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Abstract

The anoxemia theory proposes that an imbalance between the demand for, and supply of, oxygen in the arterial wall is a key factor in the development of atherosclerosis. There is now substantial evidence that there are regions within the atherosclerotic plaque in which profound hypoxia exists; this may fundamentally change the function, metabolism and responses of many of the cell types found within the developing plaque and whether the plaque will evolve into a stable or unstable phenotype. Hypoxia is characterized in molecular terms by the stabilization of hypoxia inducible factor (HIF)-1α; a subunit of the hetero-dimeric nuclear transcriptional factor HIF-1; a master regulator of oxygen homeostasis. HIF-1 expression is localized to perivascular tissues, inflammatory macrophages and smooth muscle cells adjacent to the necrotic core of atherosclerotic lesions, and regulates several genes that are important to vascular function including vascular endothelial growth factor (VEGF), nitric oxide synthase (NOS), endothelin-1 and erythropoietin (EPO). This review summarizes the effects of hypoxia on the functions of cells involved in atherogenesis, and the evidence for its potential importance from experimental models and clinical studies.

Introduction

Atherosclerosis is a chronic inflammatory process, affecting medium and large arteries, and involving lipid deposition, oxidative stress, endothelial damage, and smooth muscle cell proliferation and migration. It is associated with several established risk factors, including: hypertension, hyperglycaemia, aging and dyslipidaemia.[1] There have been a number of hypotheses of atherogenesis, including the anoxemia theory that proposes that an imbalance between the demand for and supply of oxygen in the arterial wall is a key factor in the development of atherosclerosis.[2, 3] The micro-environment within the atherosclerotic plaque is thought to be an important determinant of whether a plaque progresses, and the likelihood of clinical complications. It has previously been difficult to investigate cellular metabolism within the artery wall with sufficiently high resolution, and consequently it has not been possible to identify regions in which extreme hypoxia prevails. However, recent reports do provide substantial evidence that there are regions within the plaque in which hypoxia can be identified.[4] This state of hypoxia has the potential to fundamentally change the function, metabolism and responses of many of the cell types found within the developing atherosclerotic plaque, and this may in turn determine whether the plaque evolves into a stable or unstable phenotype. It is likely that this is mediated through effects on angiogenesis, extracellular matrix elaboration and lipoprotein metabolism. The hypoxic milieu in the atherosclerotic plaque may therefore also have implications for the putative therapeutic interventions for atherosclerosis. However, most *in vitro* studies have been conducted under normoxic conditions. The effects observed under these conditions may not accurately reflect those extant within the plaque.

The Development of a Hypoxic environment within the Atherosclerotic Plaque

Hypoxia may be defined as a physiological or pathological state in which the body, or a region of the body, is deprived of adequate oxygen supply at the tissue level to an extent that it is inadequate to meet cellular, or tissue demands. Hypoxia may be due to a reduced oxygen supply (due to insufficient blood vessel network or defects in blood vessels) or to an increased consumption of oxygen relative to the supply (this may result from a rapid increase in cell proliferation). It is usually characterized in molecular terms by the stabilization of hypoxia inducible factor (HIF)-1α; a subunit of the heterodimeric

nuclear transcriptional factor HIF-1. HIF-1, consisting of HIF-1α and HIF-1β subunits, has been described as a master regulator of oxygen homeostasis. Whilst levels of HIF-1α have been shown to be regulated by non-hypoxic stimuli such as lipopolysaccharides (LPS), thrombin, and angiotensin II (Ang II),[5] it is generally considered to be a biological marker of hypoxia and drives the expression of over 100 genes including those involved in angiogenesis and oxygen transport.[6] (see Figure 1 [7]). There are, however, some circumstances under which HIF-1α expression can be prevented.[8] Hypoxia also stabilises HIF-2α which is closely related to HIF-1α with respect to its involvement in the transcriptional activation of multiple target genes in hypoxia.[9]

In studies using human artery specimens and tissues from experimental animal models of atherosclerosis, HIF-1α has been localized to perivascular tissues, inflammatory macrophages and smooth muscle cells adjacent to the necrotic core of atherosclerotic lesions. In addition to being a marker of hypoxia, HIF-1α may directly enhance atherogenesis through several mechanisms, including: smooth muscle cell proliferation and migration, new vessel formation (angiogenesis), and altered lipid metabolism. Alteration of the HIF-1α pathway has been shown to contribute to vascular graft failure through the formation of intimal hyperplasia in which smooth muscle cells from the tunica media (the main muscle layer of the graft vessel) migrate to, and proliferate within what is termed the neo-intima.[10] HIF-1α has also been implicated in the pathogenesis of atherosclerosis, in-stent restenosis following coronary re-vascularisation, stroke, peripheral artery disease, aortic aneurysm formation and pulmonary artery hypertension [11], and also appears to be involved in the calcification of blood vessels, which often accompanies atherosclerosis. For example, Li *et al* have shown that in diabetic patients with a high coronary artery calcium (CAC) score, serum HIF-1α levels were significantly raised, and were an independent risk factor for the presence of CAC. Serum HIF-1α levels were positively correlated with other biomarkers of diabetic control and inflammation, such as C-reactive protein (CRP), Interleulin-6 (IL-6), HbA1c, fasting blood glucose (FBG), and CAC score. HIF-1α levels were therefore considered to be a significant independent risk factor, predicting the presence of CAC.[12] Whilst HIF-1 is an intracellular transcription factor, it is possible that it is released into the circulation from damaged cells, as has been reported for other transcriptional factors that include NF-κB and p53.[12-14] In healthy medium

sized arteries, the tunica intima (the innermost layer of the artery wall), is a delicate layer consisting of the endothelium, extracellular matrix, and a basement membrane comprising the internal elastic lamina (Figure 2a). During atherogenesis, the intima may thicken by the accumulation of cells and matrix, and the diffusion of oxygen can then become impaired (Figure 2b). Vasa vasorum form a network of small blood vessels lying within the tunica adventitia (the outermost layer of the artery wall). These vessels provide oxygen and nutrients to the wall of the artery. Vasa vasorum are vulnerable to hypoxia especially at the site of arterial branching as they are end arteries and the blood flow is reduced in this region. It has been hypothesized that hypoxia within the vasa vasorum is due to reduced blood flow and consequent endothelial dysfunction, local inflammation and permeation of large particles such as microbes, LDL-lipoprotein and fatty acids which are transformed by macrophages into foam cells [15, 16], which may be an initiating factor in atherosclerosis. The majority of inflammatory cells contributing to early atherosclerosis, probably enter the artery wall from the lumen, as shown by the elegant studies of Ross and colleagues.[17, 18] However, the vasa vasorum and associated micro-vessels may provide an alternate route by which leucocytes can enter the vascular wall.[19] As atherosclerosis progresses, angiogenic factors within the micro-environment of the plaque may stimulate new vessel formation. The combination of this delicate network of new vessels in juxtaposition to regions rich in inflammatory cells, that elaborate proteolytic enzymes, may contribute to intra-plaque haemorrhage and subsequent plaque rupture.[20] The involvement of vasa vasorum and intimal hyperplasia in the pathophysiology of atherosclerosis is supported by several experimental animal studies.[21, 22]

Evidence for Hypoxia within Atherosclerotic Plaque

The partial pressure of oxygen (pO_2) varies within the artery wall, even in health. The zones of hypoxia within atherosclerotic lesions (Figure 2b), may be formed because of impaired oxygen diffusion capacity as the atherosclerotic lesions enlarge, but may also be attributable to the high oxygen consumption of macrophage-derived foam cells.[3]

Hypoxia has been consistently found in atherosclerotic plaques *in vivo* in humans and animal models including knock-out mouse models (apolipoprotein E; ApoE -/- and the low density lipoprotein

receptor; LDLr -/- mice).[23] This has been demonstrated *in vivo* using various markers, including the chemical 7-(4'-(2-nitroimidazol-1-yl)-butyl) theophylline, that can be visualized using immunofluorescence.[24] Other non-invasive imaging techniques have also been applied, that directly target plaque hypoxia, and these techniques are now being further validated in human studies. The metabolic marker F-fluorodeoxyglucose (FDG), has been used to detect human atherosclerosis *in vivo* [18], and may also serve as an indirect marker of plaque hypoxia as the enhanced glucose uptake in anaerobic metabolism results in an increased uptake of the labelled FDG.[25] Imaging plaque hypoxia could provide a means of assessing putative culprit lesions that are at risk of rupture, and are consequentially liable to adverse outcomes. Advanced aortic atherosclerosis was induced in cholesterol-fed rabbits by balloon catheter injury, and F-18-fluoromisonidazole positron emission tomographic (PET) used for the *in vivo* assessment of hypoxia. This was then related to hypoxia assessed by *ex-vivo* tissue staining using pimonidazole, and immuno-staining for macrophages (RAM-11), new vessels (CD31), and hypoxia-inducible factor-1 α. Hypoxia was found to be predominantly confined to the macrophage-rich regions within the atheromatous core, whereas the macrophages close to the lumen were hypoxia negative. Intra-plaque new vessels were found predominantly in the macrophage-rich hypoxic regions (pimonidazole(+)/hypoxia inducible factor-1α(+)/RAM-11(+)).[4] In human studies, this imaging approach has been coupled with quantitative polymerase-chain reaction (qPCR) and immune-staining of plaques tissues recovered by carotid endarterectomy to determine the gene expression of HIF-1α and cluster of differentiation 68 (CD68, a marker of inflammation). HIF-1α and CD68 expression were both found to be significantly correlated with F-FDG-uptake [18], indicating an association between the presence of hypoxia, inflammation and increased glucose metabolism *in vivo.*[26]

Consequences of Hypoxia

HIF is normally only found in hypoxic cells; in normoxia, it is degraded *via* the proteasome pathway. Prolyl 4-hydroxylases (PHDs), require oxygen for their catalytic activity, and function as cellular oxygen sensors, regulating the stability of HIF. In normoxia, 4-hydroxyproline residues are formed at specific sites on the HIF-1α subunit, catalyzed by PHDs, and this leads to its ubiquitination by the von Hippel Lindau E3 ubiquitin ligase and immediate destruction in proteasomes. This prevents the

formation of a functional HIF dimer (see Figure 1 [27]). Prolyl 4-hydroxylation is inhibited in hypoxia, facilitating the formation of the HIF dimer and the activation of its target genes, including those for erythropoietin (EPO) and vascular endothelial growth factor (VEGF). It has been proposed that the induction of a pseudo-hypoxia response by inhibiting HIF prolyl 4-hydroxylases may provide a novel therapeutic target in the treatment of hypoxia-associated diseases.[28] Small-molecules, such as Roxadustat (FG-4592)[29] and ZYAN1[30], have been developed to inhibit prolyl hydroxylase domain-containing (PHD) enzymes, and cause HIF activation.[31] These agents have been specifically applied to the treatment of renal anaemia.[32] They can be administered orally are associated with an improved iron profile and an endogenous erythropoietin production near the physiological range. They are also likely to be cheaper to manufacture. The clinical trials currently underway will address whether PHD enzyme inhibitors will improve clinical end points, including cardiovascular events.[33] PHD inhibitors have been reported to reduce blood pressure[32] and plasma cholesterol concentrations.[29] Hence there is good reason to believe that some PHD inhibitors will reduce cardiovascular endpoints in patients with renal disease. Whether they will benefit a broader category of patients with high risk of cardiovascular disease is difficult to predict.

