt exposure, different chemicals for copper chelating were used. The chemicals used were Trien and Bath. HEP3B cells pre-treated with copper chelators for 4 days then exposed to Cobalt (100μM) for 6 hours. Western blot analysis for HIF-1a was performed on nuclear protein extracts as described.

Results: The copper chelators Trien and Bath had no effect on HIF-1a nuclear protein accumulation in response to cobalt (figure 4.3.4).. These data confirm that Tet had a direct effect on cobalt and that the effects seen with Tet were not as a consequence of copper chelating. Tet may act as a high affinity cobalt chelator.

Conclusion: Copper depletion had no effect on HIF-1a induction by cobalt.

Figure 4.3.4: Effect of copper depletion on HIF-1a induction by cobalt

HIF-1a

CHEMICAL	Trien	Trien	Bath	Bath	Pos
Concentration (υM)	100	400	100	800	-

Figure 4.3.4: Western blot for HIF-1a on nuclear protein extracts prepared from HEP3B cells pre-treated with copper chelators for 4 days and then exposed to Cobalt (100μM) for 6 hours. The chemicals used were Trithylenetetramine tetrahydrochloride (Trien) and 2,9-Diphenyl-1,10-phenanthrolinedisulfonic acid (Bath). HEP3B cells, without any pre-treatment were exposed to cobalt as a positive (Pos) control.

Experiment 4.4.1: Effect of copper depletion on HIF-1a induction by desferroxamine

Introduction: In experiment 4.2.2 copper depletion reduced the normal level of HIF-1a induction in response to hypoxia. In experiments 4.3.1-4 copper depletion did not affect the level of HIF-1a induction in response to cobalt. We therefore wished to assess whether the effect of copper depletion in HEP3B cells affected HIF-1a induction in response to the iron chelator desferroxamine (DSF). Experiments were undertaken using all three copper chelators. HEP3B cells were incubated with copper chelators for 4 days and then exposed to DSF (100μM) for 4 hours. HEP3B cells, without any pre-treatment were exposed to DSF as positive controls or normoxia as negative controls. Western blot analysis for HIF-1a was performed on nuclear protein extracts.

Results: Copper depletion in HEP3B cells had no effect on HIF-1a nuclear protein accumulation in response to DSF exposure (figure 4.4.1)..

Conclusion: Copper depletion had no effect on HIF-1a induction by desferroxamine.

Figure 4.4.1: Effect of copper depletion on HIF-1a induction by deferroxamine

Chemical	0	Tet	Tet	Tet	Trien	Trien	Bath	Bath	0
Concentration (υM)	Neg	20	50	100	100	400	100	800	Pos

Figure 4.4.1: Western blot for HIF-1a on nuclear protein extracts prepared from HEP3B cells pre-treated with copper depletion for 4 days and then exposed to DSF (100μM) for 4 hours. The chemicals used were N,N-Bis(2-aminoethyl)-1,3-propanedamine (Tet), Trithylenetetramine tetrahydrochloride (Trien) and 2,9-Diphenyl-1,10-phenanthrolinedisulfonic acid (Bath). HEP3B cells, without any pre-treatment were exposed to DSF as positive (Pos) controls. HEP3B cells, without any pre-treatment under normal conditions were used as negative (Neg) controls.

Experiments 4.5.1 & 2. HIF-1a induction by s-nitrosoglutathione

Introduction: From previous experiments copper depletion reduced HIF-1a induction in response to hypoxia but not cobalt or DSF. We therefore wished to assess the role of nitric oxide on HIF-1a nuclear protein accumulation. In order to do this the nitric oxide donor S-Nitrosoglutathione (GSNO) was used. In experiment 4.5.1 HEP3B cells in normal cell culture were exposed to GSNO at different concentrations (μM) for 4 hours. In experiment 4.5.2 HEP3B cells in normal cell culture were exposed to GSNO (500μM) for different time periods. Western blot analysis for HIF-1a was performed on nuclear protein extracts.

Results: Exposure of HEP3B cells to GSNO for four hours resulted in HIF-1a nuclear protein accumulation, detectable at a concentration of 100μM and maximal at 500μM (figure 4.5.1). Further experiments on time kinetics revealed HIF-1a induction to be rapid, detected at one hour, maximal at two hours and sustained for four hours (figure 4.5.2).

Conclusion: HIF-1a was induced by the nitric oxide donor GSNO

Figures 4.5.1 & 2: Effect of GSNO on HIF-1a induction

Figure 4.5.1

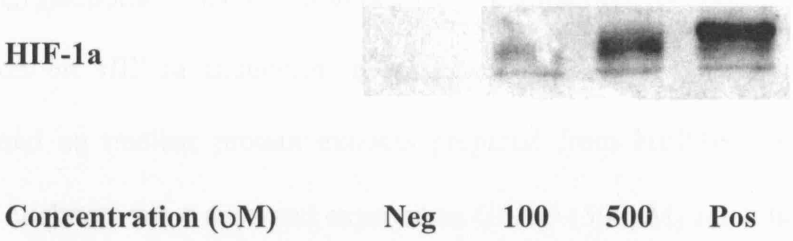
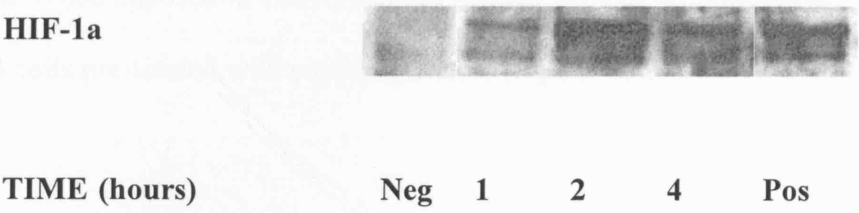


Figure 4.5.2



Figures 4.5.1 & 2: Western blot for HIF-1a on nuclear protein extracts prepared from HEP3B cells exposed to S-Nitrosoglutathione (GSNO) at different concentrations (μM) for 4 hours (figure 4.5.1). The experiment was repeated; HEP3B cells were exposed to GSNO (500μM) for different time periods (figure 4.5.2). HEP3B cells were exposed to hypoxia as positive (Pos) controls or normoxia as negative (Neg) controls.

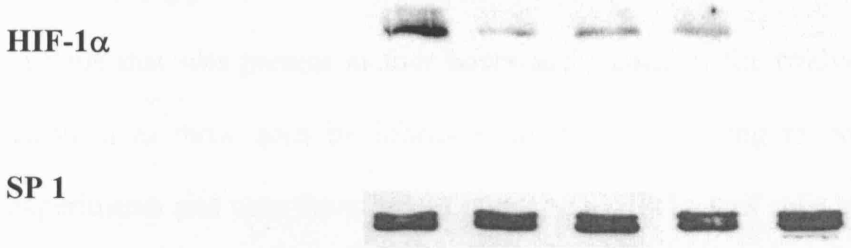
Experiment 4.5.3: Effect of copper depletion on HIF-1a induction by GSNO

Introduction: From the previous experiment the nitric oxide donor S-Nitrosoglutathione (GSNO) stimulated HIF-1a induction. To assess the effect of copper depletion on HIF-1a induction in response to GSNO, Western blot analysis was performed on nuclear protein extracts prepared from HEP3B cells pre-treated with copper chelators for 4 days and exposed to GSNO (500μM) for 4 hours. HEP3B cells, without any pre-treatment were exposed to hypoxia as positive controls or normoxia as negative controls.

Results: When exposed to GSNO HIF-1a nuclear protein accumulation was reduced in HEP3B cells pre-treated with copper chelators (figure 4.5.3).

Conclusion: Copper depletion reduced HIF-1a induction by GSNO.

Figure 4.5.3: Effect of copper depletion on HIF-1a induction by GSNO



CHEMICAL	0	Tet	Trien	Bath	0
Concentration (υM)	Pos	20	100	100	Neg

Figure 4.5.3: Western blot analysis was performed on nuclear protein extracts prepared from HEP3B cells pre-treated with copper chelators for 4 days and exposed to S-Nitrosoglutathione (GSNO) for 4 hours. Immunostaining was performed for HIF-1a. The chemicals used were N,N-Bis(2-aminoethyl)-1,3-propanedamine (Tet), Trithylenetetramine tetrahydrochloride (Trien) and 2,9-Diphenyl-1,10-phenanthrolinedisulfonic acid (Bath). HEP3B cells, without any pre-treatment were exposed to hypoxia as positive (Pos) controls or normoxia as negative (Neg) controls. Protein loading per lane was quantified by subsequent immunoblotting of the membrane for SP1 as an internal control.

Discussion

Increased copper in the TCM resulted in a stepwise increase in HIF-1a protein in the nucleus that was present at four hours and sustained for twelve. These results were identical to those seen by Martin et al. It is interesting to note that in both their experiments and ours the effect of copper on HIF-1a was seen at a concentration that appeared to exert a toxic effect on the cells in culture. The MTT assays (experiments 3.2.2) showed reduced function at 400uM copper in the TCM (500uM was used in Martins' experiment). No explanation was given in their paper to explain this (Martin, Linden et al. 2005). In the data shown here the toxic effect of increased copper remained unchanged over 24 hours. Which is in parallel with the continued expression of HIF-1a in response to icreased copper over time.

It appears that although high dose extracellular copper does not markedly increase intracellular copper concentration (data from chapter3), it does result in increased HIF-1a nuclear protein (a response that remained unchanged over time). This supports the concept that copper may exert it effect indirectly as suggested in Chapter 3. Increased copper in the TCM may generate extracellular ROS that in turn can enter the cell and induce HIF-1a expression.

The effect of copper reduction by the use of three different copper chelators was in line with the results seen in Chapter 3. There was no effect of copper depletion under normal conditions. Copper depletion reduced the level of HIF-1a nuclear protein in response to hypoxia. Again, no difference was seen between the chemicals used. The

effect of the Cu^{+} specific chelator, Bath, was identical to the other two non-specific chelators Tet and Trien. All three chemicals reduced intracellular copper by similar amounts and reduced HIF-1a nuclear protein levels in response to hypoxia by similar amounts. Which may suggest that Cu^{+} is responsible, a Cu^{++} specific chelator would be necessary to confirm this.

The experiments using cobalt to induce HIF-1a were started for simple reasons. Using cobalt to induce HIF-1a is well proven. Addition of cobalt to TCM is easier and cheaper than using a hypoxic chamber. Unfortunately as can be seen from these experiments Tet proved to be cobalt chelator in addition to copper.

Further experiments would be interesting to look at the activities of copper enzymes SOD1 and cytochrome C following copper chelation. Also for further proof of effect, cells could first be copper depleted then have exogenous copper added to confirm if the effect on HIF-1a expression on exposure to hypoxia was reversed.

The effect of the nitric oxide donor GSNO is interesting. NO is produced by a family of NO synthases (NOS) which utilise L-arginine and oxygen to generate NO. NOS isoenzymes can be broadly distinguished as being constitutively expressed e.g., Endothelial NOS, (eNOS) and inducible NOS (iNOS)) that can develop high concentrations of NO, often in response to cytokine stimulation.

The role of NO in HIF-1a regulation has been contradictory since the first reports. NO was found to inhibit HIF-1a induction or HIF-1 response to hypoxia (Sogawa, Numayama-Tsuruta et al. 1998; Huang, Willmore et al. 1999; Agani, Puchowicz et al.

2002). Contrary to these reports NO was shown to directly induce HIF-1 (Kimura, Weisz et al. 2000; Sandau, Fandrey et al. 2001). Also NO may cross talk with the PI3K-AKT inflammatory pathway and indirectly induce HIF-1 (Sandau, Faus et al. 2000). Results that are compatible with other inflammatory factors, cytokines IL-1B and TNFa, that induce HIF through involvement of the P13 / AKT3 inflammatory signalling pathway (Hellwig-Burgel, Rutkowski et al. 1999; Albina, Mastrofrancesco et al. 2001; Minet, Michel et al. 2001; Stiehl, Jelkmann et al. 2002; Hellwig-Burgel, Stiehl et al. 2005)

Unfortunately the understanding of the relationship between HIF-1 and NO has not become clearer, with studies looking at the interaction between NO and the PHD's. In human embryonic kidney cells GSNO (1000uM) promoted nuclear accumulation of HIF-1a, an effect mediated by an inhibitory effect on pHD-1, thus NO attenuated HIF-1a degradation (Metzen, Zhou et al. 2003). However, parallel studies on NO in a HEP3B cell-derived cell line (HRE7) found activation of prolyl hydroxylase and the inhibition of HIF-1a activity by NO (Wang, Sekine et al. 2002).

To make matters more confusing, established individual centres have produced results directly contradicting previous reports from the same laboratory (Genius and Fandrey 2000; Metzen, Zhou et al. 2003). Unlike the debate about the role of the mitochondria in ROS and oxygen sensing where different centres have consistently produced opposite results, perhaps reflecting a difference in culture techniques and assessment assays.

It is difficult to make reliable conclusions on this literature. In many of these experiments different NO donors were used. Chemicals that are difficult to purify, and sensitive to both light and heat. Furthermore some of the experiments were performed on specialised oxygen sensing cells from the carotid body or pulmonary vascular bed. In a series of experiments repeating many of those in the literature (as listed above) Sandau et al obtained a series of reproducible results using several NO donors that induced HIF-1a in different cell lines. They further showed that successful iNOS expression triggered HIF-1a accumulation and, to mimic an inflammatory setting macrophage derived NO activated HIF-1a in tubular LLC-PK_1 cells. These results support the hypothesis that NO, as an autocrine or paracrine factor, causes HIF-1a accumulation (Sandau, Fandrey et al. 2001). In experiments shown here the same protocol was used, GSNO was used as the NO donor. The chemical was kept at –80°C and hidden from light at all times, and experiments were performed in a darkened room. The results were the same; GSNO induced HIF-1a under normal conditions at a concentration of 100-500uM in a dose dependent manner over 2-4 hours. (Sandau, Faus et al. 2000; Sandau, Fandrey et al. 2001; Metzen, Zhou et al. 2003).

Copper has been directly linked with intracellular NO formation. Under normal circumstances NO is buffered by Nitrosothiols (RSNO) formed from NO and thiols in the presence of oxygen. Release of NO from RSNO is dependent on several catalysts including redox metals, ROS, thiols and UV light. Copper and SOD1 were shown to directly bind RSNO and catalyse rapid NO release (Jourd'heuil, Laroux et al. 1999; Singh, Hogg et al. 1999). Chelation of copper inhibited release of NO from RSNO (Mitsumoto, Kim et al. 2001).

