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Cellular hypoxia and adipose tissue dysfunction in obesity

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Expansion of adipose tissue mass, the distinctive feature of obesity, is associated with low-grade inflammation. White adipose tissue secretes a diverse range of adipokines, a number of which are inflammatory mediators (such as TNFα, IL-1β, IL-6, monocyte chemotactic protein 1). The production of inflammatory adipokines is increased with obesity and these adipokines have been implicated in the development of insulin resistance and the metabolic syndrome. However, the basis for the link between increased adiposity and inflammation is unclear. It has been proposed previously that hypoxia may occur in areas within adipose tissue in obesity as a result of adipocyte hypertrophy compromising effective O2 supply from the vasculature, thereby instigating an inflammatory response through recruitment of the transcription factor, hypoxic inducible factor-1. Studies in animal models (mutant mice, diet-induced obesity) and cell-culture systems (mouse and human adipocytes) have provided strong support for a role for hypoxia in modulating the production of several inflammation-related adipokines, including increased IL-6, leptin and macrophage migratory inhibition factor production together with reduced adiponectin synthesis. Increased glucose transport into adipocytes is also observed with low O2 tension, largely as a result of the up-regulation of GLUT-1 expression, indicating changes in cellular glucose metabolism. Hypoxia also induces inflammatory responses in macrophages and inhibits the differentiation of pre-adipocytes (while inducing the expression of leptin). Collectively, there is strong evidence to suggest that cellular hypoxia may be a key factor in adipocyte physiology and the underlying cause of adipose tissue dysfunction contributing to the adverse metabolic milieu associated with obesity.

Hypoxia: Adipose tissue dysfunction: Inflammatory adipokines: Hypoxic inducible factor-1

The biology of adipose tissue has long surpassed the boundaries that defined it as merely a storage site for metabolic fuel, in addition to its role in thermal insulation and mechanical support. White adipose tissue has been recognised to release (and take up) NEFA and secrete the enzyme lipoprotein lipase. However, its role as an important endocrine organ was realised in 1994 following the identification of leptin, the protein product of the ob gene that is mutated in the genetically-obese (ob/ob) mouse(1). This secreted protein was found to be synthesised and secreted by the adipocyte (the lipid-containing cell of adipose tissue) and thereby became one of the founder members of an extensive family of proteins, i.e. the adipokines, which are defined as proteins released from white adipocytes(2–5).

Adipokines secrete in excess of seventy-five adipokines, providing a means by which adipose tissue communicates with other organs, both centrally (brain) and peripherally (liver, skeletal muscle, etc.). The adipokines exhibit diverse functional roles, including in energy balance (e.g. leptin), insulin sensitivity and glucose metabolism (e.g. adiponectin), inflammation (e.g. TNFα), immunity

Abbreviations: HIF, hypoxic inducible factor; MCT, monocarboxylate transporters.
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(e.g. adipsin), lipid metabolism (e.g. cholesteryl ester transfer protein), blood pressure control (e.g. angiotensinogen), haemostasis (e.g. plasminogen-activating inhibitor-1) and angiogenesis (e.g. vascular epithelial growth factor). Maintaining an appropriate amount of adipose tissue is essential for optimal health. Outside ‘normal’ levels the resultant imbalance in adipokine production leads to adverse health consequences; a result of having too little (anorexia, cachexia), too much (obesity) or an inappropriate redistribution of adipose tissue (lipodystrophy).

The excessive expansion of adipose tissue mass is the distinctive feature of obesity. There has been an unprecedented rise in obesity, the incidence increasing >4-fold in the UK alone over the last three decades. The recently published Foresight report from the UK Government has indicated that at the present rate of increase (including the rise in childhood obesity) between 50% and 60% of the population could be expected to be obese by 2050. To compound the immediate problems of obesity per se (shortness of breath, back pain etc.), there is an increase in the risk of the co-morbidities associated with obesity, and more particularly of central obesity. These co-morbidities include insulin resistance (predisposing to type 2 diabetes mellitus), hypertension and CVD, which together form the cornerstones of the metabolic syndrome. There also appears to be a greater risk of developing certain cancers, including breast and colon cancer.

Obesity is characterised by a state of chronic low-grade inflammation. The elevated levels of circulatory pro-inflammatory cytokines (such as IL-6, TNFα) and acute-phase proteins (such as C-reactive protein) have been suggested as a causative link between obesity and its secondary complications, particularly insulin resistance. Importantly, some of these inflammatory mediators are also recognised as adipokines, thus consolidating an earlier phase proteins (such as C-reactive protein) have been suggested as a causative link between obesity and its secondary complications, particularly insulin resistance. Importantly, some of these inflammatory mediators are also recognised as adipokines, thus consolidating an earlier phase of acute inflammation (9–12). The elevated levels of circulatory pro-inflammatory cytokines and acute-phase proteins (such as C-reactive protein) have been suggested as a causative link between obesity and its secondary complications, particularly insulin resistance. Importantly, some of these inflammatory mediators are also recognised as adipokines, thus consolidating an earlier phase of acute inflammation.