The HIF-α subunits are also involved in other key pathways for the adaptation to hypoxia, including VEGF [34] and nitric oxide synthase (NOS) (Heikal *et al* 2016 in press).

The hypoxia/ reoxygenation cycle leads to the formation of reactive oxygen species (ROS) that may subsequently regulate HIF-1 but in a rather complex manner. It has been suggested that ROS promote angiogenesis, either directly *via* stimulation of HIF-1 genes that are involved in stimulating angiogenesis, such as NOS and NADPH oxidase or *via* the generation of active oxidation products, including lipid peroxides. ROS are associated with the development of several chronic diseases that include atherosclerosis, type 2 diabetes mellitus, and cancer (reviewed in Gorlach *et al* 2015 [35]). HIF-1α plays a key role in the progression of atherosclerosis by initiating and promoting the formation of foam cells, endothelial cell dysfunction, apoptosis, increasing inflammation and angiogenesis.[36] Although ROS have damaging effects on tissues, causing cell death, at high concentrations, lesser degrees of oxidative stress may play a positive role during angiogenesis, or other pathophysiological

processes. Angiogenesis induced by oxidative stress involves vascular endothelial growth factor (VEGF) signalling, although VEGF-independent pathways have also been identified.[37]

It has been proposed that the state of hypoxia, present in the atherosclerotic plaques of mice deficient in apolipoprotein E (ApoE-/- mice), may promote lipid synthesis, and reduce cholesterol efflux through the ABCA1 pathway; processes that are known to be mediated by HIF-1α.[23] Hypoxia has also been reported to increase the formation of lipid droplets in macrophages to promote the secretion of inflammatory mediators, and atherosclerotic lesion progression by exacerbating ATP depletion and lactate accumulation in this model of atherosclerosis.[38]

Systemic hypoxia that is, for example associated with obstructive sleep apnoea (OSA) also promotes atherosclerosis. The processes by which it may do this include effects on lipid metabolism and efflux, inflammation, altered macrophage polarization and glucose metabolism.[39] The effect of hypoxia on other mediators will be discussed further below.

Models used to assess the effects of mediators on HIF-1 expression

1. *In vitro* models

Endothelial and Endothelial Progenitor cells

Endothelial dysfunction is one of the first manifestations of atherosclerosis.[40] It is associated with a deficiency of biologically active nitric oxide (NO).[41] After chronic inhibition of endothelial nitric oxide synthase (eNOS), either pharmacologically using N^G-Nitro-L-arginine methyl ester (L-NAME) or using RNA interference, HUVECs were found to have a higher migratory capability, which was accompanied by an increased expression of VEGF and the VEGF receptor-2 (kinase insert domain receptor, KDR). Inhibition of eNOS, induced a state of pseudohypoxia (a state under which cells activate a hypoxic response in the presence of oxygen), evidenced by stabilization of HIF-1α. Furthermore, reduced NO expression was associated with significant mitochondrial dysfunction with a consequential decrease in energy production and oxygen consumption. The basal release of NO may act as a negative regulator of HIF-1α levels, and this may have important consequences for endothelial cell physiology.[42] The reported effects of NO on HIF-1 expression have been inconsistent.[43, 44] Nitrate and nitrite can be

metabolized *in vivo* to form NO and other bioactive nitrogen oxides, providing an important alternative source of NO during hypoxia when the oxygen-dependent L-arginine-NO pathway may be altered (see Figure 3).[45]

Nuclear factor-kappa B (NF-kappa B); is a redox-sensitive transcriptional factor. NF-kappa B and HIF-1 are both implicated in the expression of pro-inflammatory cytokines during intermittent hypoxia/ reperfusion (IHR). IHR is a feature of obstructive sleep apnoea (OSA) and an independent risk factor for the development of coronary and cerebral vascular diseases. *In vitro s*tudies using EA.hy926 cells (a human vascular endothelial cell line), showed that IHR induced the activation of NF-kappa B and HIF-1, enhanced the mRNA expression of pro-inflammatory cytokines and increased the activation of p38 MAPK which is critical for the production of NF-kappa B. Propofol (2,6‑di-isopropylphenol), is a potent intravenous anaesthetic agent that attenuates the IHR-induced activation of NF-kappa B dose-dependently. This effect was accompanied by decreased levels of pro-inflammatory cytokines without changing the activation of HIF-1. This effect is likely to be mediated *via* the inhibition of the p38 MAPK signalling pathway. Hence Propofol may have the potential to prevent atherosclerosis in patients with OSA by inhibiting NF-kappa B mediated inflammation in the vascular endothelium.[46]

Endothelin-1 (ET-1) is a potent vasoconstrictor that is predominantly expressed in vascular endothelial cells, as well as by other cells and tissues. Increased plasma levels of ET-1 are associated with atherosclerosis. Hypoxia is one of the most potent inducers of ET-1 gene expression in endothelial cells and may be the primary cause of the increased production of ET-1 during myocardial ischemia. Hypoxia induces the synthesis and secretion of ET-1 in isolated endothelial cells by a mechanism that is antagonized by NO and carbon monoxide and mimicked by transition metals. The induction of ET-1 by hypoxia *in vitro* has been reported to occur exclusively in early passage endothelial cells where it requires endothelial cell specific factors, new protein synthesis (synthesis inhibited by a protein synthesis inhibitor; cycloheximide) and may involve a non-heme oxygen binding enzymes.[47]

Intercellular adhesion molecule-1 (ICAM-1) is a cell surface glycoprotein typically expressed on endothelial cells, but found as a soluble form (sICAM-1) in plasma. It is thought to be a key molecule

involved for the attachment of circulating monocytes to the endothelium and their subsequent transmigration into the intima. Its levels in plasma are related to the severity of atherosclerosis and can predict future cardiovascular events. Increased expression of endothelial ICAM-1 is also related to the chronic effects of oxidative stress. Ziegelstein *et al.* found that when human aortic endothelial cells (HAECs) were exposed to hypoxia/ re-oxygenation (H/R), hypoxia alone was insufficient to increase either ROS production or ICAM-1 mRNA levels, but a 2.5-fold increase in ICAM-1 mRNA was noted after 30 min of re-oxygenation. This was not observed in a Ca^{2+} free buffer or in cells treated with a ROS inhibitor; diphenyleneiodonium. Thus, H/R upregulates ICAM-1 mRNA in HAEC *via* a Ca^{2+} and ROS-dependent mechanism. The characterization of the signalling pathways involved in H/R-induced adhesion molecule expression may result in a better understanding of the pathobiology of atherosclerosis and of the vascular biology of normal aging.[48, 49]

AMPK (AMP-activated protein kinase) is a key enzyme in the regulation of cellular energy and has cardiovascular protective effects.[50] It can detect changes in AMP levels, but may also be activated in endothelial cells by an AMP-independent mechanism, involving mitochondrial ROS, generated as a result of the interaction between NO and mitochondrial cytochrome c oxidase at low oxygen concentrations. In hypoxia, a reduction in ATP, and the breakdown of ADP to AMP could also activate AMPK.[51] AMPKα1 is responsible for the expression of genes involved in antioxidant defence, including: manganese superoxide dismutase (MnSOD), catalase, gamma-glutamylcysteine synthase and thioredoxin. Silencing of AMPKα1 has been reported to reduced mitochondrial and eNOS content, reduced cell proliferation, increased accumulation of ROS and apoptosis. Thus in HUVECs, AMPKα1 regulates both their mitochondrial content and their antioxidant defences, and is therefore likely to be beneficial in those conditions mediated by oxidative stress, and where dysfunction of AMPK may contribute to the initiation and progression of atherosclerosis.[52, 53]

Vascular endothelial cells synthesize heparan sulphates. Heparan sulphates expressed on the surface of endothelial cells, contribute to their antithrombotic properties by facilitating thrombin inactivation by anti-thrombin III; this is mediated by the heparin-like sequences within the heparan sulphate chains to which both anti-thrombin and thrombin may bind. Macrophages are proposed to be a major source of

heparan sulphate in atherosclerotic lesion, and this may be modulated by oxygen tension.[54] Furthermore, Karlinsky *et al.* showed that when bovine aortic and pulmonary endothelial cells were cultured under hypoxic conditions (3% oxygen), cell layer-associated heparan sulphate was reduced substantially (>45%). The degradation of heparan sulphate was not affected by hypoxia, however, the percentage of the heparan sulphate that bound to antithrombin III was increased significantly (>33%). The total quantity of heparan sulphates synthesized by the cultured endothelial cells was also affected during exposure to 3% oxygen, but not to the same extent; the authors therefore concluded that hypoxia may differentially influence the chain length and anti-thrombin III-binding capacity of heparan sulphate species. This would be important in helping to maintain a non-thrombogenic surface under hypoxic conditions and the hypoxia-induced modification of heparin sulphate may therefore be of relevance to the development of atherosclerotic lesions.[55]

Endothelial progenitor cells EPCs, contribute to vascular repair. They are released into the circulation from the bone marrow, and can adhere to the endothelium at sites of injury and hypoxia.[56] They can also contribute to the formation of new vessels following their differentiating into endothelial cells (ECs). Apelin is an endogenous ligand for the G protein-coupled receptor APJ, and apelin/ APJ signalling appears to be involved in a wide range of physiological and pathological functions in the cardiovascular system. It is also involved in the regulation of EPC proliferation in hypoxic conditions.[57] HIF-1α promotes the expression of apelin and APJ, which then activate the downstream PI3K/ Akt signalling pathway, a key promoter of EPC proliferation. Apelin is therefore proposed to be a novel factor in the development of atherosclerosis.[57, 58]

EPO is a 30,400-dalton glycoprotein that regulates red blood cell production. It circulates in plasma and controls the oxygen carrying capacity of the blood.[59] EPO is produced primarily in the adult kidney and foetal liver. However it is now well established that several cell types express EPO or its receptor (EPOR),[60] including vascular smooth muscle [61] and endothelial cells.[62] In these cells EPO exerts a number of effects including protection against apoptosis, [63] mitogenic and chemotactic effects (reviewed by Ribatti *et al.* [64]). It also stimulates a significant increase in circulating endothelial progenitor cells. EPO plays an important role in regulating angiogenesis.[65] Whilst EPO enhances

endothelial repair in models of vascular injury, its reported effects on neo-intima formation in animal models has been inconsistent, with some investigators reporting enhanced neo-intima formation despite complete re-endothelialization,[66] and others reporting an inhibition of neo-intima formation that was NO-dependent.[67] In human studies EPO treatment was associated with an increase in neo-intima generation in patients treated with percutaneous coronary intervention.[68] Furthermore, EPO retards the progression of atherosclerosis. EPO and one of its non-erythropoeitic analogues (HBSP; 30 µg/Kg body weight) reduces the cholesterol ester content of aortic tissue in the Watanabe heritable hyperlipidemic (WHHL) rabbits and the intima-media thickness in renal patients undergoing haemodialysis. EPO (5 U/mL) has been shown to suppress foam cell formation in murine macrophages by promoting the efficacy of cholesterol efflux.[69-71] EPO also has protective effects against stress that are related to energy metabolism where it is involved in the regulation of metabolic cell activity, cellular respiration and mitochondrial function via important energy regulation factors such as AMPK.[72]

Tissue hypoxia is the main stimulus of EPO production. Oxygen-dependent regulation of EPO gene expression is controlled primarily by the transcription factor HIF-2α rather than HIF-1α.[73]The response of vascular endothelial cells to EPO is enhanced at a low oxygen concentrations (5% or 2%); this has in particular been shown for its effect on NO release. EPOR is inducible by EPO (5 U/mL) in primary human umbilical vein endothelial cells (HUVECs) and artery (HUAECs) and cells derived from a human bone marrow microvascular endothelial line (TrHBMEC).