In experiments performed here copper depletion inhibited HIF-1a induction by GSNO. These results suggest that copper may play a role in the pathway of HIF-1a induction and suggests a role for NO in this pathway.

Chapter 5: Effect of Copper on Transcriptional Induction of HIF-1

Summary of Chapter

Introduction: In Chapter 4 we demonstrated that increased copper produced a dose dependent increase in HIF-1a nuclear protein stabilisation under normal conditions. Copper depletion reduced the normal HIF-1a nuclear protein stabilisation in response to hypoxia, a result comparable to the reduction in intracellular copper concentration seen in Chapter 3. However, no effect was seen in response to desferroxamine, but in response to the nitric oxide donor GSNO, copper depletion resulted in a significant reduction in normal HIF-1a nuclear protein stabilisation. We wished to assess the effect of increased and reduced copper on HIF-1 transcriptional activation.

Method: Transcriptional activation by HIF-1a was measured in two separate ways. Firstly by the use of a HIF-1 reporter cell line and in addition by Northern blot quantification of VEGF, a know target gene for HIF-1.

Results: Increased copper produced a dose dependent increase in HIF-1a reporter cell line activity under normal conditions. Similarly, increased copper increased VEGF mRNA. Copper depletion reduced the HIF-1a reporter cell line response to hypoxia.

Conclusion: Increased copper increased HIF-1 transcriptional activation and copper depletion reduced the normal HIF-1a induction in response to hypoxia.

Experiment 5.1.1: Effect of copper excess on HIF-1 reporter cell line

Introduction: A reporter cell line was established using Chinese Hamster Ovary (CHO) cells by stable transfection with a HIF-1a responsive luciferase reporter construct (Chapter 2). As the whole hypoxia responsive element was not incorporated there was no requirement for cofactors or co-stimulation for transcriptional activation. Hence luciferase expression was in direct response to HIF-1a binding alone, with subsequent transcriptional activation (figure 5.1.1). These HIF-1 reporter CHO cells were exposed to different concentrations of copper ($CuCL_2$) for 24 hours. The time period 24 hours was used to produce a consistent quantifiable response in luciferase activity. Transcriptional activation was measured by luciferase activity expressed in arbitrary light units. Positive and negative controls (CHO reporter cells under hypoxia or normal conditions) were run in parallel during each experiment. Number of cells per well was confirmed by cell count and subsequent protein concentration.

Results: The addition of copper resulted in a stepwise increase in luciferase activity up to a concentration of 1000μM $CuCl_2$ in TCM (figure 5.1.2). Above this concentration there was no effect, which reflects the toxic effect of copper on cell viability as previously described.

Conclusion: Copper induced HIF-1 Transcriptional activation

Figure 5.1.1: Schematic representation of HIF-1 reporter construct

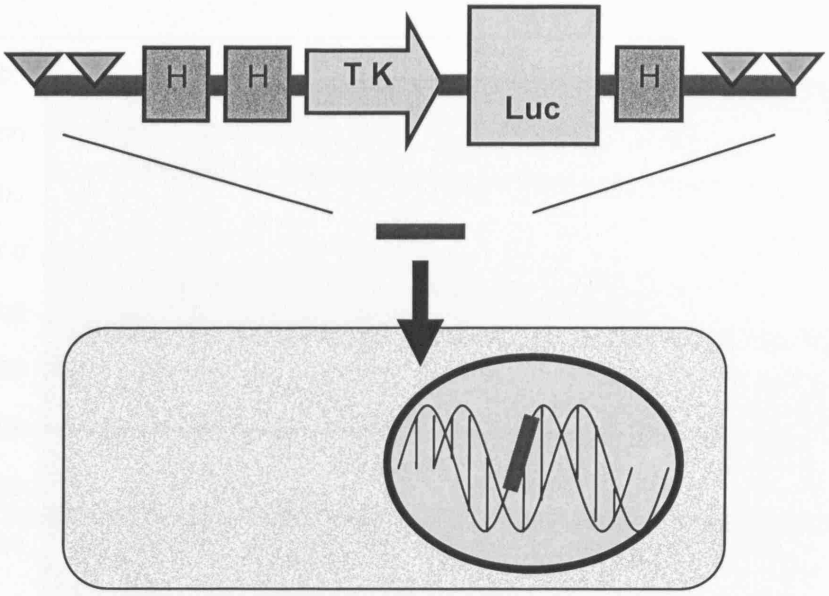


Figure 5.1.1: HIF-1 responsive luciferase reporter construct. Firefly luciferase (Luc) reporter gene was constructed using the Tyrosine Kinase promoter (TK) and three pairs of specific HIF-1a binding sites (H). As the whole hypoxia responsive element was not incorporated there was no requirement for cofactors or co-stimulation for transcriptional activation. Hence luciferase expression was in direct response to HIF-1a binding alone and subsequent transcriptional activation

Figure 5.1.2: Copper excess on HIF-1 transcriptional activation

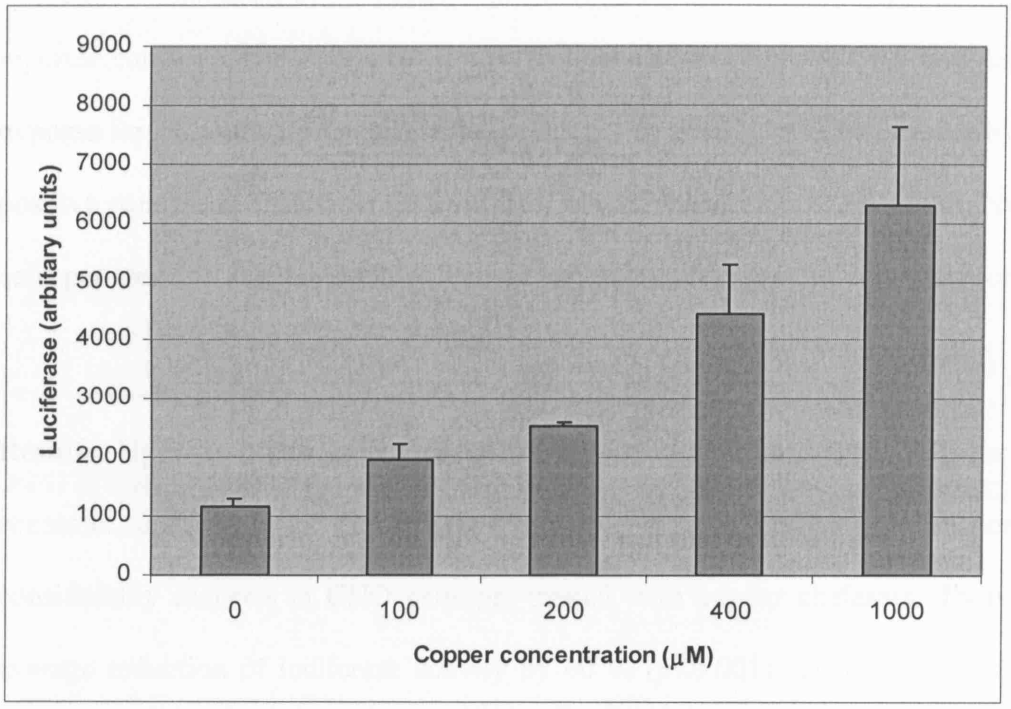


Figure 5.1.1: Chinese Hamster Ovary (CHO) cells with a stable transfection of a HIF-1 responsive luciferase reporter construct were exposed to different concentrations of copper (μM) for 24 hours. The results indicate luciferase activity expressed in arbitrary light units, as a mean and standard deviation. Statistical analysis by ANOVA confirmed significant difference in luciferase activity between different concentrations of copper ($p<0.0001$). However, on Turkey-Kramer Multiple Comparisons Test (post test) only copper concentrations of 400μM & 1000μM produced results that were significantly different ($p<0.01$) to all other concentrations.

Experiment 5.2.1: Effect of copper depletion on HIF-1 reporter cell line

Introduction: CHO cells with stable transfection of the HIF-1a responsive luciferase reporter construct were pre-treated with copper chelators for 4 days and exposed to hypoxia for 24 hours. CHO cells, without any pre-treatment were exposed to hypoxia as positive controls or incubated under normal conditions as negative controls. Number of cells per well was confirmed by cell count and subsequent protein concentration.

Results: Hypoxia produced a significant increase in luciferase activity in the reporter construct compared to the negative control (figure 5.2.1). This response was considerably reduced in CHO cells pre-treated with copper chelators. There was an average reduction of luciferase activity by 60 % ($p<0.001$). There was no difference between the chemicals used or the concentration of copper chelators in this or confirmatory experiments. Repeating this experiment with DSF showed no difference in luciferase activity in cells that had been copper depleted.

Conclusion: Copper reduction reduced HIF-1 transcriptional activation in response to hypoxia.

Figure 5.2.1: Effect of copper depletion on HIF-1 transcriptional activation

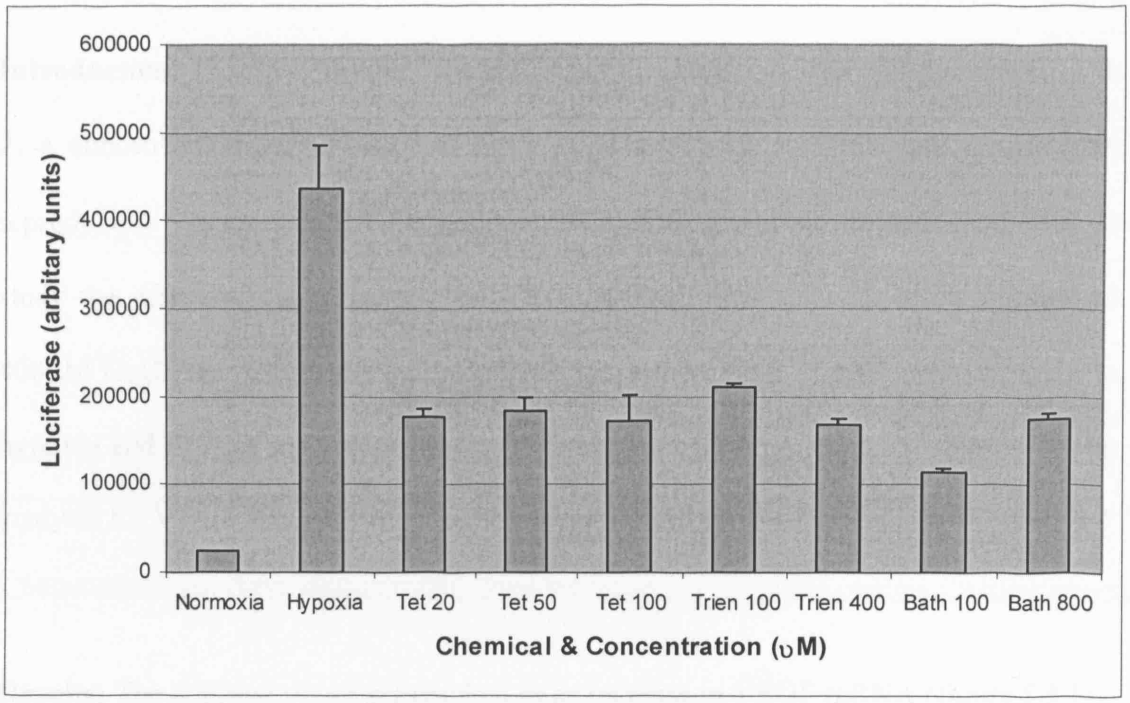


Figure 5.2.1: Chinese Hamster Ovary (CHO) cells with a stable transfection of a HIF-1 responsive luciferase reporter construct were pre-treated with copper chelators for 4 days and exposed to hypoxia for 24 hours. The chemicals used were N,N-Bis(2-aminoethyl)-1,3-propanedamine (Tet), Trithylenetetramine tetrahydrochloride (Trien) and 2,9-Diphenyl-1,10-phenanthrolinedisulfonic acid (Bath). CHO cells, without any pre-treatment were exposed to hypoxia as positive controls or normoxia as negative controls. The results indicate luciferase activity expressed in arbitrary light units, as a mean and standard deviation. Statistical analysis by ANOVA confirmed significant difference in luciferase activity in response to hypoxia in normal cells and those that had undergone copper chelating ($p<0.001$). There were no differences between the chemicals or concentrations used.

Experiment 5.3.1: Effect of copper excess on VEGF mRNA expression

Introduction: From the results of experiments in Chapter 4 and experiments 4.1.1 & 2, a concentration of 400μM $CuCl_2$ in TCM produced maximal and consistently reproducible increase in HIF-1a induction. Therefore this concentration was used to study the effect of copper excess on VEGF mRNA. HEP3B cells were exposed to 400μM $CuCl_2$ for four hours before mRNA extraction. HEP3B cells were exposed to hypoxia and DSF as positive controls or normoxia as negative controls. Northern blot analysis for VEGF was performed on mRNA as described in Chapter3.

Results: The addition of copper resulted in an increase in VEGF mRNA (figure 5.3.1).

Conclusion: Copper induced VEGF mRNA

Figure 5.3.1: Effect of copper excess on VEGF mRNA expression

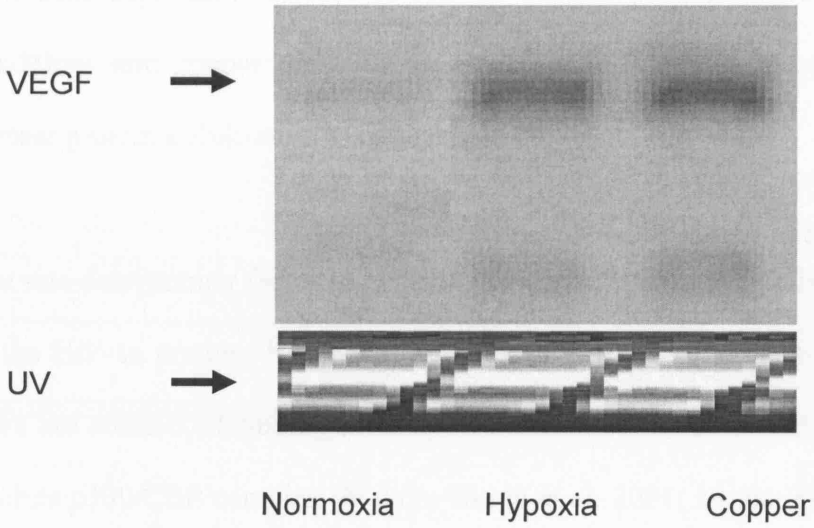


Figure 5.3.1: Northern blot for mRNA performed on HEP3B cells. HEP3B cells were exposed to 400μM $CuCl_2$ for four hours before mRNA extraction. HEP3B cells were exposed to hypoxia and DSF as positive controls or normoxia as negative controls. Northern blot analysis for VEGF was performed on mRNA as described. To confirm equal loading per lane in the gel the Ultraviolet (UV) image is given for comparison.