The hypoxia hypothesis

White adipose tissue is generally considered to be poorly vascularised. In 2002 it was demonstrated that angiogenesis is required for adipose tissue expansion. The following year, a study using a mouse cell line suggested that a hypoxic environment may be responsible for promoting the expression of angiogenic genes (vascular epithelial growth factor), thus promoting the formation of new blood vessels during adipose tissue growth. Furthermore, studies from two independent groups demonstrated that the human leptin gene is activated by hypoxia. These earlier observations formed the basis of a hypothesis proposed in 2004 that hypoxia is the key initiator in adipokine dysregulation in obesity, thereby inducing an inflammation response within adipose tissue. More precisely, localised hypoxia was suggested to develop in expanding adipocytes furthest removed from the blood supply. This proposal was enhanced by the observation that blood flow to white adipose tissue is not elevated in obese subjects, nor in contrast to lean subjects it is increased postprandially. Importantly, hypertrophic adipocytes in obese subjects can reach a size of approximately 150–200 μm in diameter, thus exceeding the normal O2 diffusion distance of 100–200 μm.

Hypoxia, defined as a deficiency of O2 in tissues, is more often associated with high altitude, ischaemic injury or tumour development. Cellular adaption to hypoxia is a highly-conserved evolutionary defence mechanism adapted by cells to conserve O2 for vital metabolic functions when levels are low or dramatically reduced. This response to low O2 levels is accomplished through the activation of specific transcription factors. The best known of these factors is hypoxic inducible factor (HIF)-1. The key element of this heterodimer is the subunit HIF-1α, which is considered to be the molecular oxygen sensor. When cellular O2 levels are sufficient, this protein is continuously synthesised but is immediately targeted for proteasome degradation by the involvement of activated prolyl hydroxylase domain oxygenases, which hydroxylate HIF-1α to provide sites for binding of the von Hippel-Lindau protein. Further hydroxylation occurs and prevents binding of the cAMP-binding protein-binding protein/p300 subunit co-factor. The presence of von Hippel-Lindau protein enables ubiquitination, thus targeting HIF-1α to the proteasome (summarised in Fig. 1). Under low O2 levels the
Hypoxia and adipose tissue

Support for the hypoxia hypothesis was provided in 2007 by the demonstration that adipose tissue is in a hypoxic state in mouse models of obesity, both genetic and diet-induced\(^\text{(40,41)}\). It was shown using \(O_2\) electrodes that the interstitial \(O_2\) partial pressure is lower \textit{in vivo} in two different fat depots of \(ob/ob\) mice\(^\text{(41)}\). The levels of \(O_2\) partial pressure reported for both lean and obese adipose tissue (48 and 15 mmHg respectively) are within the range of values described for general tissue oxygenation (40–50 mmHg) and tissue considered to be existing in a hypoxic state (e.g. retina; 2–15 mmHg)\(^\text{(31)}\).

A second technique that was used to establish the presence of hypoxia is that of a pimonidazole hydrochloride stain, again in mouse obesity models. This dye is able to perfuse into tissue and forms protein adjuncts in areas in which the \(O_2\) tension is <10 mmHg (about 1% (v/v) \(O_2\)). It was found that pimonidazole hydrochloride staining of adipose tissue from \(ob/ob\) mice is apparent when compared with lean littermates, as is the increased abundance of the probe measured by immunoblotting techniques indicating the presence of hypoxia\(^\text{(40,41)}\). Importantly, there is a substantial induction of HIF-1\(\alpha\) protein in the hypoxic adipose tissue of the obese animals\(^\text{(40,41)}\). A very recent report has suggested that mild hypoxia is also evident in adipose tissue of obese human subjects\(^\text{(42)}\).

Hypoxia and adipocytes

In order to address the concept that there is a link between hypoxia and inflammatory-related adipokines, studies in Liverpool have focused on human adipocytes using primary culture systems. Exposure of mature adipocytes to 1% (v/v) \(O_2\) has been found to induce the expression of HIF-1\(\alpha\) protein as early as 4 h, and importantly this induction is rapidly reversible; HIF-1\(\alpha\) protein levels return to basal levels within 10 min of transferring adipocytes to control \(O_2\) conditions (21% (v/v) \(O_2\))\(^\text{(43)}\). Both the mRNA and protein levels of a key hypoxia marker, the facilitative glucose transporter GLUT-1, have been found to substantially increase, indicating that these cells are indeed responsive to a hypoxic environment\(^\text{(43,44)}\). Using a candidate gene approach mRNA levels of fasting-induced adipose factor/angiotensin-like protein 4, IL-6, leptin, macrophage migration inhibitory factor, plasminogen-activating inhibitor-1 and vascular endothelial growth factor have been found to increase substantially\(^\text{(43)}\). These increased mRNA levels are accompanied by increased protein secretion in the case of IL-6, leptin, macrophage migration inhibitory factor and vascular epithelial growth factor. Furthermore, adiponectin gene expression and protein secretion decrease.