Hypoxia can also induce the mobilization of EPCs *via* a NADPH oxidase (Nox2) dependent pathway. Nox2 is an important source of ROS. This response is abolished in Nox2 knockout mice Nox2(y/-). EPO is also known to induce EPC mobilization and this too appears to be Nox2 dependent. ROS are the product of the enzymatic reaction catalysed by Nox2, and EPO was found to increase ROS production, and the migration and proliferation of EPCs derived from WT mice, but no increase of ROS formation was observed in Nox2-deficient EPCs. Furthermore, re-endothelialisation of the injured mouse carotid artery was enhanced by hypoxia, or EPO, and this effect was not observed in Nox2(y/-) mice or after transplantation of Nox2(y/-) bone marrow cells. Over-expression of the EPO receptor can increase EPO-dependent signalling. Ligand-induced EPO receptor dimerization, allows binding of

JAK2. JAK2 then creates SH2 binding sites by phosphorylating tyrosine residues on the EPO receptor. Phosphorylation of the receptor activates Rac1 and PI3K which in turn leads to the assembly of Nox2-containing NADPH oxidase that subsequently synthesize superoxide anions. STAT5 also binds to the SH2 domains of the EPO-receptor, where it is phosphorylated, dimerizes, and eventually translocates to the nucleus. It then initiates the transcription of several target genes and leads to cell proliferation, migration, and mobilization. In the absence of ROS production by Nox2, STAT5 is dephosphorylated before STAT5- mediated signalling can occur. siRNA against the redox-sensitive phosphatase SHP-2 restored EPO-mediated STAT5 induction and inhibition of SHP-2 restored EPO-induced migration in Nox2-deficient cells. It therefore appears that Nox2-derived ROS inactivate SHP-2 and thereby facilitate EPO signalling in EPCs to promote hypoxia-induced mobilization and vascular repair by these cells.[74]

Endothelial cells release ATP in response to changes in blood flow (producing shear stress), or hypoxia, where it acts on ATP receptors (P2X and P2Y) on the endothelial cells to produce NO, PGI_2 and endothelium-derived hyperpolarizing factor (EDHF), which contribute to blood vessel dilatation. ATP is also released from sensory-motor nerves during anti-dromic reflex activity to produce relaxation of some blood vessels. The purine and pyrimidine nucleosides and nucleotides have long-term (trophic) actions in promoting migration and proliferation of both vascular smooth muscle and endothelial cells *via* adenosine receptor (P1) and ATP receptors (P2Y) during angiogenesis and vessel remodelling during restenosis after angioplasty.[75, 76]

Endothelial cells express receptors for very low density lipoprotein (VLDLr). In addition to their function as peripheral lipoprotein receptors, VLDLrs have other possible biological roles including in signal transduction, angiogenesis, and tumour growth. Some recent studies suggest that the VLDLr has a regulatory role in endothelial pathology. The activation of retinal vascular endothelial cells and promotion of angiogenesis are mediated through the VLDLr. Hypoxia can trigger endothelial endoplasmic reticulum (ER) stress and apoptosis, and induce the expression of VLDLr through the interaction of HIF-1α with the hypoxia responsive element (HRE) at the VLDLr promoter. VLDLr then mediates the ER stress response and apoptosis.[77]

High density lipoproteins (HDLs) are thought to be involved to reverse cholesterol transport (transporting cholesterol from peripheral tissue back to the liver), and have therefore been proposed to have a protective role in atherosclerosis.[78] HDLs also appear to promote hypoxia-induced angiogenesis.[79] In hypoxia, recombinant HDL (rHDL) augments the HIF-1/VEGF pathway *via* scavenger receptor class B type 1 (SR-BI), leading to the modulation of the post-translational regulators of HIF-1 (PI3K/ Siahs/PHDs); crucial mediators in angiogenesis. Despite strong epidemiological and pre-clinical evidence for the athero-protective effects of HDL, recent HDL-raising clinical trials have been unable to show cardiovascular benefits.[79] Augmentation of angiogenesis and the HIF-1/VEGF pathway in hypoxia may have important implications for the targeting of HDL-raising therapies, particularly for the prevention of ischemic injury after myocardial infarction.[80]

CD40 and its ligand (CD40L) have been implicated in the pathogenesis of atherosclerosis; the interaction between CD40 and CD40L is involved in the activation of antigen presenting cells, such as macrophages. This interaction modulates the generation of endothelial ROS. It has been found that hypoxia enhances the inflammatory effect of CD40L in both endothelial and monocytic cells (THP1) and induces ROS production, the synthesis of ICAM1 and activates stress response proteins (p38 MAP kinase and HSP27), indicating that CD40L mediates the induction of oxidative stress in these cells. The interaction between CD40–CD40L is augmented in hypoxia playing a potentially important role in vascular inflammation, cellular adhesion and angiogenesis; processes involved in atherosclerosis.[81]

The retinoic acid-related orphan receptor α (RORα) is involved in regulating inflammatory and immune responses and lipid metabolism. RORα mRNA has been detected in endothelial cells and smooth muscle cells. Its expression is significantly decreased in atherosclerotic plaques. Plasma and intracellular levels of cholesterol may be important in the regulation of the transcriptional activity for RORα. Hypoxic conditions induce the expression of RORα and when cells were treated with RORα, the transcriptional activity of HIF-1α was enhanced. RORα was reported to be physically associated with HIF-1α *via* its DNA binding domain, and this was required for the RORα-induced stabilization and transcriptional activation of HIF-1α. *In vitro*, ROR ligands enhance the formation of capillary tubes by human

umbilical vascular endothelial cells suggesting a potential involvement of ROR and its ligands in the major clinical endpoints associated with atherosclerosis.[82, 83]

Atherosclerosis is associated with decreased endothelial NO production. NO normally reduces cellular oxygen consumption by competitively inhibiting cytochrome c oxidase. Endothelial NO, can therefore modulate O_2 consumption in conductance vessels and the microcirculation, and appears to regulate O_2 distribution to the surrounding tissues. Removal of NO abolishes this regulatory function. Hence endothelial NO plays a major role in facilitating the distribution of O_2, which is crucial for the adaptation of tissues, including the vessel wall itself, to hypoxia.[84-86]

Vascular smooth muscle cells

Smooth muscle cell proliferation is increased in pathological conditions, including atherosclerosis, resulting in the thickening of the vessel walls.[87] The migration of vascular smooth muscle cells (VSMCs) from the tunica media to the intima and their proliferation under the influence of growth factors such as platelet-derived growth factor is a critical step in atherogenesis.[88] The proliferation and migration of vascular smooth muscle cells is also involved in new vessel formation (angiogenesis) and this is co-ordinated by a number of factors that include VEGF.[89]

VEGF is a hypoxia-inducible angiogenic factor. It also mediates the angiogenic effects of other growth factors that are not themselves direct endothelial cell mitogens. Exposure of VSMCs to a threshold hypoxic stimulus (2.5% O_2,) causes a modest increase in VEGF mRNA levels. These effects of hypoxia are synergistic with basic fibroblast growth factor (bFGF) which also plays a role in inducing angiogenesis. This synergistic effect on VEGF expression appears to be specific as hypoxia has not been found to enhance the bFGF-induced expression of the proto-oncogene c-myc (the gene responsible for cell proliferation). bFGF may therefore promote angiogenesis both by a direct effect on endothelial cells and indirectly by the up-regulation of VEGF in VSMCs. The synergy demonstrated between hypoxia and bFGF suggests that multiple diverse stimuli may interact *via* the up-regulation of VEGF expression in VSMCs to amplify the angiogenic response.[90] The increased expression of VEGF following exposure to hypoxia is also enhanced by platelet-derived growth factor-BB (PDGF-BB). The

synergy between hypoxia and PDGF-BB was again found to be selective for VEGF expression as hypoxia had no effect on the PDGF-induced upregulation of the proto-oncogene c-myc.[91]

Moderate hypoxia (5% O_2) has also been reported to enhance the proliferative responses of human pulmonary artery SMC (HPASMC) to mitogens including PDGF, bFGF, and epidermal growth factor (EGF). Moderate hypoxia also elicited increased cellular HIF-1α levels, and enhanced PDGF-, bFGF, and EGF-induced expression of HIF-1α. These effects were abrogated by the knocking-down of HIF-1α using specific small interfering RNAs but this failed to inhibit the co-mitogenic effect of hypoxia. Hence HIF-1α promotes proliferative responses of human VSMC to bFGF, PDGF, and EGF by mechanisms that may involve HIF-1 dependent expression of cyclin A, but HIF is apparently not crucial to the enhancement of bFGF, PDGF, and EGF-induced proliferation of VSMC that occurs during hypoxia.[92]

Hypoxia increases cytosolic calcium concentration, enhances prostaglandin (PG) release and synthesis of platelet-activating factor (PAF) in endothelial cells and increases neutrophil adherence. Hypoxia may also change the balance between endothelial cell-derived growth inhibitors and activators for SMCs, shifting it to a more pro-proliferative activity which could explain the proliferation of SMCs observed *in vivo* in some pathological conditions, such as atherosclerosis. Michiels *et al.* investigated the influence of hypoxic endothelial cell-conditioned media on SMC proliferation. HUVECs exposed to hypoxia, elaborate mitogenic factors that had a pro-proliferative effect on SMC that was not inhibited if the HUVECs were treated with the protein synthesis inhibitor, cycloheximide, but was blocked if prostaglandins (PGs) synthesis was inhibited during hypoxia. Therefore it appears that PGs and bFGF are responsible for this pro-proliferative activity and their effect is additive.[87]