Discussion

In the previous chapter we showed that increased copper excess in cell culture resulted in a dose dependent increase in HIF-1a nuclear protein stabilisation under normal conditions and copper depletion significantly reduced the expected rise in HIF-1a nuclear protein stabilisation to hypoxia.

The rate-determining factor in hypoxic gene induction by HIF-1 is nuclear stabilisation of the HIF-1a protein. HIF-1 is not regulated at a post-transcriptional level. However, there are some confounding factors. Modulation of the co-factor binding is seen, FIH inhibits p300/CBP complex (Mahon, Hirota et al. 2001; Lando, Peet et al. 2002). Also the p35srj gene, a HIF-1 gene product may also inhibit HIF-1 transcriptional activation (Bhattacharya, Michels et al. 1999). Therefore although we have shown that copper affects HIF-1a nuclear protein accumulation it is also necessary to prove that it can then complex with ARNT and proceed unimpeded to hypoxic gene transcription. We therefore wished to assess whether Copper excess or copper depletion effected HIF-1 transcriptional activation.

Transcriptional activation was measured in two separate ways. By use of stable transfect to create a reporter cell line, also by Northern blot quantification of VEGF a know target gene for HIF-1.

The stable transfection was undertaken in CHO cells and the reporter construct contained six isolated HIF-1 binding sites. As the whole hypoxia responsive element was not incorporated there was no requirement for cofactors or co-stimulation for

transcriptional activation. Hence luciferase expression was in direct response to HIF-1 binding alone and subsequent transcriptional activation. The results seen here for HIF-1 transcriptional activation in CHO cell reporter construct were the same as the data seen from Western blot for HIF-1a nuclear protein. Copper excess in cell culture resulted in a dose dependent increase in luciferase activity under normal conditions. Copper depletion significantly reduced the expected rise in luciferase activity compared to normal conditions under hypoxia. Results that are the same as those seen by Martin's group (Martin, Linden et al. 2005). The Northern blot for VEGF confirmed that copper did not affect transcriptional co-factors and copper increased HIF-1 and hypoxia gene transcription.

These results confirm that altering copper concentration in cell culture affects the HIF-1 pathway of gene activation and gene transcription.

Chapter 6: HIF-1 expression in animal models of Copper Toxicity and Copper Deficiency

Summary of Chapter

Introduction: In Chapters 4 & 5 we showed that increased copper increased HIF-1 induction at protein and at transcriptional level in cell culture. Similarly reduced copper concentration in cell culture reduced the normal levels of HIF-1 induction. We wished to assess the effect of increased and reduced copper on HIF-1 expression in an animal model.

Methods: Immunohistochemistry, with staining for HIF-1a, was performed on liver sections from rats. Different animal groups were examined. Firstly the normal rat was compared with rats subjected to hypoxia in order to establish a baseline level of response and to act as a control group. The effect of copper was then studied in two separate groups; the copper toxic rat and rats fed a copper deficient diet. All experiments involved included serial sections (3-7) from each sample and experiments were repeated a minimum of three times.

Results: Staining for HIF-1a was poor despite different enhancement protocols and results were not consistently reproducible between batches. Increased staining for HIF-1a was seen in hypoxic compared with normal rats. The copper toxic model did not show increased HIF-1a expression compared with their normal counterparts. Staining for HIF-1a was increased in rats fed a copper deficient diet. However, these rats were also anaemic and this may represent a confounding factor.

Conclusion: Reproducible results could not be established using established immunohistochemistry protocols for HIF-1a.

Introduction

In previous chapters we established that increased copper was associated with increased HIF-1 induction under normal conditions. Reduced intracellular copper concentration in cell culture reduced the level of HIF-1 induction in response to hypoxia. However, it was difficult to correlate the effect of copper excess, which did not raise intracellular copper concentration, with the increased nuclear HIF-1a expression. We therefore wished to assess whether these results were reproducible outside cell culture in normal physiology, therefore in an animal model,.

The first experiment looks at HIF-1a expression by immunohistochemistry (IHC). Male Wistar rats exposed to hypoxia were compared to those under normal conditions. These experiments reproduced those undertaken in cell culture and served two purposes; to establish a baseline for HIF-1a expression and detection by IHC and thus establish positive and negative controls, secondly to validate the IHC protocol. The subsequent experiments look at HIF-1 in animal models of copper toxicity and copper depletion.

Animal models used

In these experiments we wished to explore the effects of increased and reduced copper levels in an animal model.

These experiments were performed in collaboration with two other centres (Appendix 2). This was done to minimise the number of animals used, all the samples analysed in

this chapter were recived as 'gifts' from experiments performed for other research projects. For experiments on copper toxicity; the Long-Evans Cinnamon (LEC) rat is an established rodent model for Wilson's disease. A defective copper transporting P-type ATPase causes a failure of intracellular copper transport in the liver. The copper toxicity that results gives the rats a characteristic 'cinnamon' coloured coat. Rather than develop a colony of LEC rats we were able to receive samples from an established colony. These animals were undergoing a separate research programme. We were able to receive fresh frozen liver samples, as described in Methods.

Separate to the samples recived from the LEC colony we were able to receive samples from one other centre that was looking at the effect of hypoxia and separately copper deficiency in the male Wistar rat. There is no established rat model for copper deficiency. The protocol was therefore established for the experiments that group was undertaking. Ideally for the copper depletion experiments it would have been interesting to look at the effect of rats fed a copper deficient diet over time i.e. copper depletion for 1, 2, 3, and 4 weeks with six animals in each group. However, it did not seem ethically reasonable to undertake these experiments in light of the results given here.

Immunohistochemistry and Antibody availibility

The protocol used for IHC staining was well established and was previously reported from our group and others (details are provided in the methods chapter). At the time of experiments only two antibodies against HIF-1a were available. The first was a

commercially available Novus E2 monoclonal antibody. The second a gift from Dr. R. Wenger, Zurich, was a polyclonal chicken antibody. Detection in both protocols was performed using diaminobenzidine (DAB). Since these experiments many more antibodies have become commercially available, these are now well proven and validated between groups. At this time only Professor Wenger's chicken antibody had been validated in the literature and that was predominantly internally by that group.

Throughout the IHC experiments performed, two main problems were encountered. Firstly lack of effective HIF-1a staining and excessive staining of background peroxidase activity. Essentially these two problems cumulated in results showing poor contrast between stained nuclei and surrounding tissue.

In order to overcome these problems discussion took place within the department and histology departments at Birmingham and Zurich. Also DAKO were contacted and the protocol altered at their suggestions. Rather than list all experiments performed to modify the protocol, one is included here and another in the next chapter. Chapter 7 looks at HIF-1a expression in human tissue. Many of the IHC protocol adjustment experiments were undertaken in parallel. Many experiments took place to try and modify the protocol. The main protocol changes employed can be divided in four groups. All experiments involved included serial sections (3-7) from each sample and experiments were repeated a minimum of three times.

Blocking

Background endogenous peroxidase activity was reduced by blocking with normal sheep serum (NSS) or Bovine Serum Albumin (BSA) in TBS + 0.1% Tween for 30min. Dilute hydrogen peroxide was also tried.

Antibody Incubation

Two antibody protocols were used. Concentrations of primary and secondary antibodies were varied. Primary incubation was normally for 24hrs at 4°C however different durations (48hrs) and temperatures were tried.

Washes

The washing steps were normally in TBS. These were changed to include tween or 1% NSS and the duration of washes was varied.

Detection

Peroxidase reaction was detected by incubation with DAB for 10min at room temperature, prepared as per manufacturers instructions with 45mg of sodium azide and filtered 2x before use. Following discussion with DAKO it was not possible to use alternative detection methods. The DAB concentration was varied, duration of incubation varied, washes performed between serial incubations with DAB and different temperatures of DAB used. Commercially available 'liquid DAB' was also tried.

Experiment 6.1.1: IHC for HIF-1a in normal and hypoxic rat liver

Introduction: Male Wistar rats were reared under normal conditions. Animals had free access to water and standard rat chow. Experimental rats (n=4) were exposed to hypoxia (7000m equivalent) for 8 hours. Following ethanasia liver tissue was retrieved. Tissue was fresh frozen and mounted on OCT and stored at –80°C.

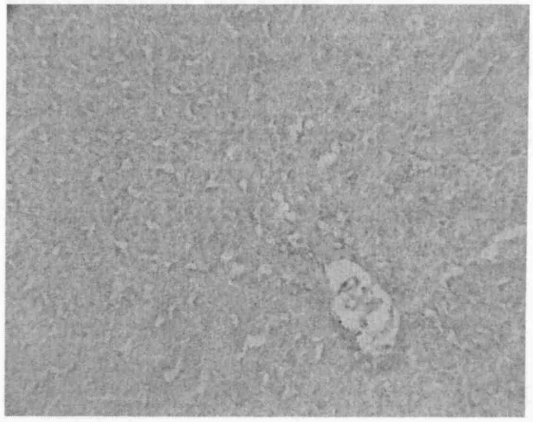
Methods: Sections were stained using the monoclonal antibody protocol with a primary antibody concentration of [1:50]. Additional sections were blocked for 30mins in 10% normal sheep serum. Negative controls were performed with no primary antibody. Antibody staining for HIF-1a was confirmed by a positive control. Following detection one section was counterstained with haematoxylin (CS).

Results: No staining was seen for HIF-1a in the control group (figure 6.1.1a). Considerable background staining was seen in all specimens. A blocking step improved quality and contrast (figure 6.1.1b). Sporadic nuclear staining for HIF-1a was seen in hypoxic tissue (figure 6.1.1c). The increased in HIF-1a staining was not uniform either in sequential sections or between different samples in the experimental group.

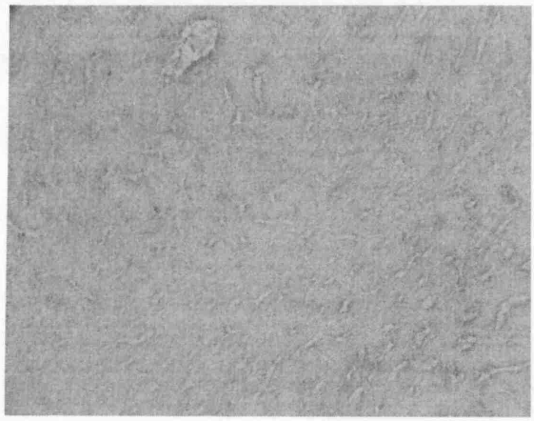
Conclusion: HIF-1a may be increased in hypoxic rat liver

Figures 6.1.1 (a-d): IHC for HIF-1a in normal and hypoxic rat liver

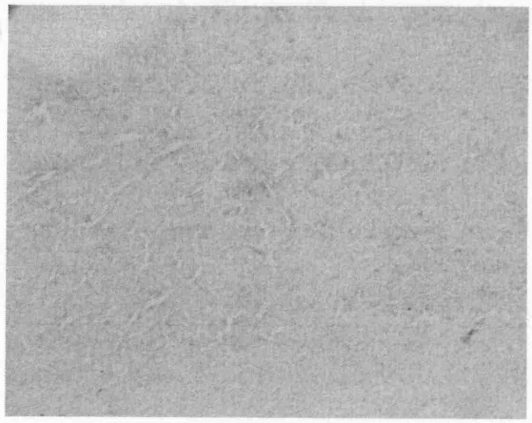
a. Normal Rat



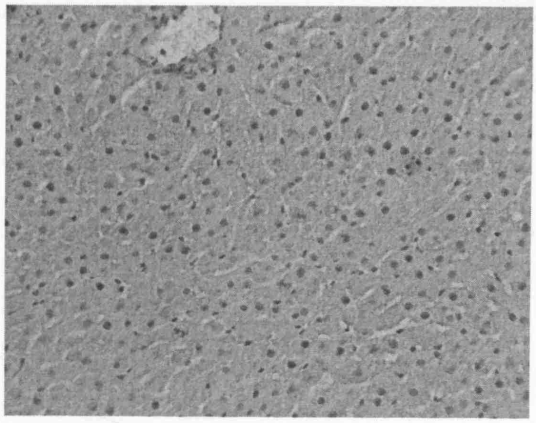
b. Normal Rat (Blocking step)



c. Hypoxic rat



d. Normal Rat CS



Figures 6.1.1 (a-d): IHC was performed on liver samples for HIF-1a on male Wistar rats kept under normal conditions (control group) and those exposed to hypoxia. Counterstaining with Haematoxylin was performed on a sequential section. Images were taken at 25x magnification. The introduction of a blocking step reduced background peroxidase activity in the control group (a, b). Increased nuclear staining for HIF-1a was seen in rats exposed to hypoxia (c), but when compared to a counterstained section (d) staining was sporadic.

Experiment 6.2.1: IHC for HIF-1a in copper toxic rats

Introduction: The Long-Evans Cinnamon (LEC) rat is an established rodent model for Wilson's disease. A defective copper transporting P-type ATPase causes a failure of intracellular copper transport in the liver with consequent copper toxicities. LEC rats are known to develop hepatitis and liver cancer spontaneously.

Methods: Inbred LEC rats and their normal counterparts, Long-Evans Agouti (LEA) rats, were bred under specific pathogen-free conditions. Animals had free access to water and standard rat chow. All rats were weighed. Following ethanasia liver tissue was retrieved. Tissue blocks were immediately frozen in isopentene, pre-chilled by immersion in liquid nitrogen, before storage at $^{-}$80°C. Sections were stained using the monoclonal antibody protocol with primary antibody concentration of [1:50]. Additional sections were blocked for 30mins in 10% normal sheep serum. Negative controls were performed with no primary antibody. Antibody staining for HIF-1a was confirmed by a positive control. Following detection one section was counterstained with haematoxylin (CS).