Proteoglycan are important constituents of the arterial wall extracellular matrix, and participate in normal vascular homeostasis and function, as well as in the trapping and modification of serum-derived lipoproteins. The majority of proteoglycans in the artery wall are produced by vascular endothelial cells and SMCs. The proteoglycans secreted by smooth muscle cell cultures exposed to hypoxia/ re-oxygenation are increased substantially compared to cells exposed to continuous normoxia. The newly

synthesized proteoglycans synthesized by SMCs exposed to either condition did not differ in their charge densities or molecular weight but did differ in glycosaminoglycan (GAG) composition. In the cells exposed to hypoxia /reoxygenation there was a 60% increase in a proteoglycan sub-fraction that bound LDL with very high affinity. Hypoxia followed by re-oxygenation is perhaps a more important signal for smooth muscle cell changes, including induction of synthesis of a proteoglycan species with very high affinity binding for plasma LDL.[93]

Human coronary artery smooth muscle cells (CASMCs) cultured under hypoxic conditions (1% O2, 5% CO2) have been found to express increased levels of VEGF, VEGF receptor-1 (VEGFR-1) and HIF-1α. Hypoxia was associated with enhanced [H^3]-thymidine incorporation, which was completely inhibited by a neutralizing antibody against VEGF and attenuated by a neutralizing antibody against NADPH-cytochrome P-450 reductase (NPR), which contributes to the stabilization of HIF-1α. The effects of hypoxia were also attenuated in NPR-knock down cells, and enhanced in NPR-over-expressing cells. Hypoxia-induced proliferation of CASMCs is mediated by an increased expression of VEGF and VEGFR-1, acting via an autocrine mechanism. The expression of these two molecules is dependent on the stabilization of HIF-1α, which is regulated by NPR. It has therefore been suggested, that hypoxia as well as NPR are implicated in the pathogenesis of progressive atherosclerosis.[94]

Thrombospondin-1 (TSP-1) is also associated with the formation of atherosclerotic lesions, acute vascular injury, hypercholesterolemia, and hypertension. The administration of antibodies against TSP-1 has been shown to significantly accelerate re-endothelialization and reduce neo-intima formation in balloon-injured rat arteries. TSP-1 has been reported to be a potent inducer of the migration of bovine pulmonary artery smooth muscle cells and human vascular smooth muscle cells (VSMCs). When human coronary artery smooth muscle cells (CASMCs) were cultured under hypoxic conditions, mRNA and protein levels of thrombospondin-1 (TSP-1), and mRNA levels of integrin β(3) were increased with the increase in HIF-1α protein. DNA synthesis and migration of the cells were stimulated under these conditions, and a neutralizing anti-TSP-1 antibody apparently suppressed the migration, but not DNA synthesis. The migration was also inhibited by the tripeptide Arg-Gly-Asp (RGD) that binds to integrin β(3). Furthermore, the migration was completely suppressed in HIF-1α-knockdown cells

exposed to hypoxia and enhanced in HIF-1α-overexpressing cells. These results suggest that hypoxia enhances the migration of CASMCs, and that the migration is elicited by TSP-1, the induction of which is dependent on the stabilization of HIF-1α.[95]

Macrophage migration inhibitory factor (MIF) has emerged as a key factor in vascular re-modelling and in the development and progression of atherosclerosis. MIF is an essential, upstream component of the inflammatory cascade and has a critical role in several inflammatory conditions. It can be expressed by vascular endothelial cells, VSMCs and macrophages. Increased expression of vascular MIF is associated with foam cell transformation during atherogenesis. MIF is expressed in atherosclerotic lesions, and it has been suggested to be involved in atherosclerotic plaque development. Several pro-atherosclerotic mediators such as oxidized LDL, CD40-L and angiotensin II are able to stimulate MIF expression. MIF mRNA and protein were found to be up-regulated early after exposure to moderate hypoxia (3% O_2) in cultured human VSMCs. This is HIF-1α dependent, since knockdown of HIF-1α was found to inhibit the hypoxia induced induction of MIF gene and protein expression. This up-regulation of MIF was attenuated by antioxidant treatment and by inhibition of extracellular signal-regulated kinase (ERK). Under moderate hypoxia (3% O2), both cell proliferation and cell migration were increased in VSMC cells. Blocking the MIF by specific small interference RNA to MIF (MIF-shRNA) resulted in the suppression of proliferation and migration of VSMCs.[96]

Leptin is a adipose tissue derived hormone involved in weight regulation.[97] Leptin has now been reported to be hypoxia-inducible [98] and may be involved in the pathogenesis of atherosclerosis by initiating leukocyte and macrophage recruitment to the endothelium.[99] Leptin also has several other pro-atherogenic properties; it stimulates the proliferation of VSMCs and their production of metalloproteinase. In addition, leptin promotes the production of proliferative and inflammatory cytokines, it increases platelet aggregation and enhances the secretion of pro-atherogenic lipoprotein lipase by cultured human and rodent macrophages causing endothelial dysfunction through the induction of AngII, ROS, and the JNK pathway.[100]

An adverse intrauterine environment during a critical period in foetal development may increase subsequent risk of cardiovascular disease in adulthood.[101] Chronic hypoxia during gestation is considered to be one of the most common intrauterine stressors and Lv *et al.* showed that exposure of neonatal rat aorta smooth muscle cells (NRSMCs) to 2% oxygen enhanced their proliferation in a time-dependent manner, and decreased their rate of apoptosis. This was associated with reduced levels of levels of the pro-apoptotic proteins, BNIP3 and bax, and an increase in the anti-apoptotic factor bcl-2 under hypoxic conditions. Specific down-regulation of HIF-1α partly abolished the proliferative effect of hypoxia.[102]

Dipeptidyl peptidase 4 (DPP4) is a 110 kDa glycoprotein, expressed on several cell types and the expression of which is upregulated following exposure to moderate hypoxia (3% O_2) Matrix metalloproteinase (MMP)-1, -2 and 14 are involved in the shedding of DPP4 from human vascular smooth muscle cells both constitutively as well as under hypoxic conditions. It has been reported that increased DPP4 shedding from SMC occurs due to a complex interplay between different MMPs in cell type-specific manner [103, 104], and that DPP-4 inhibitors, such as alogliptin have anti-atherosclerotic properties.[105]

Obstructive sleep apnoea (OSA) is characterized by intermittent hypoxia (IH), and is associated with an increased risk of cardiovascular disease. IH induces VSMC proliferation, possibly mediated in part by members of the epidermal growth factor family (EGF), such as epiregulin (ER), amphiregulin (AR) and neuregulin-1 (NRG1), and the EGF receptor (erbB2) may be involved in the IH-induced VSMC proliferation.[106] IH induces the expression of HIF-1 dependant genes, including VEGF and NF-Kappa B as well as the release of reactive oxygen species.[107] However, there is still debate as to whether HIF-1α is activated during OSA and IH.

Low-density lipoprotein receptor-related protein (LRP1, also termed the apoE receptor) plays an important role in lipoprotein metabolism and vascular function. In VSMCs exposed to hypoxia (1% O_2) there was a time-dependent induction of LRP1 mRNA and protein expression, with maximum levels being observed at 12 to 24 hours following exposure. This effect was mediated by HIF-1α and was

found to result in increased cholesteryl ester (CE) accumulation due to an increased uptake of modified (aggregated) low-density lipoprotein (agLDL). Blockade of HIF-1α expression inhibited the up-regulatory effect of hypoxia on LRP1 expression and agLDL-derived intracellular CE accumulation, suggesting that both LRP1 overexpression and increased CE accumulation in hypoxic VSMCs are dependent on HIF-1α.[108]

Resistin is a cysteine-rich adipose-derived peptide hormone that in humans is encoded by the *RETN* gene.[109] It produces pro-inflammatory effects in the vascular wall.[110, 111] It promotes endothelial cell activation and causes endothelial dysfunction of porcine coronary arteries. Resistin was reported to have a potential pro-atherogenic effect by increasing the expression of pro-inflammatory cytokines in vascular endothelial cells, and through its promotion of VSMC proliferation. Hypoxia significantly increases the expression of resistin protein and mRNA in rat aortic SMCs. The specific extracellular signal-regulated kinase (ERK) inhibitor PD98059, antioxidant N-acetylcysteine, and ERK siRNA all attenuated the induction of resistin protein by hypoxia. Resistin also increases reactive oxygen species production that could be blocked by pre-treatment with N-acetylcysteine. Hence it appears that the hypoxia-induced increase in resistin is mediated through ROS, ERK mitogen-activated protein (MAP) kinase and nuclear factor of activating T cells pathway.[112]

Monocyte/ macrophage

Angiogenesis is increased in lipid-rich plaque. Oxidized low-density lipoprotein (ox-LDL) is generated in lipid-rich plaque by oxidative stress. It triggers an inflammatory response to cause endothelial dysfunction. However, ox-LDL can activate HIF-1α in monocytes in a hypoxia-independent manner. It has been hypothesized that HIF-1α activation in monocyte-macrophages could transmit pro-angiogenic effects of ox-LDL linking hyperlipidemia, inflammation, and angiogenesis in atherosclerosis. Ox-LDL has been reported to strongly induce HIF-1α and VEGF in monocyte-macrophages and increase endothelial tube formation in co-cultured endothelial cells. HIF-1α inhibition reversed this effect. There was a direct pro-angiogenic effect of ox-LDL in an *in vivo* angiogenesis assay. Again, HIF-1α inhibition abrogated the pro-angiogenic effect of ox-LDL. In a rabbit model, administration of a low-lipid diet

significantly reduced the expression of both HIF-1α and VEGF, resulting in decreased plaque neo-vascularization.[113] Ox-LDL appears to be a pro-angiogenic agent linking hyperlipidaemia, inflammation, and angiogenesis in atherosclerosis. Hence this effect is dependent on macrophages and on the induction of the HIF-1α pathway converting monocyte–macrophages into potent pro-angiogenic cells. This mechanism could underlie the previously reported property of ox-LDL as a marker of atherosclerosis progression in patients with coronary artery disease.[113]

Monocytes migrate from the circulating blood where the oxygen tension is high, to the arterial wall where the oxygen tension is low. This change in the micro-environmental oxygen concentration may affect the metabolism of LDL by the monocyte-derived macrophage. Whilst the specific binding and association of LDL are not changed by hypoxia, the degradation of LDL is reduced to 60% of its rate in normoxia, and the rates of cholesterol esterification and cellular accumulation are increased. The secretion of ApoE is not altered under hypoxia, and therefore it is likely that ApoE-independent cholesterol efflux is reduced. Therefore, hypoxia affects the intracellular metabolism of LDL, stimulates cholesterol esterification, and enhances cholesteryl ester accumulation in macrophages.[114]