Results: The average weight of the LEA rats was heavier than the average weight of the LEC rats (229.78g (sd 9.826) v 187.35g (sd 17.483) p = 0.0134) (table 6.2.1). No staining for HIF-1a was seen in either the LEA or LEC liver tissue (figures 6.2.1).

Conclusion: No staining for HIF-1a was seen in either the LEA or LEC tissue.

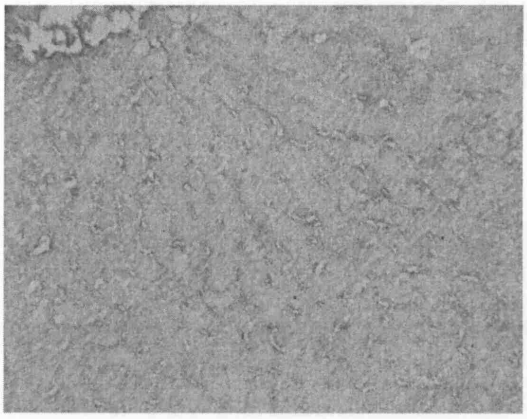
Table 6.2.1: Weight of copper toxic rats and their normal counterparts.

Rat	Weight of rat (grams)	Rat	Weight of rat (grams)
LEA 1	215.7	LEC 1	185.7
LEA 2	237.8	LEC 2	167.6
LEA 3	234.9	LEC 3	210.2
LEA 4	230.7	LEC 4	185.9
Average	**229.78**	**Average**	**187.35**

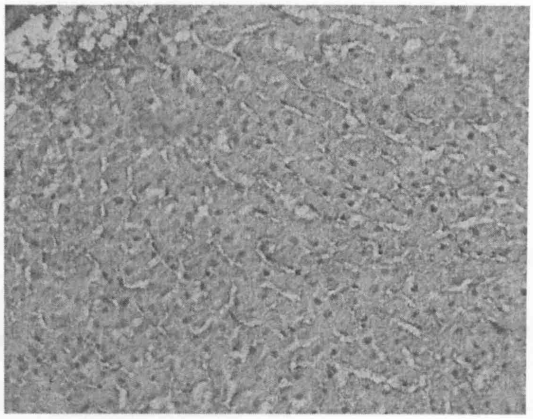
Table 6.2.1: The Long-Evans Cinnamon (LEC) rat and their normal counterparts, Long-Evans Agouti (LEA) rats, were bred under specific pathogen-free conditions. Animals had free access to water and standard rat chow. All rats were weighed. Following ethanasia liver tissue was retrieved. *The average weight of the LEA rats was heavier than the average weight of the LEC rats (229.78g (sd 9.826) v 187.35g (sd 17.483) unpaired T-Test, p = 0.0134)*

Figures 6.1.2 (a-d): IHC for HIF-1a on LEA & LEC rats

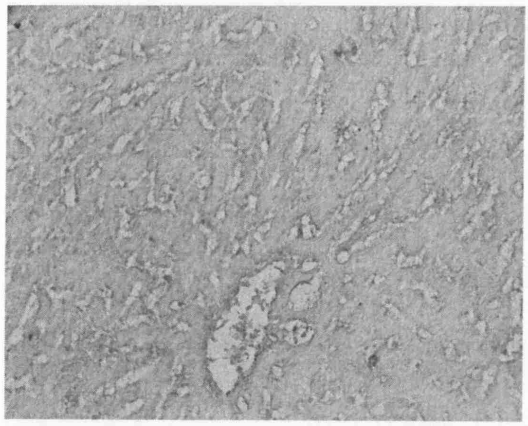
a. LEA Rat



b. LEA Rat CS



c. LEC Rat



d. LEC Rat CS

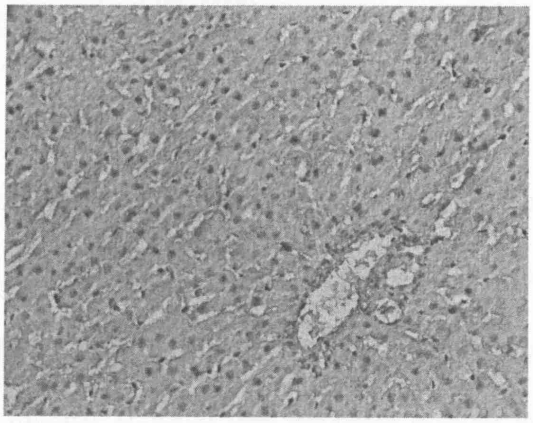


Figure 6.1.2 (a-d): IHC was performed on liver samples for HIF-1a on Long Evans Agouti (LEA) & Long Evans Cinnamon (LEC) rats. Sequential sections were blocked for 30mins in 10% normal sheep serum or counterstained with haematoxylin (CS). Antibody staining for HIF-1a was confirmed by a positive control. Images were taken at 25x magnification. No nuclear staining was seen in either LEA or LEC specimens.

Experiment 6.3.1: IHC for HIF-1a in copper deficient rats

Introduction: The Mottled and Brindled mouse are established models for copper deficiency there was no established rat model. In order to produce copper deficiency male Wistar rats were reared with a copper deficient diet.

Methods: Animals had free access to water and were fed varying degrees of a copper deficient diet (table 6.3.1). Following ethanasia liver tissue was retrieved. Tissue was fixed in 4% formalin solution then mounted on paraffin blocks. Sections were stained using the monoclonal antibody protocol with primary antibody concentration of [1:50]. Additional sections were blocked for 30mins in 10% normal sheep serum. Following detection one section was counterstained with haematoxylin.

Results: Increased staining for HIF-1a was seen in all specimens compared with experiment 6.1.1. HIF-1a nuclear staining was significantly increased in the copper deficient rats 2 & 3 (figure 6.3.1a-e) where the pattern of staining exhibited a zonal distribution across the liver acinus. HIF-1a nuclear staining was maximal around the central vein and reduced around the portal triad. Staining was sequentially reduced in rats 4 to 9 (figures 6.3.2 f-k). There was no difference in results between monoclonal or polyclonal IHC protocols used.

Conclusion: HIF-1a expression was increased in copper deficient rats. Expression was zonal in distribution, maximal around the central vein of the liver acinus.

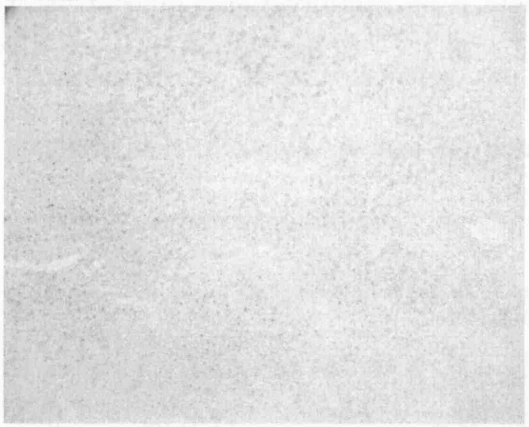
Table 6.3.1: Feeding protocol for male Wistar rats fed a copper deficient diet.

Rat Number	Copper Deficient Diet (Days)	Normal Diet (Days)
Control	0	42
2	40	0
3	42	0
4	42	4
5	42	8
6	42	14
7	42	21
8	42	46
9	42	74

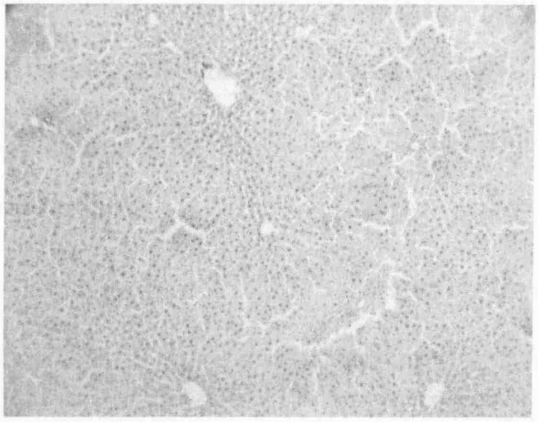
Table 6.3.1: In order to produce copper deficiency male Wistar rats were reared with a copper deficient diet. Animals had free access to water and were fed varying degrees of a copper deficient diet before reintroduction of a standard rat chow.

Figures 6.3.1 (a-e): IHC for HIF-1a in copper deficient rats

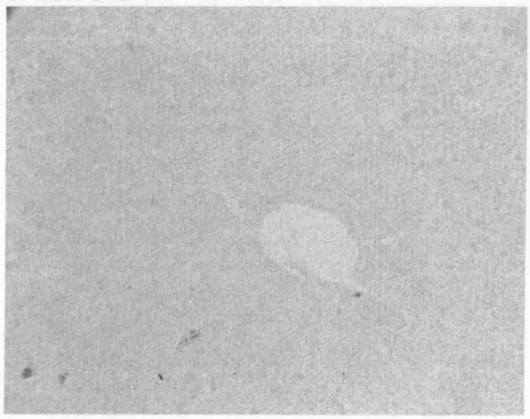
a. Rat 1



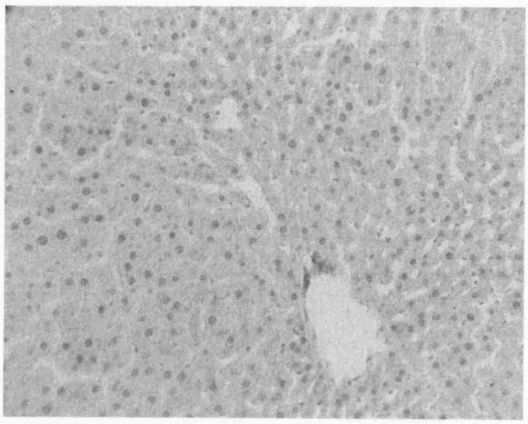
b. Rat 2



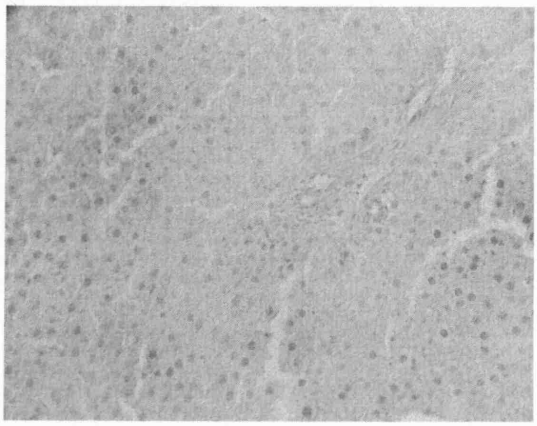
c. Rat 1 CV



d. Rat 2 CV



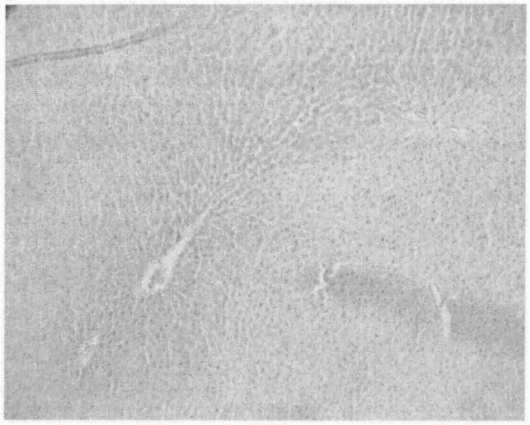
e. Rat 2 PT



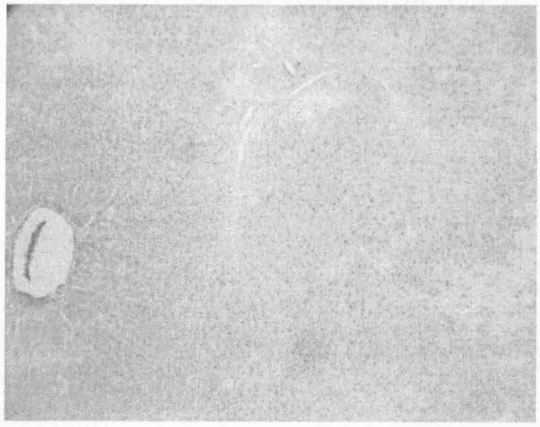
Figures 6.3.1 (a-e): IHC was performed on liver samples for HIF-1a on male Wistar rats fed a copper deficient diet as shown in table 6.3.1. Counterstaining with Haematoxylin was performed on a sequential section. Increased HIF-1a expression was seen in the copper deficient rats 2 (b) compared to the control (a) at 10x magnification. When the images were enlarged to, 25x magnification, this difference was more pronounced around the central vein (CV) in each specimen. Minimal staining was seen in figure c compared to increased nuclear staining seen with figure d. In the copper deficient rats (2 & 3) a zonal pattern of HIF-1a staining was seen with increased HIF-1a staining around the central vein (d) compared to the area around the portal triad (PT) (e). There was no difference in staining between rats 2 & 3.