Transmigration of monocyte-derived macrophages into the sub-endothelial space is also a crucial step in atherogenesis. Pro-inflammatory macrophages ingest ox-LDL *via* scavenger receptors and become foam cells, thereby promoting plaque formation. Foam cell formation is a key event in both early and late atherosclerotic lesions.[18] Although a number of proteins may contribute to this process, scavenger receptors A (SRA), lectin-like ox-LDL receptor (Lox-1), and cluster of differentiation 36 (CD36) have been demonstrated to be the most relevant for cholesterol uptake.[115] mRNA and protein expression of SRA and CD36 are upregulated by ox-LDL, but are decreased following exposure of macrophages to hypoxia.[116] In contrast, Lox-1 mRNA and protein levels were shown to be upregulated under hypoxic conditions.[116] In this study it was also shown that there was an increase in the lipid content of macrophages after exposure to 0.2% oxygen or treatment with the hypoxia-mimetic dimethyloxalylglycine (DMOG). Blocking of the Lox-1 receptor decreased the hypoxic induction of ox-LDL uptake and lipid content of macrophages. RNAi-mediated knock-down of HIF-1α in macrophages was found to attenuate the hypoxic induction of Lox-1. Hence hypoxia increases the lipid

uptake by macrophages and differentially regulates the expression of ox-LDL receptors. Lox-1 plays a major role in hypoxia-induced foam cell formation which is, at least in part, mediated by HIF-1α. It has been proposed that HIF-1α is involved in Lox-1 upregulation, thereby enhancing ox-LDL uptake. [116]. Furthermore, Burke *et al* showed that RNA interference for HIF-1α (HIF-1α-siRNA) inhibited foam cell formation in the human monoblastic cell line (U937) treated with ox-LDL while several atherosclerosis-related genes, such as cyclooxygenase-2 (COX-2), vascular cell adhesion molecule-1 (VCAM-1) and interleukin-1 β (IL-1 β) were down regulated. Hence the induction of HIF-1α by atherogenic factors may be involved in coordinating the cellular events that culminate in the formation of atherosclerotic lesions.[117]

ATP-binding cassette transporter A1 (ABCA1) plays an important role in reverse cholesterol transport (RCT), a process that is involved in atherogenesis. During atherogenesis, lipid loaded macrophages are exposed to regions of local hypoxia that may influence RCT and affect the lipid efflux of macrophages in the arterial wall. HIF-1 binds to the HRE region of the ABCA1 promoter and the HIF-1 complex increases ABCA1 promoter activity and ABCA1 expression. Primary human macrophages exposed to hypoxia or constitutively expressing active HIF-1α have been found to respond with a potent change in ABCA1 expression, which shows a strong correlation with HIF-1β expression. Furthermore, ABCA1-mediated cholesterol efflux was found to be regulated by HIF-1β under hypoxia. *In vivo*, in macrophages prepared from human atherosclerotic lesions, HIF-1β acted as a major regulator of ABCA1 expression under hypoxia. HIF-1β availability could therefore determine ABCA1 expression and cholesterol efflux in macrophages under hypoxia.[118]

The AMP-activated protein kinase (AMPK) was initially identified as the kinase that phosphorylates 3-hydroxy 3-methylglutaryl coenzyme A reductase (HMGCoA reductase, the rate-limiting enzyme for cholesterol biosynthesis). AMPK is activated by increased concentration of AMP. Hypoxia stimulates AMPK in activated monocytes. Stimulation of macrophages with the anti-inflammatory cytokines interleukin-10 and transforming growth factor-β (TGFβ) resulted in the rapid phosphorylation of AMPK, whereas stimulation of macrophages with lipopolysaccharide resulted in AMPK dephosphorylation and inactivation. AMPK directs signalling in macrophages in a way that suppresses

pro-inflammatory responses and promotes macrophage polarization to an anti-inflammatory functional (M2) phenotype.[119]

Several mechanisms are involved in controlling monocyte and macrophage motility in response to hypoxia. Chemokinesis (the motility of cells) is enhanced in human monocyte derived macrophages (HMDM) after 24 h exposure to low O_2 tension (0.5%) compared to cells grown under normoxia. Cell surface heparan sulphate proteoglycan (HSPG) bind cytokines, chemokines, and growth factors and regulate a wide variety of biological activities, including cell migration, cell adhesion, proliferation, receptor interaction, coagulation, and lipoprotein binding and uptake. Macrophages appear to play a role in the synthesis of cell membrane HSPG associated with CVD. There are two types of cell membrane HSPG; the transmembrane syndecans and the GPI-anchored-glypicans. HMDM exposed to hypoxia for 24 hours had a lower mRNA expression of syndecan-1 and -4 compared with cells exposed to normal cell culture conditions. Protein levels of syndecan-1 were also decreased significantly and cells exposed to hypoxia had lower mRNA expression for key enzymes involved in HS biosynthesis.[54]

A critical step in the development of atherosclerotic plaques appears to be the retention of apoB-100-containing lipoproteins in the arterial wall, and this is partially mediated by the interaction between positively charged regions of apoB-100 and the negatively charged glycosaminoglycan (GAG) chains of proteoglycans (PG) in the intima. Macrophages secrete PG, such as versican, that can alter the retention of lipoproteins and the activity of enzymes, cytokines, and growth factors involved in atherogenesis. They can secrete other PG including perlecan and serglycin, and macrophage colony stimulating factor (MCSF). Versican has been found to be co-localized with HIF-1α in macrophage-rich areas in human advanced atherosclerotic lesions. *In vitro*, versican and perlecan mRNA expression increased after exposure to hypoxia. Furthermore, siRNA knockdown of HIF-1α and HIF-2α in the THP-1 cell line showed that hypoxia-induced versican and perlecan mRNA expression were inhibited, and this was mediated *via* HIF signalling. Versican expression was co-regulated by HIF-1α and HIF-2α but expression of perlecan was influenced only by HIF-1α. Hence oxygen concentration is an important modulator of PG expression in macrophages, partially explaining the pro-atherogenic role of macrophages in atherosclerotic lesions.[120] Furthermore the secreted GAGs from human monocyte-

derived macrophage (HMDM) exposed to hypoxia, were longer, more sulphated and had a higher affinity for LDL compared to those produced under normoxia. These results indicate that hypoxia induced changes in macrophage GAG biosynthesis which have important consequences for the interaction with LDL and may contribute to the development of atherosclerosis in the hypoxic intima.[121]

Within inflamed tissues such as atherosclerotic plaques, macrophages are chronically exposed to hypoxic conditions. In stress conditions, such as those associated with inflammation, Akt regulates several signalling pathways and mediates cell survival. Macrophages also express metalloproteinases (MMP) that promote matrix remodelling and atherosclerotic plaque instability. Whilst moderate hypoxia (approximately 2% to 5% O2) does not affect macrophage differentiation (assessed by scavenger receptor expression), it was found to be associated with activated Akt and inactivated glycogen synthase kinase (GSK)-3 (a negative effector of Akt), allowing nuclear translocation of β-catenin. Catenin is a transcriptional factor that plays a critical role in a variety of cell processes including angiogenesis. Hypoxia was associated with an induction of mRNA expression of the β-catenin-associated genes, including: MMP-7, CD44, and c-Myc. RNAi of TCF7L2, a cofactor of β-catenin, was found to suppress MMP-7 expression induced by hypoxia. Inhibition of Akt phosphorylation by the PI3 kinase inhibitor, LY294002, abolished hypoxia induced GSK-3 β inactivation, β-catenin activation, and MMP-7 expression. Macrophages under hypoxia were found to be more resistant to ox-LDL-induced apoptosis. Therefore the induction of macrophage products in chronically hypoxic tissues such as atherosclerotic plaques and adipose tissue may further amplify the pro-inflammatory milieu within these tissues.[122]

Toll-like receptors (TLRs) play an important role in triggering immune and inflammatory responses by detecting invading microbial pathogens and endogenous danger signals. They elicit both potentially protective and detrimental effects in atherosclerosis where the outcome of TLR signalling is dependent on the agonist and responding cell type.[123] Increased expression of TLR4 is implicated in exacerbating the inflammatory responses in ischemic tissue injury and chronic diseases. TLR4 expression is upregulated by hypoxic stress mediated by HIF-1α in macrophages. PI3K/Akt was shown to be necessary for HIF-1α stabilization on the early stage of hypoxia through inhibition of GSK-3β.

Treatment with pharmacological inhibitors of PI3K and Akt, or knockdown of Akt expression by siRNA, were found to block the increase of TLR4 mRNA and protein levels in macrophages exposed to hypoxia. Phosphorylation of Akt by hypoxic stress preceded the nuclear accumulation of HIF-1α. HIF-1α-mediated up-regulation of TLR4 expression was blocked by LY294002. Furthermore, the antioxidant, sulforaphane (SFN) suppressed hypoxia induced up-regulation of TLR4 mRNA and protein by inhibiting PI3K/Akt activation and the subsequent nuclear accumulation and transcriptional activation of HIF-1α. However, p38 was not involved in HIF-1α activation and TLR4 expression induced by hypoxic stress in macrophages. These data indicate that PI3K/Akt contributes to the hypoxia-induced increase in TLR4 expression in macrophages at least partly through the regulation of HIF-1 activation and that SFN contributes to protection against ischemic tissue injury by down-regulation of TLR4 expression in macrophages.[124]

Inflammation is involved in all phases of atherogenesis, and proinflammatory cytokines and chemokines secreted from the immune cells involved in the inflammatory response are involved in the progression of the atherosclerotic process.[125] Among the cytokines that have received particular attention are interleukin-1α (IL-1α) and IL-1β.[126] Hypoxia enhances pro-IL-1 β protein, but not mRNA expression in lipopolysaccharide (LPS)-stimulated human macrophages. It also retards the autophagic degradation of pro-IL-1 β, and thus prolongs its effective half-life, promoting its intracellular accumulation. Furthermore, hypoxia increases the expression of NLRP3, and augments caspase-1 activation in LPS-primed macrophages. Consequently, human macrophages exposed to hypoxia, secrete increased levels of mature IL-1 β compared to normoxic macrophages after treatment with crystalline cholesterol. In human atherosclerotic plaques, derived from carotid endarterectomy specimens, IL-1 β was localized predominantly in macrophage-rich regions that also express activated caspase-1 and the hypoxia markers HIF-1α and hexokinase-2. Hence hypoxia appears to potentiate the expression of IL-1 β in the context of atheroma.[127]

The link between macrophage glycolysis and their pro-inflammatory activity is probably mediated by HIF-1α and the transcriptional induction of 6-phosphofructo-2 kinase, 2, 6-biphosphatase (PFKFB3).