Figures 6.3.2 (f-k): IHC for HIF-1a in copper deficient rats

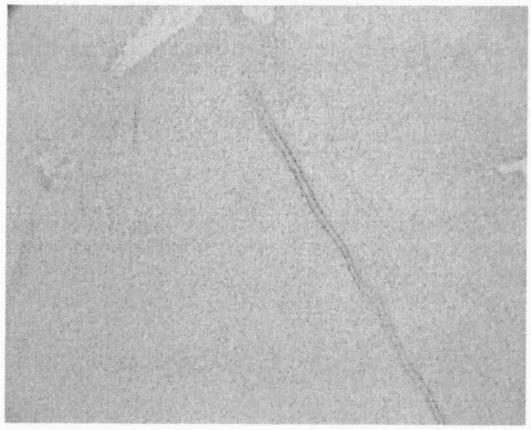
f. Rat 4



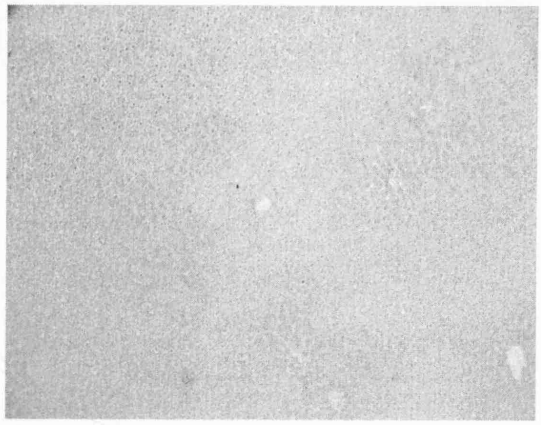
g. Rat 5



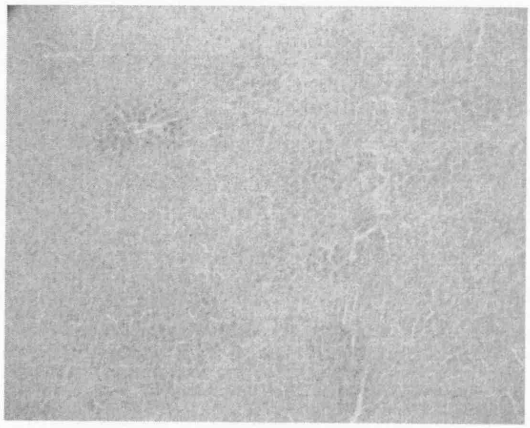
h. Rat 6



i. Rat 7



j. Rat 8



k. Rat 9

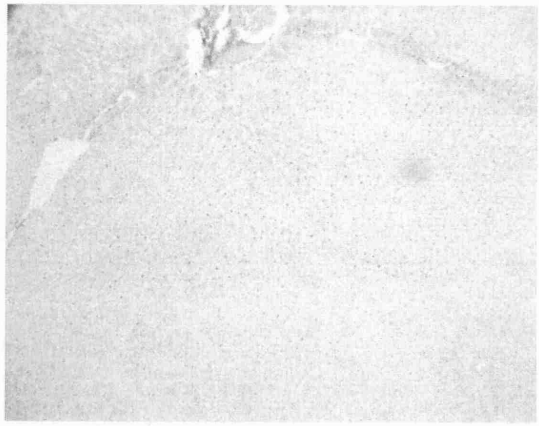


Figure 6.3.2: IHC performed on liver samples for HIF-1a on male Wistar rats fed a copper deficient diet as shown in table 6.3.1. Images were taken at 10x magnification. Compared to the copper deficient rats 2 & 3 where increased HIF-1a expression was seen (b) compared to the control (a) less staining for HIF-1a was seen in rats 4 to 9 (f – k). Some increased (dotted) staining can be seen in nuclei of some of the sections. However this is not as clear as the previous sections.

Discussion

The experiments performed in Chapters 3 & 4 revealed consistent results suggesting a role for copper in HIF-1a induction. Increased copper in cell culture induced HIF-1a protein stabilisation, nuclear translocation and transcriptional activation. Reduced copper has the opposite effect.

The IHC experiments were performed on normal and rats exposed to hypoxia in order establish a baseline in the rat for positive and negative controls in subsequent experiments. A baseline expression of HIF-1a was expected in normal liver tissue, reflective of the 'housekeeping function'. The question was, whether the IHC protocol would be sensitive enough to detect it. Work from the Keitzmann group had shown uniform expression of HIF-1a throughout the liver acinus under normal conditions (Kietzmann, Cornesse et al. 2001). However, others had not shown HIF-1a detectible by IHC in normal tissue (Zhong, De Marzo et al. 1999).

The hypoxia experiment was a repetition of published experiments shown to significantly increase HIF-1a in tissues of animals exposed to hypoxia (Camenisch, Tini et al. 1999; Stroka, Burkhardt et al. 2001). In the experiments performed here the results produced less staining than expected. No HIF-1a was seen in normal rat liver. Some HIF-1a staining was seen in the hypoxic tissue (figure 6.1.1c), although these results were variable and thus interpretation is subjective. Other groups had similar problems while trying to assess HIF-1a in human tumours (Talks, Turley et al. 2000). Increased HIF-1 expression when assessed by IHC was only seen in half of human tumours studied (Zhong, De Marzo et al. 1999). In a study looking at cervical cancer,

clinical outcome was worse in HIF-1 staining tumours. No difference was found in HIF-1 staining between stage or grade of disease and considerable variability of staining was seen (Birner, Schindl et al. 2000). The reason for poor staining in these experiments is similar, probably reflecting the quality of the primary antibody available at that time. The antibodies were not reliably effective in IHC (Professor C. Pugh – personal communication). As an alternative to IHC for HIF-1a, different groups have used downstream genes as a marker of HIF-1 in tumour specimens (Lal, Peters et al. 2001) or specific tumour cell lines (Yoon, Buchler et al. 2001). Alternatively insitu hybridisation for iNOS or VEGF could be performed.

The LEC Rat is the established rodent model for Wilson's disease. An interesting feature of LEC rat is increased incidence of hepatoma formation. A similar feature is seen in Wilson's disease, where untreated patients have a 100x relative risk of developing hepatocellular carcinoma. ROS and hydroxyl radicals generated by copper are suggested to trigger the mechanism of carcinogenisis (Terada and Sugiyama 1999; Yamamoto, Hirose et al. 1999; Theophanides and Anastassopoulou 2002). The use of the LEC rat therefore seemed logical to assess the effect of copper excess in the animal model. It was expected that HIF-1a expression would be increased in the LEC rat. Unfortunately no HIF-1a expression was seen in any of the LEA or LEC specimens. In these experiments, tissue samples were from an established colony of LEC rats (Professors M. L. Schilsky & S. Gupta, NY). The tissue samples were rapidly retrieved and fresh frozen, therefore it was assumed the results seen here were not a consequence of processing. One possibility could reflect the problems with sensitivity of HIF-1a IHC as highlighted before. Another, that HIF-1a is not over-expressed in the setting of chronic copper toxicity. HIF-1 has dual function; a housekeeping role with a baseline

expression reflecting normal cellular function, or an acute phase response to stimulus as seen in hypoxia. The effect of chronic stimulation of HIF-1 has not been studied extensively. In animals subjected to chronic hypoxia the initial increase in HIF-1a induction returned to normal over time (Chavez, Agani et al. 2000). The initial exposure to hypoxia would induce HIF-1a and trigger an acute response via induction of angiogenesis, polycythemia and metabolic changes. These responses during prolonged hypoxia would be able to restore normal oxygen tension, thus removing the tissue hypoxia (LaManna, Chavez et al. 2004). The effect may be the same with chronic copper exposure, with adaptation to the presence of chronic copper toxicity. Therefore the results seen here may be a true representation of HIF-1a expression in the setting of chronic copper toxicity. Experiments should be repeated with a third group for comparison of LEA rats exposed to acute copper toxicity. At the time of these experiments no ELISA was available for the assessment of HIF-1 protein in rat tissue.

The results from the copper deficient rat experiments showed increased expression of HIF-1a. These results confirm that the IHC protocol used was working and HIF-1a could be detected in the cell nucleus. The obvious question is why did copper deficient rats have increased HIF-1a expression? Results that are opposite to those in cell culture. One answer could be that this was a processing problem. The tissue blocks were fixed in formalin rather that fresh frozen in liquid nitrogen. The tissue may have been subjected to hypoxia prior to, or during fixation (Talks, Turley et al. 2000). However, against this argument is the copper deficient rats (rats 2 & 3) had the greatest increase in HIF-1a expression. This decreased sequentially in those rats fed a normal diet.

An alternative explanation was that rearing rats with a copper deficient diet made the animals very sick and in particular anaemic. Factors that resolved, on return to normal diet. The presence of anaemia would significantly induce HIF-1a and consequently EPO production and this may represent a confounding factor, causing false positive results in these experiments. The experiments may represent the HIF-1a response to anaemia rather than copper deficiency.

An interesting result was that the increased HIF-1a expression exhibited a zonal pattern in the liver acinus. The area around the central vein is relatively hypoxic compared to that around the portal triad. It is therefore logical to assume that HIF-1a would be expressed in this area. Previous experiments in normal liver have demonstrated uniform expression of HIF-1a across the acinus (Stroka, Burkhardt et al. 2001) . It may be that although HIF-1 protein is uniformly expressed across the liver acinus under normal conditions, it increased in a zonal pattern under situations of stress following acute stimulation and subsequent HIF-1a induction. This explanation for a zonal pattern of HIF-1a expression would in turn lead to a zonal pattern of gene transcription and is in keeping with work undertaken by the Keitzmann group. They suggested this in an elegant series of experiments that showed a zonal regulation of gene function responsible for glucose metabolism, in response to oxygen (Kietzmann, Cornesse et al. 2001; Kietzmann, Krones-Herzig et al. 2002; Kietzmann 2004).

Chapter 7: HIF-1 expression in the Human Liver

Summary of Chapter

Introduction: We have shown, in cell culture, that copper excess increased HIF-1 induction at protein and at transcriptional level. Similarly reduced copper concentration in cell culture reduced the normal levels of HIF-1 induction. Results in the animal model of copper toxicity and copper depletion were inconclusive. We wished to assess HIF-1 expression in human liver.

Methods: Three sets of experiments were undertaken. First the effect of copper excess on HIF-1a induction was assessed in primary human hepatocytes. Second, IHC with staining for HIF-1a was performed on liver samples from patients with; normal liver, cirrhotic alcoholic liver disease, Wilson's' disease and ischaemia reperfusion injury (IRI) following liver transplantation. Finally an ELISA for HIF-1a on liver tissue from patients with Wilson's' disease was performed.

Results: HIF-1a nuclear protein was increased in primary human hepatocytes exposed to copper excess. IHC staining for HIF-1a was poor despite different enhancement protocols. However liver with IRI following transplantation showed increased expression of HIF-1a nuclear protein. ELISA showed no difference for HIF-1a compared to controls in patients with Wilson's disease.

Conclusion: Primary hepatocytes showed increased HIF-1a expression in response to copper excess. Increased HIF-1a expression was not seen in patients with Wilson's disease. HIF-1a expression may be increased in patients with IRI.

Introduction

In previous chapters we established that increased copper was associated with a minimal rise in intracellular copper concentration and was associated with increased HIF-1 induction. Reduced intracellular copper concentration in cell culture reduced the level of HIF-1 induction in response to hypoxia. In Chapter 6 the experiments on IHC in the animal models unfortunately yielded poor results except in some of the copper deficient rat samples, whose results were conflicting. These may a problem of the antibody effectiveness, as only two antibodies were available at the time of theses experiments (as described in Chapter 6, Introduction).

In the experiments performed here we explore the effects of increased and reduced copper levels in human tissue. The single biggest factor affecting this part of the research was the Alder Hay Hospital scandal in 2000. Initially this project aimed to assess HIF-1a and downstream gene expression throughout the liver transplant process. The plan was to retrieve liver biopsies at all stages in the transplant process; from the donor before and after cold perfusion, in the explanted liver, and in the recipient following implantation and on day seven post operatively. The objective was to correlate clinical and molecular data. Following the Alder Hay Hospital scandal in the press and the ensuing aftermath there was a complete embargo on all submissions to the local ethics committee. At the Queen Elizabeth Hospital, Birmingham patients, following liver transplantation routinely had a protocol biopsy on day seven post-operatively. The Liver Labs had an established ethical approval to retrieve additional liver biopsies from these patients for the purpose of research. A large tissue bank of

biopsies was kept with over 1000 samples and these were important to the departmental research programme. At the start of this research considerable concern over the biopsy tissue bank and the continuation of research biopsies prevented submission of further projects involving human tissue retrieval from the department. Consequently the initial proposal for research was abandoned. Furthermore considerable restraint was placed on all access to primary human hepatocyes and the biopsy tissue bank. Fortunately some access was allowed, and these experiments are described in this chapter.

The first experiment looks at HIF-1a expression in primary hepatocytes this was undertaken to confirm that the response seen in HEP3B cells was indicative of non-immortalised cells. Healthy liver tissue was retrieved at the resection margins from a patient undergoing operation for colorectal hepatic metastases. Extracted hepatocytes were plated and allowed to rest overnight. The normal lifespan for these cells was a couple of days, therefore copper depletion by incubation of the primary hepatocytes with the copper chelators was not possible as this process took four days.

In order to assess HIF-1a in human liver samples IHC was performed. The IHC experiments of Chapter 6 were undertaken in parallel with those reported here. The same protocols were used with the two antibodies against HIF-1a that were available at that time. The aim was to look at HIF-1a expression in normal human liver and liver samples taken from patients undergoing liver transplant for alcoholic liver disease (ALD) and end stage cirrhosis. The reasons for using ALD samples were; these samples were taken insitu from the liver before it was explanted and the samples were snap frozen. Increased HIF-1a expression was expected in liver samples from patients

with end stage ALD for two reasons; severe cirrhosis and scarring would distort the acinii and result in areas of local tissue hypoxia, also that HIF-1 would be in increased in the focal areas of nodular regeneration, which are vascular and undergoing increased angiogenisis. These experiments would then serve to establish a baseline for HIF-1a expression and detection by IHC in human liver so that comparison could be made with samples from patients with Wilson's disease and IRI following liver transplant.

As with Chapter 6, the same problems were encountered and there was no difference in results seen for IHC in human or rat tissue. Throughout the IHC experiments two main problems were encountered; lack of effective HIF-1a staining and excessive staining of background peroxidase activity. In Chapter 6 we described how this was explored, the protocol adjustments made and experiments performed. The human and rat IHC was performed at the same time, either together or in batches. Although we consistently showed increased HIF-1a staining in the copper deficient rats (2, 3) results were less consistent in other samples. The antibodies used were confirmed effective as the same aliquots were used in parallel for the cell culture Western Bolts. All the protocol adjustment experiments are not shown in full here and an example is given that represents the work undertaken.

The main objective for this chapter was to explore HIF-1a expression in patients with copper toxicity (Wilson's Disease) and in patients with IRI following transplantation. IHC was performed on both sets of samples and an ELISA was undertaken on the Wilson's patients. ELISA was not performed on the IRI due to volume of tissue required for the experiment.

Experiment 7.1.1: Effect of copper excess on HIF-1a induction:

Introduction: To assess whether response seen in HEP3B cells was indicative of non-immortalised cells, the experiments of Chapter 4 were repeated with primary human hepatocytes. Primary hepatocytes were extracted and plated in TCM then allowed to settle overnight. Experiment 4.1.1 was repeated and primary hepatocytes were exposed to different concentrations of copper for a period of four hours. Western blot analysis for HIF-1a was performed on nuclear protein extracts. Positive and negative controls were run in parallel during each experiment.

Results: HIF-1a protein was detected in nuclear extracts of primary hepatocytes exposed to copper at a concentration of 400μM $CuCl_2$ in TCM (figure 7.1.1). Small amount of HIF-1a nuclear protein was seen at lower concentrations but not with the same effect as with HEP3B cells.