Tawkol *et al* showed that hypoxia could potentiate macrophage glycolytic flux and a proportional up-regulation of pro-inflammatory activity, that is dependent on both HIF-1α and PFKFB3.[128]

In macrophages exposed to hypoxia, there is an up-regulation of several genes, including: VEGF, the glucose transporter 1 (GLUT-1), matrix metalloproteinase-7 (MMP-7), neuromedin B receptor, and the DNA-binding protein inhibitor. These genes may be important for the survival and functioning of macrophages in hypoxic diseased tissues.[129] Other immune cells are found within atherosclerotic lesions, namely dendritic cells (DCs) and T cells, and are also proposed to contribute to atherogenesis.[125] DCs can be found in healthy arterial vessels but accumulate especially in the areas of atherosclerotic plaque. In atherosclerosis, DCs are thought to play an important role in controlling cell recruitment, migration, lipid uptake, and T-cell responses. Hypoxia, ox-LDL, tumor necrosis factor-α, and inhibition of endothelial NO synthase may all augment adhesion of DC to the endothelium where HIF-1 triggers DC activation. [130, 131]

miRNAs are a class of endogenous small RNAs that act as inhibitors of post-translational gene expression or promote RNA degradation by duplex-formation within the 3'-UTR of target mRNAs. They play an important role during a wide range of cellular processes by fine-tuning gene expression.[132] Monocytes migrate from the circulation, where the oxygen tension is high, into areas with a high inflammation, such as the atherosclerotic plaque. There, they differentiate into macrophages. During this process there is a change in the expression pattern of the HIF-1α and HIF-2α genes. This is associated with a down-regulation of microRNAs encoded by the miR-17-92 cluster. miRNAs from the miR-17-92 cluster (mi-17 and -20a), can regulate HIF-1α and HIF-2α by targeting their 3'-UTR. These miRNAs are repressed during the differentiation of monocytes into-macrophages. miR-over-expression in human macrophages demonstrates the important role of this microRNA-mediated regulation of the HIF-system for adaption of macrophages to hypoxia. The HIF-system is activated during the differentiation of monocyte into macrophages. This activation is in part mediated by a miRNA-dependent mechanism, which seems to be crucial for the adaption of macrophages to hypoxia.[133]

2. Animal models

Rodent models

a. Altering HIF-1 expression

HIF-1α is the regulatory subunit of a transcriptional complex, which controls the recruitment of multipotent progenitor cells and tissue repair in ischemic tissue by inducing stromal cell- derived factor (SDF)-1 α expression. In VSMCs, HIF-1α can be activated under normoxic conditions by platelet products. Previous studies have investigated the effect of altering the local expression of HIF on atherosclerotic plaque development in murine vascular injury models.

The expression of HIF-1α has been investigated in a wire-induced vascular injury model in the hyperlipidaemic ApoE(-/-) mice. In this model, HIF-1α expression was increased in the tunica media, predominantly in the SMCs, as early as 1 day after injury. Nuclear translocation of HIF-1α and co-localization with stromal cell derived factor (SDF-1α; a pro-inflammatory chemokine upregulated in injured vessels and responsible for cell migration and recruitment of new smooth muscle progenitor cells) was detected in neo-intimal cells after 2 weeks. HIF-1α mRNA expression was induced at 6 hours after injury as determined by real-time RT-PCR. Inhibition of HIF-1α expression by local application of HIF-1α-siRNA reduced the neo-intimal area by 49% and significantly decreased the neo-intimal SMCs content compared to treatment with the control- siRNA. The expression of HIF-1α and SDF-1α were reduced in neo-intimal cells of the HIF-1α-siRNA treated arteries. HIF-1α expression therefore appears to be directly involved in the formation of the neo-intimal after vascular injury and mediates the up-regulation of SDF-1α, which may affect the stem cell-dependent repair of injured arteries.[134] In experiments in which the local expression of the different HIF-subunits (HIF-1α, HIF-2α) was increased using adenoviral infection, or reduced by using a dominant-negative mutant, the extent of neo-intima formation was reduced in the dominant negative mutant mice, and this was associated with decreased expression of several established HIF-target genes, including: VEGF-A and its receptors Flt-1 and Flk-1. In contrast, the local overexpression of HIF-1α and HIF-2α was associated with a further increase in the injury induced neo-intima formation.[135]

HIF-1 is detectable in the nuclei of endothelial cells (ECs) covering murine and human atherosclerotic lesions. When the HIF-1α gene was specifically deleted in the ECs from ApoE knockout mice (EC-HIF-1α (-/-)), the degree of atherosclerotic lesions, the lesional content of macrophages, and the expression of CXCL1 (responsible for the accumulation of macrophages in the atherosclerotic lesion) in ECs were all reduced in the lesions produced by partial ligation of the carotid artery or in the aorta following diet-induced atherosclerosis in EC-HIF-1α (-/-) compared with control mice. In parallel *in vitro* studies, it was reported that mildly ox-LDL or lysophosphatidic acid 20:4 increased endothelial CXCL1 expression and monocyte adhesion in the atherogenic lesion *via* the induction of HIF-1. In the mice with endothelial HIF-1α deficiency there was a down-regulation of the micro RNA miR-19a, which controls endothelial function and neo-vascularization; and HIF-1-induced miR-19a expression that mediated the up-regulation of CXCL1 in mildly ox-LDL-stimulated ECs.[136]

HIF-1 has also been reported to attenuate the inflammatory response by regulating T cell activation and cytokine production. When HIF-1α was over-expressed in ApoE knockout murine lymphocytes, antibody array analysis revealed a pattern consistent with a shift in the cytokine translation pattern from a T helper 1 to T helper 2 phenotype. Normally, the mice in this model develop atherosclerotic lesions within the aortic sinus and these lesions were reduced in the mice with T cells over-expressing HIF-1α. These findings were associated with a reduced expression of IFN-gamma in CD4+ spleen-derived lymphocytes and aortae of these mice representing a novel immune-modulatory approach in atherosclerosis.[137]

It has been also reported that HIF-1 expression is upregulated in CD11c(+) antigen presenting cells (APCs) within atherosclerotic plaques of LDL receptor-deficient (Ldlr(-/-)) mice and that conditional deletion of HIF-1α in CD11c(+) APCs in high-fat diet-fed Ldlr(-/-) mice increased the formation of atherosclerotic plaques, and increased lesional T-cell infiltration. This suggests HIF-1 has a protective role during atherogenesis. It was further reported that HIF-1 directly modulates Signal Transducers and Activators of Transcription 3 (Stat3). A reduction in STAT3 expression was found in HIF1-deficient APCs and aortic tissue, together with an upregulated interleukin-12 expression and expansion of type 1 T-helper (Th1) cells. Over-expression of STAT3 in bone marrow cells of HIF-1α-deficient APCs

reversed the enhanced atherosclerotic lesion formation and reduced Th1 cell expansion in chimeric Ldlr (-/-) mice. Furthermore, deletion of HIF-1α in the lysine motif; LysM (+) bone marrow cells (the most prominent motif for binding to peptidoglycans) in Ldlr (-/-) mice, did not affect lesion formation or T-cell activation. In human atherosclerotic lesions, HIF-1, STAT3, and interleukin-12 protein were found to co-localize with APCs. HIF-1 therefore appears to inhibit APC activation and Th1 T-cell polarization during in Ldlr(-/) mice and to attenuate the progression of atherosclerosis.[138]

Vascular injury can be induced by placement of an external vascular polyethylene cuff, which is associated with the formation of a neo-intima.[139] When this model of vascular injury was used in HIF-1-(T-cell)-deficient mice, there was an enhanced neo-intimal response in the mutant mice, and significantly greater infiltration of inflammatory cells into the adventitia. The authors suggest that the mechanism of augmented vascular remodelling in the mutant mice appeared to be due to enhanced production of cytokines by activated T cells and increased antibody production in response to a T-dependent antigen in the mutant mice.[140]

Using an *ex-vivo* rat tail artery model maintained for 4 days in hypoxic or normoxic culture and then assessed for contractility, oxygen consumption, and lactate production in oxygenated medium, hypoxia was found to be associated with depressed basal energy turnover, impaired mitochondrial capacity, and altered Ca^{2+} homeostasis, but did not affect contractile energetics.[141]

b. Intermittent Hypoxia

As discussed earlier in the review, intermittent hypoxia (IH) is a feature of obstructive sleep apnoea (OSA), and is associated with increased cardiovascular mortality and pulmonary hypertension. This may be attributable either to more extensive lesions, or lesions that are more unstable in subjects with OSA. In a murine model of IH, using a quantitative proteomic method showed that a total of 163 proteins were identified as having changed significantly, of which 34 showed significant differences between genders, and which may be related to vascular injury induced by chronic IH. Twenty up-regulated proteins and 14 down-regulated proteins were observed in female mice compared to male mice.[142]

When ApoE-deficient (ApoE(-/-)) mice were fed on a high cholesterol diet, and then subjected to IH for up to 12 weeks, it was reported that at 4 weeks, IH was associated with an increased plaque size in the aortic sinus and the descending aorta. At 12 weeks, atherosclerosis progressed in all groups, but more rapidly in the descending aorta of the IH-exposed animals. Plaque composition was similar between IH and controls. In the mice exposed to IH, blood pressure rose with time compared to the mice kept in normoxia, and there were relatively stable increases in serum lipids and arterial stiffness.[143]

Intermittent hypoxia may cause endothelial dysfunction in part by the local increase in ROS and reduction of the peripheral repair capacity. This is supported by the finding that anti-inflammatory; Infliximab and anti-oxidant; L-glutathione prevent hypoxia-induced vascular and extravascular changes.[144]

The hormone adiponectin has been reported to have anti-atherosclerotic properties. Severe obstructive sleep apnoea-hypopnea syndrome (OSAS) is associated with hypo-adiponectinemia and a nocturnal reduction in circulating adiponectin concentrations. Exposure of mice to 3-weeks of sustained hypoxia (10% O_2) resulted in a significant accumulation of adiponectin in the pulmonary arteries. Pulmonary arterial wall remodelling, was found to be greater in adiponectin-knockout mice than wild-type mice under hypoxia. Over expression of adiponectin was associated with significantly decreased hypoxia-induced pulmonary arterial wall thickening and right ventricular hypertrophy.[145] In rats exposed to IH for 8 hours/day, for 5 weeks, cardiac dysfunction was induced as measured by echocardiograph. Adiponectin treatment was associated with amelioration in these indices of cardiac dysfunction and also reduced expressed proteins associated with endoplasmic reticulum (ER) stress as well as the expression of ROS.[146]

ApoE-KO mice, and mice deficient in both ApoE and p50 genes (ApoE-p50-DKO), were exposed to IH or sham conditions. IH caused atherosclerosis in ApoE-KO mice fed on a normal chow diet in a time-dependent manner. IH caused more severe atherosclerotic lesions in the ApoE-p50-double KO (DKO) mice on a normal chow diet. ApoE-KO and ApoE-p50-DKO mice exposed to IH for 30 and 9 weeks, respectively, displayed similar areas of atherosclerotic lesions on cross sections of aortic root.

P50 gene deletion (in ApoE-p50-DKO mice) significantly augmented IH-induced serum levels of tumour necrosis factor-α and IL-6, aortic tumour necrosis factor-α, and inducible NOS expression and aortic infiltration of Mac3-positive macrophages (a marker of proliferation). IH caused a greater elevation in serum cholesterol concentrations in ApoE-p50-DKO than in ApoE-KO mice. IH was found to be associated with a down-regulation of hepatic LDL receptor and HMG-CoA reductase expression in the ApoE-p50-DKO but not in ApoE-KO mice. NF-κB p50 appeared to protect against IH-induced atherosclerosis by inhibiting vascular inflammation and hypercholesterolemia.[147]

In male ApoE-deficient mice subjected to intermittent hypobaric hypoxia (IHH) for 8 weeks for 8 hours a day, plasma lipid levels, and plaque size did not differ from control mice. However, the IHH-treated mice exhibited significantly decreased plaque collagen content, and increased matrix metalloproteinase (MMP)-9 protein expression; both features of atherosclerotic plaque instability. Tissue inhibitor of MMP (TIMP)-2 expression was decreased after exposure to IHH. Hence, IHH may promote atherosclerotic plaque instability in ApoE-deficient mice by changing the balance of MMPs and TIMPs.[148] In order to explore the early impact of IH on endothelial function and atherogenesis, ApoE-/- mice were exposed to a 6-week-intermittent hypoxia either immediately (early pre-atherosclerosis) or after 5 weeks of high-cholesterol diet (advanced pre-atherosclerosis), with mice maintained under normoxia serving as controls. Endothelial function was assessed *ex vivo*. Flow cytometry was used to assess blood plasma CD31+/ annexin V+ endothelial micro-particles as well as sca1/flk1+ endothelial progenitor cells in blood and bone marrow. IH was found to cause impaired endothelial function and integrity. Peripheral repair capacity expressed as the number of endothelial progenitor cells in blood was attenuated under IH, despite the elevated number of these cells in the bone marrow. In contrast, endothelial function, as well as micro-particle and endothelial progenitor cell levels were similar under IH versus control in more advanced pre-atherosclerosis.[149]

When endothelial cells were co-cultured with lymphocytes isolated from rats that had been subjected to either IH, or normal conditions, there was a significant decrease in apoptosis regulators, Bcl-2 level and the Bcl-2/Bax ratio, and a significant increase in apoptosis as assessed by Bax and caspase-3, and an increase in measures of oxidative stress (MDA, SOD, and CAT) and inflammation (TNF-α, IL-8,

CRP, and ICAM-1). These effects were partially restored by pre-treatment with tempol (a membrane permeable free radical scavenger) and exacerbated by intermittent hypoxic co-incubation.[150]

Supplementation with the n-3 polyunsaturated fatty acid, docosahexanoic acid (DHA) which has anti-inflammatory properties, prevented IH-induced atherosclerosis acceleration in ApoE -/- mice. This was associated with a decrease in aortic MMP-2 gene expression.[151]

c. Miscellaneous hypoxia-related studies in Murine models

Whilst advanced murine and human plaques are hypoxic it is unclear whether plaque hypoxia is causally related to atherogenesis. It is possible to partially ameliorate the hypoxia in atherosclerotic plaques by breathing hyperoxic carbogen gas. LDL receptor-deficient mice (LDLR-/-), fed on a Western-type diet, were treated with carbogen (95% O_2, 5% CO_2) or air, and the effect on plaque hypoxia size, and phenotype was studied. Pimonidazole was used as an indicator of tissue hypoxia. Carbogen increased arterial blood pO_2 5-fold and reduced plaque hypoxia in advanced plaques of the aortic root and arch. The effect of repeated carbogen exposure on atherosclerosis in LDLR-/-mice fed on a Western-type diet included: a reduction in plaque hypoxia (-40%), necrotic core size (-37%), and TUNEL+ (terminal uridine nick-end labeling positive) apoptotic cell content (-50%) and increased efferocytosis of apoptotic cells by cluster of differentiation 107b+ (CD107b, MAC3) macrophages (+36%) in advanced plaques of the aortic root. Plaque size, plasma cholesterol, hematopoiesis, and systemic inflammation were unchanged. *In vitro,* hypoxia hampered efferocytosis by bone marrow-derived macrophages.[152]

Following a myocardial infarction there is a mobilization of endothelial progenitor cells (EPCs) from the bone marrow. This appears to be affected by the circadian gene period2 (per2) that is known to regulate the function of EPCs. Flow cytometry revealed a decreased number of circulating EPCs in per2(-/-) mice after MI. *In vitro,* per2(-/-) EPCs showed decreased migration and tube formation capacity under hypoxia. Western blot analysis revealed that there was an inhibition in the activation of extracellular signal-regulated kinase and Akt signaling in the bone marrow of per2(-/-) mice and inhibited PI3K/Akt expression in per2 (-/-) EPCs under hypoxia.[153]

A number of microRNAs function as tissue protective factors in ischemia-reperfusion (I/R) and/or hypoxia-reperfusion (H/R)-induced myocardial injury. miR-21 has an anti-apoptotic role in I/R-induced myocardial damage *in vivo* and in H/R-induced H9C2 cell death *in vitro*. Increased expression of miR-21 upregulates Akt signalling activity by suppressing the expression of phosphatase and tensin homolog (PTEN; an Akt negative regulator) and the increased activity of Akt signalling further inhibits apoptosis partially by increasing the ratio of B-cell lymphoma 2(Bcl-2)/Bcl-2-associated X protein, which further suppressed the expression of caspase-3. Hence miR-21 has a protective role in I/R- and H/R-induced cardiocyte apoptosis *via* the PTEN/Akt-dependent mechanism.[154]

Rabbit models

The presence of hypoxia within atherosclerotic lesions has been confirmed using 7-(4'-(2-nitroimidazol-1-yl)-butyl)-theophylline (NITP) which binds to foam cells in cholesterol-fed rabbits.[24]

Removal of the carotid artery adventitia from rabbits can induce the formation of an intimal hyperplastic lesion. In rabbits fed on a normal diet, the size of the lesion (measured as the intimal: medial ratio) was maximal by day 14 and thereafter, regressed toward control dimensions by day 28. In rabbits fed on a high cholesterol (HC) diet, the lesion was again maximal by day 14. Although some regression was seen, the lesion persisted to day 42. The authors proposed that lesion formation may be initiated following the development of arterial wall hypoxia, secondary to excision of the adventitial vasa vasorum.[155]

Micro-angiogenesis in the arterial wall has been observed during the development and progression of atherosclerosis. The inter-relationship between inflammation, oxidative stress and hypoxia has been investigated with respect to the process of early atherosclerotic micro-angiogenesis in the cholesterol-fed rabbit. The microvessel density (MVD) in the HC group was significantly higher ($P<0.01$) than that observed in the normo-cholesterolemic rabbits. Whilst HIF-1α levels did not change significantly in either of the two groups the levels of inflammatory markers and antioxidants were significantly different between the two groups ($P<0.05$). These results suggest that during the initial stages of the hyperlipidemia-induced atherosclerosis, micro-angiogenesis may be associated with an increase in

inflammation and a decrease in the levels of antioxidants which promote this process even before any tissue hypoxia is detected.[1]

Ex vivo, organ bath experiments show that segments of aortae from control, cholesterol-fed, or Watanabe hereditary hyperlipidemic (WHHL) rabbits have substantially different responses when the experiments were conducted in hypoxic versus normoxic conditions. These experiments looked at responses to: aggregating platelets, 5-hydroxytryptamine (serotonin; 5-HT), norepinephrine, endothelin, and prostaglandin F2α. The contractions under hypoxic conditions in the atherosclerotic rings were not dependent on the presence of an intact endothelium as they could not be antagonized by blockers of α-adrenoceptors, 5-HT2 receptors, histamine receptors, thromboxane receptors, and muscarinic cholinoreceptors. Inhibitors of cyclooxygenase, lipoxygenase, Na+, K+-ATPase, and free radical scavengers or an activator of endothelium-derived relaxing factor did not significantly affect the hypoxic contraction. The absence of effect of several inhibitors of protein synthesis appears to rule out the involvement of endothelin, angiotensin II, and bradykinin. The contraction under hypoxic conditions was not influenced by the omission of Ca^{2+} from the medium or by inhibition of Ca^{2+} influx but was prevented by blockade of intracellular Ca^{2+}. The inhibitors of NOS (nitro-L-arginine, 100 μM) and the guanylyl cyclase (methylene blue, 10 μM) both enhanced the initial contractile responses to 5-HT to a similar extent as hypoxia and completely prevented the hypoxic contraction in the atherosclerotic tissues. Hypoxia decreased cGMP but not the cAMP levels in atherosclerotic aortae with and without endothelium. These data indicate that there are unusual vasoconstrictor responses in atherosclerotic arteries; this constrictor response depends on the availability of intracellular Ca^{2+} and seems to be due to the further inhibition of an already impaired cGMP production, most likely due to inhibition of NOS present in non-endothelial cells of the atherosclerotic blood vessels.[156]

Balloon injury in the iliac-femoral arteries of hypercholesterolaemic rabbits fed with a 0.5% cholesterol diet or a conventional diet was used as a model of atherosclerosis in which macrophage-rich or a smooth muscle cell (SMC)-rich neo-intima was created. Several metabolites of the glycolytic and pentose phosphate pathways (an alternative route of glucose catabolism) were found to be increased, and the tricarboxylic acid (TCA) cycle in the iliac-femoral arteries with macrophage-rich neo-intima, compared

with those that were not injured and those with SMC-rich neo-intima. Pimonidazole immunoreactivity was closely localized with the nuclear translocation of HIF-1α and hexokinase II expression in macrophage-rich neo-intima. *In vitro*, THP-1 macrophages stimulated with lipopolysaccharides (LPS) and interferon-gamma (IFN- γ) were cultured under normoxic and hypoxic conditions. The levels of glycolytic and pentose phosphate pathway (4 of 6) metabolites increased in LPS and IFN- γ stimulated macrophages under hypoxic but not normoxic condition.[157]

Whilst it has been reported that hypoxia increases, and hyperoxia reduces experimental atherosclerosis, it is unclear if repetitive hypoxic and hyperoxic insults affect intimal thickening after arterial injury. In a rabbit model of intermittent repetitive hypoxia or hyperoxia, hypoxia markedly increased the intima-to-media ratio in the abdominal aorta (AA) whereas hyperoxia had no effect on AA disease but increased intimal thickening in thoracic aorta (TA). Hyperoxia promoted positive arterial re-modelling in both TA and AA, resulting in larger luminal size. The cholesterol content in AA was increased by hypoxia and decreased by hyperoxia, but decreased by both treatments in TA. Lipophilic antioxidants and the proportion of arterial lipids that was oxidized were not altered by either hypoxia or hyperoxia. Hence, intermittent repetitive hyperoxia is not protective but promotes arterial disease in normal and injured arteries independent of lipid peroxidation.[158]

Porcine models

In Yucatan miniature pigs the oxygenation profiles across the superficial femoral arteries has suggested a relatively poorly oxygenated media (a trough value of approximately 25% of the intimal oxygenation has been reported) with a progressive rise in oxygenation toward the intimal and adventitial surfaces. The vasa vasorum are required to supply the outer media with oxygen, and occlusion of the adventitial vasa vasorum by flush ligation of the arterial branches that supply them resulted in a focal, intimal hyperplastic lesion, composed predominantly of smooth muscle cells which have migrated from the media or was a product of some migration that resulted in proliferation; a process initiated by hypoxia.