Conclusion: Copper excess induced HIF-1a in primary hepatocytes.

Figure 7.1.1: HIF-1a induction by copper in primary hepatocytes

Figure 7.1.1: Western blot analysis performed on nuclear protein extracts prepared from primary human hepatocytes exposed to different concentrations of $CuCl_2$ in TCM for four hours. Immunostaining was performed for HIF-1a. Primary hepatocytes were exposed to hypoxia as positive (Pos) controls or normoxia as negative (Neg) controls. A second positive control was HEP3B cells exposed to hypoxia for four hours.

Experiments 7.2.1: IHC for HIF-1a in normal human liver.

Introduction: We wished to establish a baseline for HIF-1a expression in the in the normal liver, so that comparison could be made in subsequent experiments. Normal liver samples were retrieved at the resection margins from patients undergoing operation for colorectal hepatic metastases. Tissue was fresh frozen in isopentene in the gaseous phase of liquid nitrogen, then mounted on OCT and stored at $-80^{o}C$.

Methods: The protocol adjustments described in Chapter 6 were undertaken in parallel with experiments performed on human samples in this chapter. One set of protocol experiments is given here (table 7.2.1) where several staged changes were made to the protocol described in Methods.

Results: Figures 7.2.1(A1-C3) show the results. The uses of a blocking step improved the quality of IHC in the human liver as it did in the rat sections in Chapter 6. Increased incubation times for the primary or secondary antibodies did not enhance HIF-1a detection and varying the detection protocol did not enhance HIF-1a staining in these sections or those from patients with ALD (data not shown). Similarly no difference was seen between the monoclonal or polyclonal antibodies used.

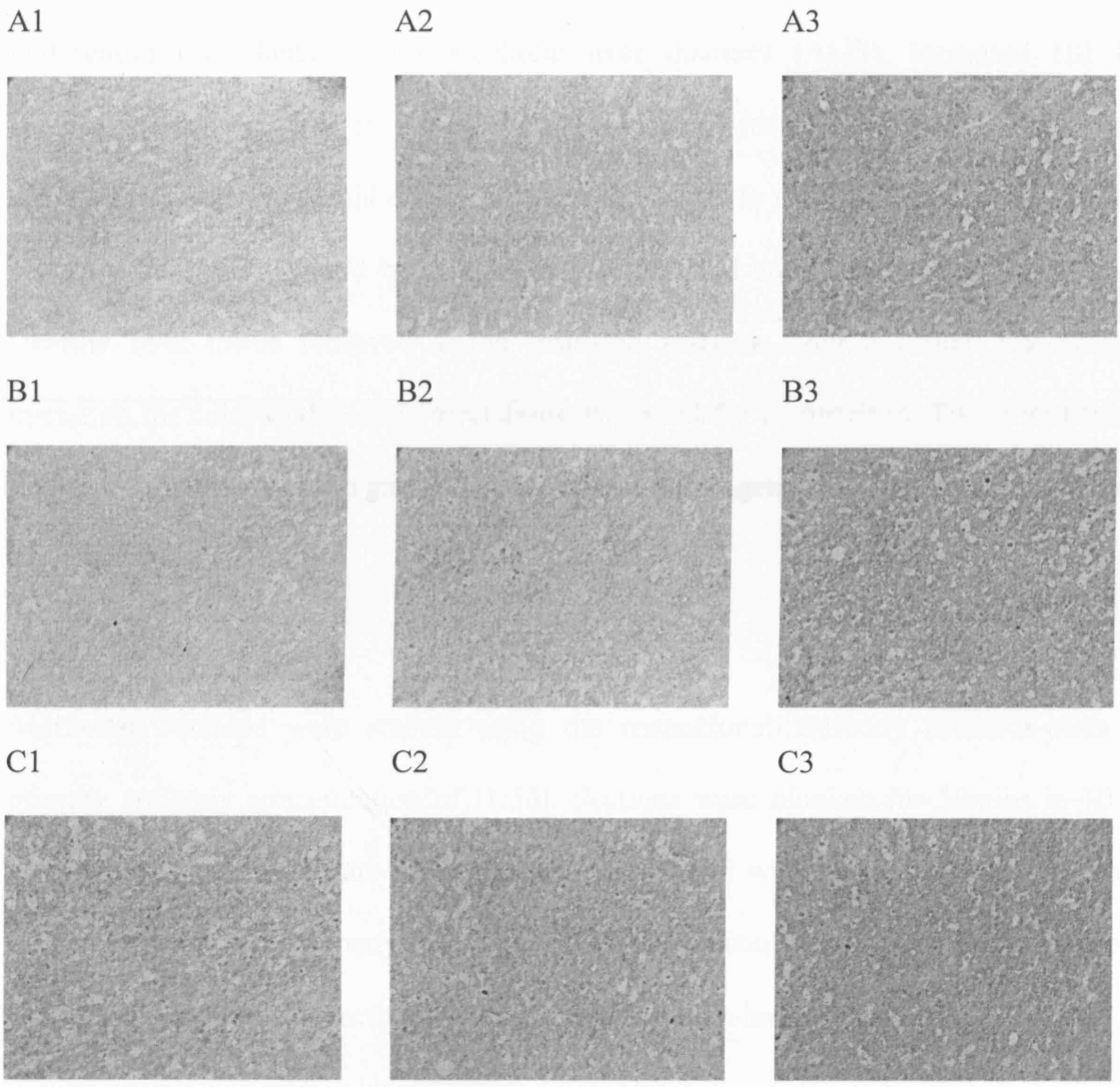
Conclusion: It was not possible to achieve detection of HIF-1a in normal human liver.

Table 7.2.1: Changes in the IHC protocol for detection

Image	Block	Protocol	Detection
A1	10% NSS	Normal	10 min DAB
A2	10% NSS	Normal	2 x 5min DAB
A3	10% NSS	Normal	Liquid DAB
B1	10% NSS	5 min washes	10 min DAB
B2	10% NSS	5 min washes	2 x 5min DAB
B3	10% NSS	5 min washes	Liquid DAB
C1	10% NSS	No Tween	10 min DAB
C2	10% NSS	No Tween	2 x 5min DAB
C3	10% NSS	No Tween	Liquid DAB

Table 7.2.1:. Changes in detection protocol for HIF-1a IHC. All samples were initially blocked with 10% normal sheep serum (NSS) and antibody incubation was as described in Chapter 2. Prior to detection the washes were altered as shown and the detection with DAB as then altered to try to improve the staining of nuclei for HIF-1a.

Figures 7.2.1 (a-c): IHC for HIF-1a using different detection protocols



Figures 7.2.1 (A1- C3): IHC was performed on human liver samples for HIF-1a from patients with normal liver tissue. Images were taken at 25x magnification. The introduction of a blocking step was previously shown to be effective in reducing background peroxidase activity in experiment 6.0.1.). The polyclonal antibody protocol was used at a concentration of 1:50. Variation of the wash part of the protocol or changes in the detection part had no effect on HIF-1a detection. HIF-1a was not seen in sections taken from normal human liver.

Experiment 7.2.2: IHC for HIF-1a in alcoholic liver disease.

Introduction: Tissue samples were collected from livers removed from patients undergoing transplantation for alcoholic liver diseases (ALD). Increased HIF-1a expression was expected in a liver with severe ALD for two reasons; as the severe cirrhosis and scarring would distort the acinii and result in areas of local tissue hypoxia, secondly that HIF-1 would be in increased in the focal areas of nodular regeneration. Healthy liver tissue retrieved at the resection margins from a patient undergoing operation for colorectal hepatic metastases, was used for comparison. Tissue was fresh frozen in isopentene in the gaseous phase of liquid nitrogen, then mounted on OCT and stored at $-80^{o}C$.

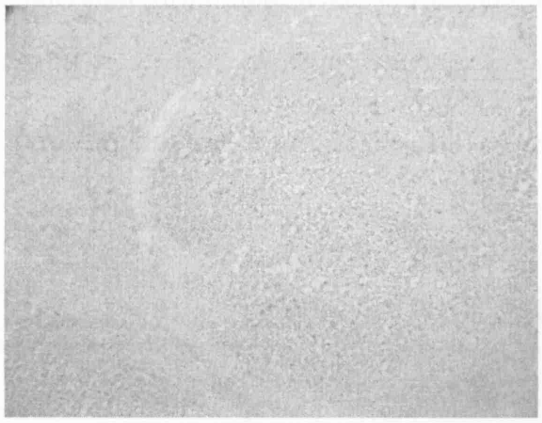
Methods: Sections were stained using the monoclonal antibody protocol with a primary antibody concentration of [1:50]. Sections were blocked for 30mins in 10% normal sheep serum. Negative controls were performed with no primary antibody and normal human liver. Antibody staining for HIF-1a was confirmed by a positive control. Following detection one section was counterstained with haematoxylin (CS).

Results: There was mild positive staining for HIF-1a in the regenerative nodules for HIF-1a compared to normal liver (figure 7.2.2). The positive control produced obvious staining for HIF-1a.

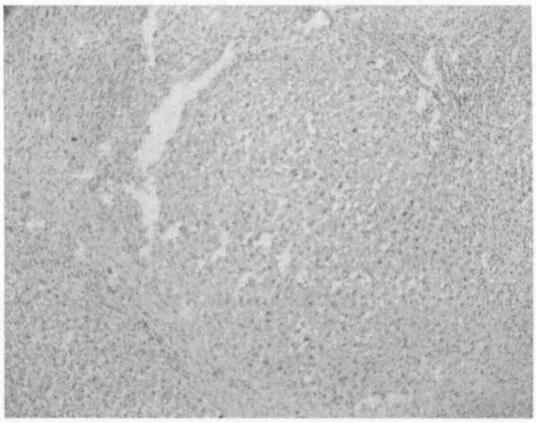
Conclusion: HIF-1a expression was marginal in may be increased in ALD.

Figures 7.2.2 (a-d): IHC for HIF-1a in ALD

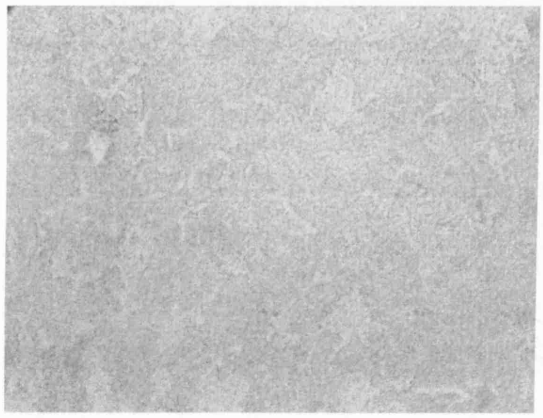
a. ALD



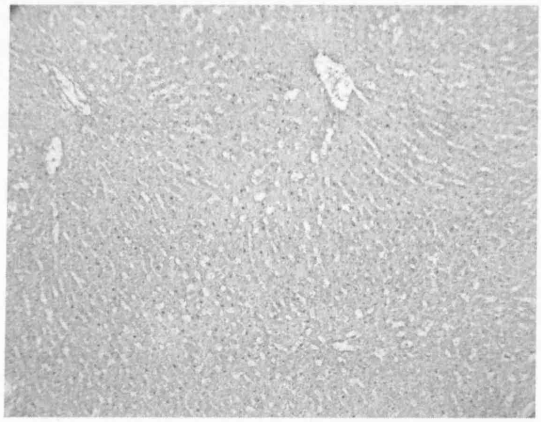
b. ALD CS



c. Normal Liver



d. Rat Liver (2) - Positive control



Figures 7.2.2 (a-d): IHC was performed on human liver samples for HIF-1a from patients with ALD and those with normal liver tissue. The monoclonal antibody protocol was used. Primary antibody concentration was [1:50] and counterstaining with Haematoxylin was performed on a sequential section. Images were taken at 25x magnification. HIF-1a was marginally increased in samples with ALD in the areas of nodular regeneration (a, b) compared to normal human tissue (c) where no HIF-1a expression was seen. A positive control of copper deficient rat (2) tissue was used (d).

Experiment 7.2.3: HIF-1a IHC in Wilson's disease

Introduction: In the previous decade several patients at the QEH Birmingham had undergone orthotopic liver transplantation for end stage liver disease secondary to the Wilson's disease. Samples of liver tissue from the explanted livers were kept in the tissue bank at the 'liver labs' at the University of Birmingham.

Methods: Both antibody protocols were used. The IHC protocol remained the same with inclusion of a blocking step with 10% NSS.

Results: Specimens were difficult to process and section. Problems arose from shattering of tissue. No significant staining was seen for HIF-1a, although some sections did show scattered nuclear staining for HIF-1a (figures 7.2.3 (a-d)).

Conclusion: No significant staining was seen for HIF-1a in Wilson's disease.

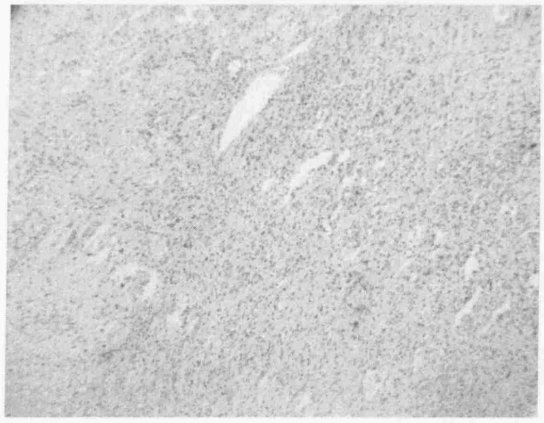
Figures 7.2.3 (a-d): IHC for HIF-1a in Wilson's disease

a. Polyclonal

b. Polyclonal (CS)

c. Monoclonal.

d. Monoclonal (CS).



Figures 7.2.3 (a-d) IHC for HIF-1a performed on liver biopsies from patients with Wilson's disease. Both the monoclonal and polyclonal antibody protocols were used. Primary antibody concentration was [1:50] and images were taken at 25x magnification. No difference was noted between the protocols and little staining was seen for HIF-1a, some scattered nuclear staining was seen (a, c) but this was clearly marginal when comparing to the counterstained sections (b, d).

Experiment 7.3.1: ELISA for HIF-1a in human liver.

Introduction: Following difficulties with the IHC protocols and failure to achieve reproducible results, alternative methods of detecting HIF-1a in human tissue were sought. One option was to process tissue for Western Blot, unfortunately limited tissue availability prevented this. So a new commercially available ELISA for HIF-1a was used. Healthy liver and tissue from explanted livers from patients with Wilson's' disease were compared.