Coronary arteries also contain a network of adventitial vasa vasorum. These have been visualized in the cholesterol-fed pig, using a novel computed tomography technique. Two different types of vasa vasorum were defined: first-order vasa vasorum ran longitudinally parallel to the vessel and second-order originated from first-order vasa vasorum circumferentially around the vessel wall. In the hypercholesterolemic coronary arteries, there was a significant increase in the area of the vessel wall and in the density of vasa vasorum. The latter occurred especially by an increase of second-order vasa vasorum and was associated with a loss of the normal spatial pattern. Hence adventitial neo-vascularization of the vasa vasorum occurs in experimental hypercholesterolemic coronary arteries before the development of vascular lesion. This occurs particularly by an increase of second-order vasa vasorum and a loss of orientation of the normal vasa vasorum pattern, which may be a part of the early atherosclerotic remodelling process.[159]

3. *Ex Vivo* human studies

Markers of hypoxia and inflammation have been previously identified by microarray analysis, in atherosclerotic carotid arteries with low to moderate stenosis. A notable overexpression of HIF-1α in inflammatory and hypoxic areas of carotid arteries in all types of lesions (type II-V lesions) taken from patients with carotid stenosis of < 50% was found.[160]

Heat shock proteins (HSPs) are molecular chaperones which are essential for cell survival.[161] Hypoxia was reported to markedly increase the expression of several HSPs in human saphenous vein and the internal mammary artery from patients undergoing coronary artery bypass surgery. A 6 h period of oxygen deprivation resulted in elevated levels of HSP60, and HSP72 and of HSP73 in the saphenous vein. In the internal mammary artery, HSP73 expression was found to be significantly enhanced.[162]

The precise cellular and molecular mechanisms regulating adventitial vasa vasorum neo-vascularization, which occurs in the pulmonary arterial circulation in response to hypoxia, remain unknown. In the vessel wall, endothelial cells and mural cells, including fibroblasts, pericytes, and smooth muscle cells, make physical cell-cell contacts, suggesting a role for intracellular communication in the regulation of vascular growth and function. The cell-cell communication between endothelial

cells and non- endothelial cells may represent a critical process in the initiation, stabilization, and maturation of new vessels. Adventitial fibroblasts (AdvFBs) and vasa vasorum endothelial cells (VVECs) were isolated from the adventitia of pulmonary arteries. Hypoxia activated pulmonary artery AdvFBs exhibited pro-angiogenic properties and influenced the angiogenic phenotype of the VVEC, in a process involving endothelin-1 (ET-1); a pro-angiogenic regulator of neo-vascularization in many pathological conditions. AdvFBs, either in experiments using co-culture, or conditioned media, stimulated VVEC proliferation or augmented the self-assembly and integrity of cord-like networks that formed when VVECs where cultured on Matrigel. When co-cultured on Matrigel, AdvFBs and VVECs self-assembled into heterotypic cord-like networks, a process augmented by hypoxia but attenuated by either selective endothelin receptor antagonists or oligonucleotides targeting prepro-ET-1 mRNA. Hence hypoxia-activated AdvFBs exhibit pro-angiogenic properties and communicate with VVECs, in a process involving ET-1, to regulate vasa vasorum neo-vascularization occurring in the adventitia of pulmonary arteries in response to chronic hypoxia.[163]

Increased HIF-1α expression is observed in human atherosclerotic plaque with different phenotypes. The expression of HIF-1α appears to be associated with the presence of a large extracellular lipid core and macrophages and co-localizes with VEGF. HIF-1α is also associated with an inflammatory phenotype and with VEGF expression. HIF-1α expression is upregulated in activated macrophages under normoxic conditions suggesting that plaque (immune) inflammatory reactions might also be involved in plaque HIF-1α expression.[164] Intra-plaque angiogenesis is a typical feature of hypoxic tissue and expression of HIF. Sluimer *et al.* have demonstrated the presence of hypoxia in human advanced atherosclerotic lesions. Hypoxia was assessed by infusing pimonidazole before carotid endarterectomy. The mRNA and protein expression of HIF-1α, HIF-2α, HIF-responsive genes (VEGF, glucose transporter [GLUT]1, GLUT3, hexokinase [HK]1, and HK2), and microvessel density were determined in a series of non-diseased and atherosclerotic carotid arteries using microarray, quantitative reverse transcription polymerase chain reaction, *in situ* hybridization, and immunohistochemistry. Pimonidazole immune-histochemistry showed hypoxia was particularly present within the macrophage-rich core of the lesions and correlated with the presence of angiogenesis, thrombus formation, and

expression of CD68, HIF, and VEGF. The mRNA and protein expression of HIF, its target genes, and microvessel density increased from early to stable lesions, but no changes were observed between stable and ruptured lesions. The HIF pathway was associated with lesion progression and angiogenesis, suggesting its involvement in the response to hypoxia and the regulation of human intra-plaque angiogenesis.[165]

OSA is known to be a risk factor for coronary heart disease. Peripheral blood was taken from adults with suspected OSA and mRNA was prepared from the isolated monocytes for the analysis of C-C chemokine receptor 2 (CCR2; a chemokine that mediates monocyte chemotaxis). Monocytic CCR2 gene expression was found to be increased in severe OSA patients and higher levels were detected after sleep. *In vitro* experiments showed that IH increased the CCR2 expression in THP-1 monocytic cells even in the presence of TNF-α and CRP and also promoted the monocyte chemotactic protein-1 (MCP-1)-mediated chemotaxis and adhesion of monocytes to endothelial cells. Both ERK and p38 MAPK were confirmed to be involved in the signalling pathway for the induction of CCR2 in monocytes by IH. These results strongly suggest an important role of CCR2, in atherosclerosis in patients with OSA.[166]

Neuro-immune guidance cue netrin-1 (Ntn1), initially characterized in the specialized migration of neurons during development, has also been found to inhibit macrophage migration from atherosclerotic plaques, and hence enhances chronic inflammation. Netrin-1 and its receptor uncoordinated-5-B receptor (Unc5b) are expressed in macrophages in hypoxic regions of human and mouse plaques. Ntn1 and Unc5b mRNA are also upregulated in macrophages treated with ox-LDL or inducers of oxidative stress, including hypoxia. These responses are abrogated by inhibiting HIF-1α, indicating a causal role for this transcription factor in regulating Ntn1 and Unc5b expression in macrophages. J774 macrophages overexpressing active HIF-1α express increased levels of netrin-1 and Unc5b and have a reduced migratory capacity compared with control cells. This can be restored by blocking the effects of netrin-1. Netrin-1 protects macrophages from apoptosis under hypoxic conditions in a HIF-1α-dependent manner *via* regulating macrophage trafficking and accumulation during hypoxia in atherosclerosis. Therefore, HIF-1α regulation of netrin-1 is involved in promoting macrophage accumulation and survival in atherosclerotic plaque and provides further evidence of a role for hypoxia

in sustaining inflammation. Local inhibition of such factors may have a therapeutic value for the resolution of inflammation in atherosclerosis and other chronic inflammatory disease.[167, 168] A high proportion of stable plaques have been reported to have neo-vascularization. Regional expression of hypoxia- and thioredoxin- related genes in different parts of stable atherosclerotic carotid plaques has been investigated. The expressions of HIF-lα, VEGF-A, thioredoxin, and thioredoxin interacting protein were analysed in carotid endarterectomy samples using RT-PCR on laser micro-dissected regions. The expression of all four genes was significantly lower in the medial region at the mRNA level. High expression of these genes was noted in the shoulder region and sites of neo-vascularization, with no significant difference between the two, and these expression patterns were related to macrophage infiltration. Hypoxia- and thioredoxin-related genes are significantly overexpressed in human stable carotid atherosclerotic plaques and strongly correlate with macrophage infiltration rather than neo-vascularization. Macrophage infiltration may lead to overexpression of these genes providing a mechanism of transcriptional activation of several other target genes contributing to angiogenesis, atherosclerotic progression and vascular remodelling in stable carotid plaques.[169]

Plaque haemorrhage in carotid atherosclerosis may promote plaque progression, and rupture that may result in cerebrovascular disease. HIF-1α induces angiogenesis *via* the expression of VEGF and E26 transformation-specific-1 (Ets-1). A higher incidence of plaque haemorrhage is observed in plaques associated with symptoms than in those without. HIF-1 α, VEGF, and Ets-1 expression is co-located in the deep layer of plaque, where angiogenesis is also well developed. Symptomatic plaques were found to have a higher expression of VEGF than asymptomatic plaques. Plaques with haemorrhage were associated with a higher incidence of plaque ulceration and higher expression of Ets-1 than those without haemorrhage. Significantly higher expression levels of VEGF and Ets-1 were observed in plaques with haemorrhages, ulcers and severe stenosis. Hypoxia-inducible angiogenic proteins in human carotid atherosclerosis therefore appear to promote intra-plaque angiogenesis, which can induce plaque haemorrhage and progression.[170]

Genetic determinants of the response to Hypoxia

Patients with chronic ischemic heart disease develop coronary artery collaterals (new vessels) due to myocardial hypoxia. However, there is a marked variability in the tendency to develop these collaterals. Whilst this variability is likely to be multifactorial, genetic factors are thought to be involved. Genetic variations in HIF-1α has been reported to influence the development of coronary artery collaterals in patients with significant coronary artery disease.[171] They have also been reported as predisposing to abdominal aortic aneurysm.[172]

Conclusions

There is now substantial evidence that there are areas of profound hypoxia within atherosclerotic lesions. There is also good evidence that hypoxic states, such as that associated with obstructive sleep apnoea may be involved in the aetiology of cardiovascular disease. HIF-1 appears to be an important mediator of the pro-atherogenic cellular response to hypoxia. Hypoxia has important effects on intermediary metabolism, cholesterol disposal and the inflammatory response within the artery wall. It also appears to promote changes in the cellular and extracellular composition of the artery wall that may impact on the response to injury.

Declaration of Interest

The authors declare no potential conflict of interests with respect to the research, authorship, and/or publication of this article.

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