Methods: Tissue was processed in liquid nitrogen as described in Chapter 2. ELISA was performed on protein extracts according to manufacturers guidelines.

Results: The ELISA showed increased HIF-1a in the two positive controls used (figure 7.3.1). No increased HIF-1a was seen in the liver samples from patients with Wilson's disease.

Conclusion: No HIF-1a was detected in liver from patients with Wilson's disease.

Figure 7.3.1: Human liver ELISA for HIF-1a

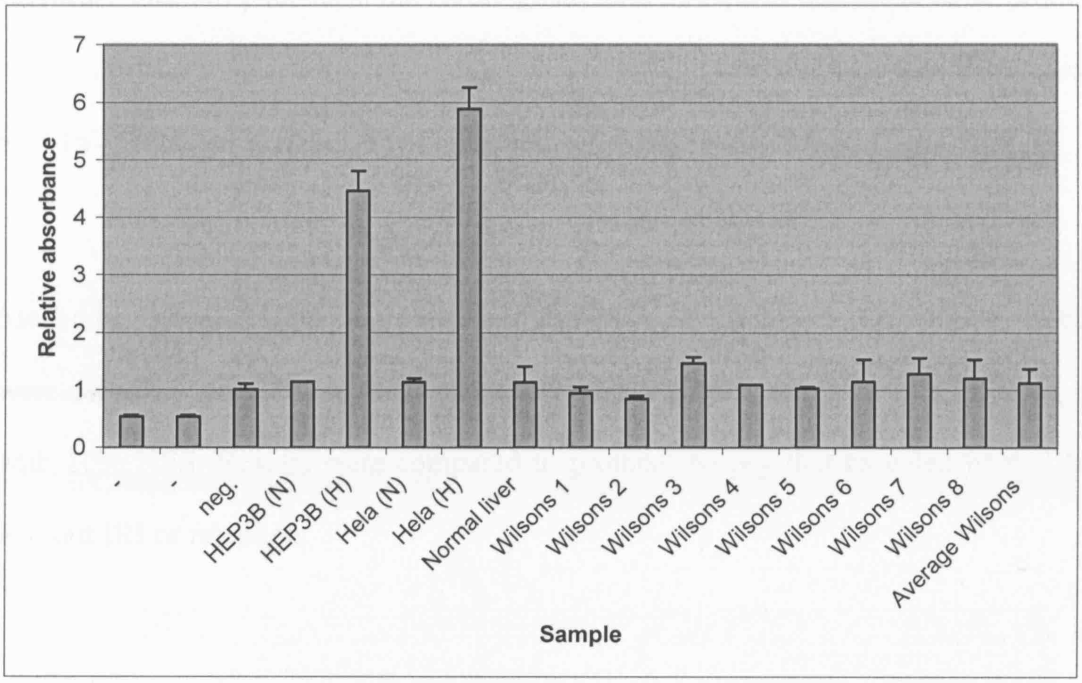


Figure 7.3.1. ELISA for HIF-1a protein on tissue samples from patients undergoing transplantation for end stage liver disease secondary to Wilson's disease. HEP3B and HELA cells under normoxia and hypoxia were used as positive and negative controls. There was no difference in relative absorbance comparing normal liver tissue with all the Wilson's samples (unpaired t-test; p=0.955)

Experiment 7.4.1: HIF-1a IHC in IRI

Introduction: All patients at the Birmingham liver transplant unit underwent 'protocol' biopsy of the transplanted liver on day seven following operation. We wished to assess HIF-1a expression in patients with histologically diagnosed IRI.

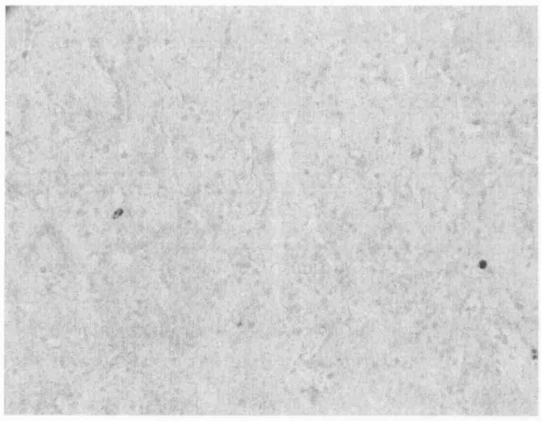
Methods: Biopsies from four patients with histologically proven IRI and no rejection were assessed. The IHC protocol remained the same with inclusion of a blocking step with 10% NSS. Results were compared to protocol biopsy that revealed normal liver without IRI or rejection.

Results: Increased staining was seen for HIF-1a in all sections studied (figures 7.4.1 (a-d)).

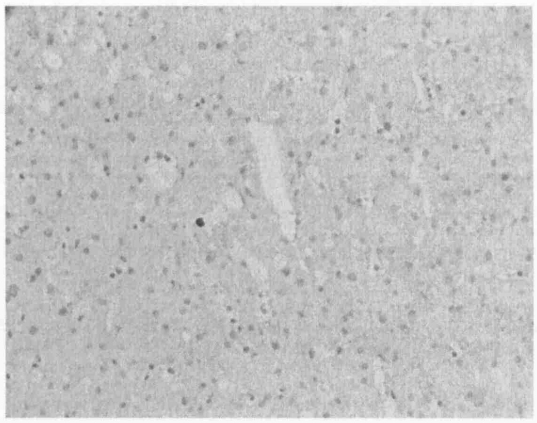
Conclusion: HIF-1a was increased in IRI compared to normal liver.

Figures 7.4.1 (a-d): HIF-1a IHC in IRI

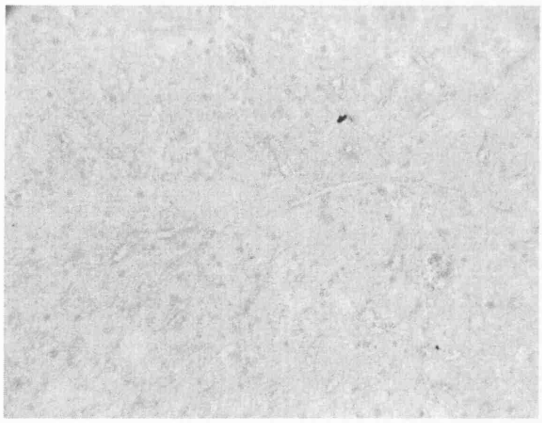
a. IRI



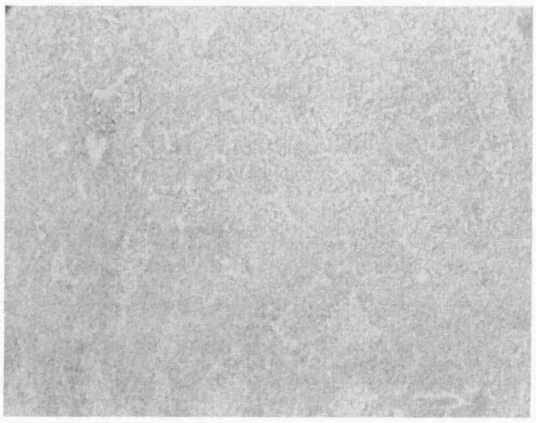
b. IRI CS



c. IRI



d. Normal



Figures 7.4.1: IHC for HIF-1a performed on liver biopsies from patients with Ischaemia Reperfusion Injury. The monoclonal antibody protocol was used. Primary antibody concentration was [1:50] and images are taken at 25x magnification. Normal human liver was used here as a negative control.

Discussion

The experiments performed in Chapters 3 & 4 revealed consistent results suggesting a role for copper in HIF-1a induction. Copper excess in cell culture induced HIF-1a protein stabilisation, nuclear translocation and transcriptional activation. To assess whether this response seen in HEP3B cells was indicative of non-immortalised cells the experiments were repeated with primary human hepatocytes. The response seen did demonstrate some increased HIF-1a nuclear protein on exposure to copper for four hours. However, the response was not as marked as that seen in HEP3B cells. There may be several reasons for this. Primary hepatocytes were extracted from surgically removed liver segments from patients with benign liver tumours or secondary hepatic deposits from colonic carcinoma. The excised liver would first be examined by a histopathologist for assessment of resection margin, disease diagnosis, stage and grade. Following this the remaining tissue was available, with consent, for research. Consequently several hours' delay occurred before extraction of primary hepatocytes. This delay, warm ischaemia and extraction process may affect molecular response of primary hepatocytes. But contrary to this HIF-1a induction was as expected in response to hypoxia suggesting the functional response of the extracted hepatocytes to be intact. An alternative reason may be increased susceptibility to copper toxicity or altered dose response to copper. The latter is less likely as dose response to hypoxia, DSF, Cobalt and other stimuli are similar in all previous studies published. Altered time kinetics may also explain the difference, however the response to hypoxia was the same as HEP3B cells following hours of exposure. Difficulty in extraction of primary hepatocytes, high demand and adequate supply of healthy liver tissue meant that experiments could only be repeated a few times to address these questions.

The results in normal human liver and from patients with ALD were disappointing. A baseline level of HIF-1a expression was expected, reflecting a housekeeping role in the normal liver, as discussed in Chapter 6. The hypothesis was that in ALD with severe cirrhosis liver tissue would be hypoxic, also areas of regeneration as seen in the IHC sections may express HIF-1a (French 2004; Medina, Arroyo et al. 2004). Although some increased staining could be seen in the regenerating nodules this was not marked. Despite enhancement of the detection and adjustments to the protocol results were uniformly poor. Sampling and sectioning of the tissue specimens cannot be at fault as in both cases tissue was retrieved and frozen within minutes.

Results from Wilson's disease tissue were again disappointing. Wilson's disease is rare. Only eight patients had received OLT for end stage liver disease secondary to Wilson's disease at the Queen Elizabeth Hospital Birmingham in the previous twelve years. One biopsy from each was available for analysis, and access to this tissue was limited. The tissue specimens themselves were extremely hard and brittle, consequently obtaining sections for IHC was difficult, as the tissue tended to shatter or crumple on cutting in the cryostat. Furthermore as IHC had proven unreliable for analysis of HIF-1a, persistence along this line of investigation was abandoned. Another method of analysis could have been to perform Western Blot on nuclear protein extracted from frozen tissue specimens. However, this would have required use of all the biopsy material, which was not an option. The ELISA used was commercially available, both positive controls produced expected results. Therefore, although this commercial ELISA is not referenced in the literature, one can assume the results achieved are representative and not a false negative consequence of processing.

Results from this ELISA suggest no increase in HIF-1a expression in liver tissue from patients with Wilson's disease. This is unfortunate as it contradicts the results in copper excess cell culture. While this may be a true reflection on HIF-1a expression in the Wilson's liver there are several reasons to explain these results. As discussed with the IHC there may be processing issues. HIF-1a nuclear protein stabilisation is very sensitive with complete abolition of response seen in minutes. The specimens were old and collection of the samples from explanted livers may have been delayed. Hepatectomy can take several hours, the samples being retrieved once this liver had been removed. However, this is an unlikely explanation as if anything the retrieval process is only likely to induce ischaemia of the tissue and if anything produce false positive results by induction of HIF-1a in response to tissue ischaemia.

As discussed in Chapter 6 HIF-1a is an acute phase response protein. The role of HIF-1 is to mediate an adaptive response to acute hypoxia. This response by HIF-1 is initiated instantaneously and the effect mediated over hours and days. If the response is effective the stimulus will be nullified and HIF-1 expression will return to normal. Thus chronic stimulation of HIF-1 results in adaptation and a normal level of HIF-1a expression (Chavez, Agani et al. 2000; Tissot van Patot, Bendrick-Peart et al. 2004).

Chronic copper toxicity results in destruction of normal liver architecture and subsequent cirrhosis. It may be that with chronic exposure HIF-1a is not over expressed as it was in the cell culture experiments, which only looked at effect up to 24 hours of copper exposure.

Despite two chapters of negative results representing many months of work it was good to see increased HIF-1a expression on the IHC in IRI. A result that helps to verify that the IHC protocol used in Chapters 6 & 7 did work. However, staining was sporadic and varied between batches assessed. To overcome this slides were independently assessed and scored by a histopathologist (0 (negative), 1+ (negative), 2+ (borderline), or 3+ (positive)). Data were assessed in biopsies taken at day 7 following liver transplantations, and normal liver was compared with liver showing rejection or IRI. Results showed that HIF-1a might be increased in rejection and IRI (T. Richards. Hepatology 2002). However, when looking at the IRI biopsies alone compared to normal this did not reach significance and repeating the IHC resulted in variability between batches.

The reason for increased HIF-1 in IRI may be related to hypoxia. However, HIF-1 has been shown to be upregulated by several cytokines, IL2 and TNF also the P13 – AKT3 cell signalling pathway involved in inflammation. It is therefore more likely that any increased HIF-1 seen in IRI here was related to the inflammatory process of IRI.

Summary of Conclusions

Following restoration of blood supply to ischaemic tissue IRI can result in increased postoperative complications and reduced operative success. HPC is an effective mechanism to reduce the effects of IRI. Research has predominantly occurred in animal models of IRI, but more recently human trials in liver and aortic surgery have shown beneficial effect of HPC in preventing both local and systemic complications.

The molecular response to hypoxia is mediated by the transcription factor HIF-1 that stimulates an array of genes to enable cellular adaptation and survival. HIF-1 is increased following HPC and may be responsible for preparing the cell to prevent and reduce the effects of IRI.

HIF-1 expression is directly related to oxygen levels and HIF-1 appears to be controlled by ROS, although the exact mechanism remains a matter of much debate. Under normal circumstances ROS are tightly regulated and controlled by specific enzymes and buffers. A key component of the buffers is a transition metal ion core, in particular copper, that is able to exist in two oxidative states. Further copper is also highly reactive in isolation and readily able to facilitate ROS production by Fenton chemistry. Both ROS and Fenton chemistry are involved in the pathway of HIF-1 activation and expression.

The research presented here aimed to assess a mechanism to artificially affect HIF-1 expression by altering copper levels in cell culture and to assess the affect of altered copper levels on HIF-1 expression in vivo.

In Chapter 3 increased copper excess produced a small rise in intracellular copper concentration, however this was not in proportion to the concentration of exogenous copper used and was not dose dependent. In fact at higher concentrations of copper excess there was less effect on intracellular copper concentration. Copper depletion produced a significant reduction in intracellular copper concentration that was in proportion to previous reports. The results for increased exogenous copper may reflect the tight regulation of intracellular copper in the cell. All intracellular copper is protein bound with no free intracellular copper. The cell does not tolerate copper excess; any increase in copper is met by active transport and excretion of copper from the cellCopper depletion would result in depletion of copper metallo-enzymes, which are crucial in ROS processing.

Increased copper had a toxic effect on cellular mitochondrial function at high doses, as assessed by MTT assay. The toxic effect was less when assessed by trypan blue exclusion. As it appears that increased exogenous copper did not result in increased intracellular copper concentration. This may reflect the generation of extra-cellular ROS by copper mediated Fenton chemistry. ROS that then cross the cell membrane and reduce mitochondrial function. As trypan blue exclusion reflects cell membrane integrity, one of the final markers of cell death is a compromised membrane, these results may reflect the absolute level of copper toxicity tolerated by the cell.

In Chapter 4 increased copper produced a significant and dose dependent increase in HIF-1a nuclear protein stabilisation, the rate determining step in HIF-1 activation. These results with those from Chapter 3 suggest that extracellular copper excess must affect a mechanism of HIF-1 induction either mediated at the cell membrane or by a secondary factor that crosses the membrane, as the copper excess does not result in and increase in copper inside the cell.

Copper depletion significantly reduced HIF-1 induction in response to hypoxia. The level of reduction corresponded to the level of intracellular copper reduction as seen in Chapter 3. This suggests that intracellular copper concentration is directly involved in the pathway of HIF-1 induction. Copper depletion had no effect on HIF-1 induction by cobalt or DSF. Cobalt affects HIF-1 by a hypoxia independent pathway possibly not involving ROS. Iron is an essential co-factor with oxalogluterate in prolene hydroxylation of HIF-1 thereby preventing pVHL binding. Both Cobalt and DSF mechanisms of HIF-1 induction may therefore not be involved in the ROS pathway of HIF-1 activation and explain the lack of effect by copper depletion. The results seen with the nitric oxide donor GSNO are interesting. The role of NO in regulation of HIF-1 is confounding. Reports exist both for NO inducing HIF-1 and NO inhibiting HIF-1. Significant debate exists over the effect of NO either by altering redox balance or directly by peroxynitrite production. Either way consensus is achieved in that NO does directly affect ROS with the cell. In experiments performed here GSNO produced an increase in HIF-1 induction, a response reduced by copper depletion. These results reinforce the role for copper in the ROS pathway of HIF-1 induction.

Using a reporter cell line the results of Chapter 5 were confirmed. Increased copper significantly increased HIF-1 activation and consequent transcription. Copper depletion reduced the HIF-1 response to hypoxia, but not DSF.

In vivo assessment of HIF-1 in animal and human models of copper imbalance was unfortunately poor. The experiments performed in Chapter 6 aimed to assess whether copper excess or depletion had an effect on HIF-1 in rodent models. The LEC and LEA rodents unfortunately yielded no significant results. Chronic copper depleted mice were extremely unwell and anaemic, confounding factors that may well have induced HIF-1. Whether there was an effect remains difficult to conclude. This may reflect difficulty with HIF-1 IHC techniques and antibodies at the time. However an interesting finding in the copper deficient models was a zonal expression of HIF-1a, a finding that may reflect an acute response of HIF-1a to stimulus.

Similar findings were seen in experiments on human tissue. Much of this work was performed in parallel to that in Chapter 6. Samples were readily available from explanted alcoholic liver disease tissue, samples that should be exposed to significant hypoxic insult both as a consequence of the disease process and also from the operation. These samples were used to test variations in the protocol to try and enhance detection of HIF-1 and reduce background endogenous peroxidase activity, unfortunately to no avail. Using a different technique of ELISA for analysis of several biopsies from patients with Wilsons disease, again revealed negative results. Unfortunately, it was difficult to make any conclusions from these results; whether they reflect the consequence of endstage liver disease, age of the samples or a reflection of chronic copper toxicity.

The main conclusion that can be drawn from this work is that copper appears to have a functional role in the hypoxic induction of HIF-1. This probably reflects involvement of copper enzymes in the ROS pathway of oxygen signal transduction.

Future Experiments

Key experiments should be repeated with copper depletion followed by treatment with copper supplementation to establish whether the effect of copper depletion is reversed by normalising intracellular copper concentration.

Throughout this research the assumption has been made that intracellular copper depletion directly produced a reduction in intracellular copper metalloenzymes or their function. Further analysis is necessary to assess this quantitatively by measuring intracellular concentration of CuSOD, and copper based cytochromes. Also qualitative analysis of redox enzymes should be performed including; CuSOD, glutathione and metallothione. Also analysis of intracellular ROS and ideally to quantitately assess the Fenton reaction, a key step in the hypothesis for this work.

HIF-1 is the key transcription factor for over 60 genes. The pathways involved in hypoxic response appear to involve a paradox. HIF-1 mediates the Pasteur effect enabling cells to survive in a low oxygen environment (a housekeeping effect) whereas it also mediates angiogenisis and upregulation of cellular function to improve oxygenation (an acute response). It would be interesting to establish a quantitative gene profile (mRNA) to establish which pathways of response are activated by HIF-1 under different conditions.

Clinically chronic exposure to either copper excess or copper depletion is of little functional relevance. The experiments performed here merely attempted to assess whether copper excess or depletion had an effect on HIF-1. Whether there was an effect

remains difficult to conclude. Chronic copper depleted mice were extremely unwell and anaemic, confounding factors that may well have induced HIF-1. LEC and LEA rodents yielded no significant results. It would have been interesting to repeat these experiments in LEA & LEC rodents and assess the HIF-1 expression in response to hypoxia. Seperately the effect of acute copper toxicity should be looked at in the male Wistar rat.

To specifically address whether copper excess or copper depletion can aid in preventing IRI in vivo experimentation could be performed. To look at organ function animals could be pretreated prior to visceral clamping. Outcome could then be assessed both clinically, by organ function and at a molecular level by analysis of sequential biopsies.

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Appendix 1 - Materials

Reagents

AMPS	10% Ammoniumperosidisulphate	(100mg/ml)
DEPC water	Diethyl Pyrocarbonate	(0.1% in ddH_20)
Denhardts (50x)	ddH_20	500ml
	Polyvinylpyrrolidone	5g
	Ficoll (Type 400, Pharmacia)	5g
	Bovine serum albumin	5g
DTT (1M)	Dithiothreitol, 3.09g in 20ml sodium acetate (pH 5.2)	
EDTA (0.5M)	Ethylenediaminetetra-acetate	(pH 8.0)
	Disodium EDTA	186.1g/L
HEPES (2x)	ddH_20	100ml (pH 7.05)
	HEPES	1.0g
	NaCL	1.6g
	KCL	0.074g
	Na_2HPO_4	0.027g
	Dextrose	0.2g
PBS	Phosphate buffered saline	1L (pH 7.4)
	NaCL	8.0g
	KCL	0.2g
	Na_2HPO_4	1.44g
	KH_2PO_4	0.24g
MOPS (10x)	(3-(N-Morphilino)propanesulfonic acid) (pH 7.0)	
	MOPS	41.86g

	Sodium acetate (50mM)	4.1g
	EDTA (10mM)	50ml
	Autoclave, the solution turns yellow	
NaCL (5M)	Sodium Chloride	292.2g/L
SDS (10%)	Sodium dodecyl sulphate	(pH 7.2)
	Heated to 68°C to assist dissolution	
SSC (20x)	ddH_20	1000ml (pH 7.0)
	Sodium Chloride	175.3g
	Sodium Citrate	88.2g
SSPE (20x)	ddH_20	1000ml (pH 7.4)
	NaCL	175.3g
	Na_2HPO_4	27.6g
	EDTA	7.4g
Tris (1M)	Tris base	121.1g/L
TBS	Tris buffered saline	(pH 7.4)
	NaCL	8.0g
	KCL	0.2g
	Tris base	3g

Chemicals

All Chemicals supplied by Sigma-Aldrich Co. Irvine UK.

Name	MSDS name	Cas Number
Bathocuproine	2,9-Diphenyl-1,10-phenanthrolinedisulfonic acid	52698-84-7
Cobalt	Cobalt Chloride hexahydrate 99%	7791-13-1
Copper	Cupric Chloride	13468-85-4
Desferoxamine	Deferoxamine methanesulfonate salt, 95% (TLC)	138-14-7
GSNO	S- Nitrosoglutathione	57564-91-7
Tetraamine	N,N-Bis(2-aminoethyl)-1,3-propanedamine, 97%	4741-99-5
Trien	Trithylenetetramine tetrahydrochloride, 97%	4961-40-4

Western blot

Cell lysis buffer (CLB)

Reagent	Stock	Concentration used	Amount per 100ml
Tris (pH8.0)	1M	10mM	1ml
EDTA (pH8.0)	0.5M	1mM	200µl
NaCl	5M	150mM	3ml
NP-40	10%	0.01%	100µl

Protease inhibitor cocktail for CLB

Protease inhibitor	Stock	Concentration used	Amount per 10ml CLB
Leupeptins	1mg/ml	1µl	5µl
Pepstatin A	1mg/ml	1µl	5µl
PMSF	100Mm	1mM	50µl
Na_3Vo_4	100Mm	1mM	50µl
Aprotinin	1mg/ml	1µl	5µl

Nuclear extraction buffer (NEB)

Reagent	Stock	Concentration used	Amount per 10ml
HEPES (pH7.9)	100mM	20mM	2ml
EDTA (pH8.0)	0.5M	1mM	20µl
NaCl	0.5M	400mM	800µl

Protease inhibitor cocktail for NEB

Protease inhibitor	Stock	Concentration used	Amount per 1ml NEB
PMSF	100mM	1mM	10µl
DTT	100Mm	1mM	10µl

SDS-polyacrylamide gel

System used: Invitrogen Ltd. Paisley, UK.
Mini-Protean II Electrophoresis Cell
(BioRad, Hemel Hempstead, UK)

7.5% SDS-polyacrylamide gel

Component	Resolving Gel	Stacking Gel
40% Acrlyamide:Bisacrlyamide	1.925	0.5
Resolving buffer	2.5	-
Stacking buffer	-	4.5
ddH_20	5.475	-
AMPS	0.1	.05
TOTAL volume (ml)	10	5.05

Resolving Buffer

ddH_20	500ml (pH 9.0)
Tris-HCL	1.5M
TEMED	0.4%
SDS	0.4%

Stacking Buffer

ddH_20	500ml (pH 6.8)
Tris-HCL	0.14M
TEMED	0.11%
SDS	0.11%

Running Buffer

ddH_20	1000ml
Glycine	14.4g
Tris base	3.0g
SDS	1.0g

Transfer buffer stock

ddH_20	1000ml
Glycine	14.5g
Tris base	29g
SDS	1.85g

Transfer Buffer

Stock	200ml
ddH_20	600ml
Methanol	200ml

Enhanced chemiluminescent (ECL) assay

10ml	100mM TRIS-HCL (pH 8.5)
3µl	30% H2O2
25µl	90mM Courmic Acid in DMSO
50µl	250mM Luminol in DMSO

Northern blot

RNA Formaldehyde gel

Component	Amount
Agarose	2.6g
MOPS (10x)	20ml
DEPC-treated water	145.6ml
Formaldehyde	34.4ml

- Apparatus soaked in 0.3M HCL for 5 hours before use.
- Components boiled in microwave to dissolve the gel then allowed to cool to 60°C before the formaldehyde is added.

Loading buffer

Formamide	250µL
Formaldehyde	75µL
10X MOPS	50µL
Tracking dye	50µL
EtBr (10mg/ml)	5µL

Tracking dye

ddH_20	10ml
*BPB	0.5mg
*Xylene cyanol	0.5mg
Glycerol	50%
EDTA	1mM
	*Sigma 3269

Hybridization buffer (2x)

SSPE (20x)	50ml

Denhardts (50x)	20ml
SDS (10%)	10ml
0.1% DEPC	20ml
Store at -20^0C	

Pre-hybridization mix

5ml 2x hybridization buffer

5ml formamide

100µl salmon sperm*

Hybridization mix

2.5ml hybridization buffer

2.5ml dextran sulphate in formamide

50µl salmon sperm*

50µl radio-labeled probe

*denatured at 95^0C for 5 min and cooled on ice for 1 min.

HIF-1a ELISA tissue extraction

Buffer C1

Reagent	Stock	Concentration used	Amount per 100ml
HEPES (pH 7.9)	500mM	10mM	2ml
KCL	1M	10mM	1ml
EDTA (pH 8.0)	1M	0.1mM	20µl
EGTA	500mM	0.1mM	20µl

Protease inhibitor cocktail

Protease inhibitor	Stock	Concentration used	Ratio to C1
DTT	100Mm	1mM	1:100
PMSF	100Mm	0.5mM	1:200
Leupeptins	1M	1mM	1:1000
Na_3Vo_4	100Mm	1mM	1:100
Pepstatin A	1µg/µl	1µg/ml	1:1000

Buffer C2

Reagent	Stock	Concentration used	Amount per 10ml
HEPES (pH 7.9)	500mM	20mM	400µl
NaCl	5M	400mM	800µl
EDTA (pH 8.0)	500mM	1mM	20µl
EGTA	500mM	1mM	20µl
Glycerol	100%	10%	1ml

Protease inhibitor cocktail

Protease inhibitor	Stock	Concentration used
DTT	100Mm	1mM
PMSF	100Mm	1mM
Leupeptins	1M	1mM
Na_3Vo_4	100Mm	1mM

Appendix 2 - Collaboration for animal experiments

Experiments on hypoxia and copper deficiency in male Wistar rats were undertaken in collaboration with: -

Dr David Tosh.

Centre for Regenerative Medicine, Department of Biology & Biochemistry,

University of Bath, Claverton Down, Bath, UK.

(Home Office licence PPL 30/1324; chief investigator Dr D. Tosh).

D.tosh@bath.ac.uk

http://www.bath.ac.uk/bio-sci/tosh.htm

Experiments on copper toxicity in male LEC and LEA rats were undertaken in collaboration with: -

Dr Sanjeev Gupta MD.

Department of Medicine, Albert Einstein College of Medicine,

Bronx, New York 10461, USA.

Animals were reared in the Animal Facilities for Experimental Medicine, Albert Einstein college of Medicine

sanjvgupta@pol.net.

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