Similar results have generally been observed when cells are treated with the hypoxia mimetic CoCl\(_2\), which is able to stabilise HIF-1\(\alpha\) under normal \(O_2\) levels by inactivating the cellular prolyl hydroxylase domain hydroxylases\(^\text{(43)}\). Each of the hypoxia-sensitive genes has been found to be dependent on HIF-1 activation, with the exception of IL-6, which appears to be HIF-1 independent. Interestingly, expression of the key pro-inflammatory cytokine TNF\(\alpha\) is not hypoxia sensitive. Overall, these findings on human adipocytes parallel those observed for mouse 3T3-L1 and 3T3-F442A fat cells\(^\text{(40,41,45)}\) and imply that hypoxia may be the underlying cause of tissue inflammation and cellular dysfunction in obesity.

The response of human adipocytes to hypoxia has been further investigated using a commercial high-throughput real-time PCR strategy (PCR arrays). A collection of eighty-four genes related to hypoxia signalling were used to screen human adipocytes subjected to hypoxia for 24 h\(^\text{(46)}\). Of the twelve genes that were found to be up regulated, including those genes identified using the candidate gene approach described earlier (leptin, vascular epithelial growth factor etc.), the largest change that was observed was that for a previously-unreported adipocyte-related gene, metallothionein-3, the mRNA level of which was found to increase >600-fold over 24 h compared with untreated cells. This response is selective for this specific member of the metallothionein gene family, as the related gene, MT-2A, is minimally affected by hypoxia\(^\text{(46)}\). The role of metallothionein-3 in adipocytes is still unclear, but the gene is highly expressed in the brain and is thought to protect the cells against hypoxic damage\(^\text{(47)}\).

Hypoxia and adipocyte metabolic function

The metabolic consequence of reduced cellular \(O_2\) levels within the adipocyte extends beyond that of altered adipokine expression. One of the initial steps in the response of cells to hypoxia is adaptation at the mitochondrial level by both reducing the amount of the high \(O_2\)-consuming

prolyl hydroxylase domain enzymes are inactivated, thus stabilising HIF-1\(\alpha\) by prevention of von Hippel-Lindau protein binding and enabling binding of the cAMP-binding protein-binding protein/p300 subunit co-factor.

The second subunit, HIF-1\(\beta\), a constitutively-expressed protein that is \(O_2\) insensitive, is able to bind to HIF-1\(\alpha\) and form the active transcription factor. HIF-1 is involved in the activation of over seventy genes directly via binding to cis-acting hypoxic response elements; these genes involve a multiplicity of functions including angiogenesis, glucose metabolism, apoptosis, cellular stress, extracellular matrix re-modelling and inflammation\(^\text{(35)}\).

Three \(\alpha\) subunits have been described, each being derived from a different gene. Whereas the main focus has been on HIF-1\(\alpha\), attention has recently been given to HIF-2\(\alpha\). The \(\alpha\) subunit forms the transcription factor HIF-2, expression of which appears to be more tissue selective than HIF-1 and may respond at different cellular \(O_2\) levels\(^\text{(36)}\). Little is known about HIF-3\(\alpha\) and its splice variants, but there is some suggestion that one of these variants may act as a negative regulator\(^\text{(45)}\). In addition to HIF-2, other transcription factors have been shown to mediate responses to low cellular \(O_2\) levels, including NF-xB and cAMP-response element-binding protein\(^\text{(38,39)}\). NF-xB is more readily recognised as mediating inflammatory signalling pathways, such as those associated with TNF\(\alpha\), but would also appear to play a role in hypoxia, particularly intermittent hypoxia\(^\text{(39)}\).
process of oxidative phosphorylation and simultaneously improving its efficiency (48). The main site of O₂ consumption is at complex IV, comprising cytochrome c oxidase subunit 4. However, this process is not fully efficient and some leakage can occur at complex III, resulting in the generation of reactive oxygen species. Under low O₂ conditions the mitochondrial protease LON is up regulated and acts to degrade the cytochrome c oxidase subunit 4–1 subunit, which is subsequently replaced through the up-regulation of cytochrome c oxidase subunit 4–2; this subunit is more efficient for the consumption of O₂ (48). These events have also been demonstrated with human adipocytes (B Wang, IS Wood and P Trayhurn, unpublished results).

As a result of reduced oxidative phosphorylation the cell switches to anaerobic glycolysis for its energy production. However, in order to compensate for the lower efficiency of ATP production, there is an increase in demand for glucose via up-regulation of GLUT-1 expression and insertion into the plasma membrane. As mentioned earlier, GLUT-1 is considered to be a molecular marker for cellular hypoxia. Indeed, the levels of GLUT-1 mRNA and protein have been shown to increase in human adipocytes in response to low O₂ tension (43,44). Furthermore, an increase in glucose transport has been observed in hypoxic human adipocytes (44), and more recently also in murine fat cells (49,50). What is not clear from the measurement of glucose uptake is the extent to which other glucose transporters may be involved in the response to hypoxia. Adipocytes have been shown to express a range of other transporters, gene expression has been found to be increased for GLUT-3 and GLUT-5 family (51,52). Of these transporters, GLUT-1 is considered to be a molecular marker for cellular hypoxia (53,54). Of these transporters, gene expression has been found to be increased for GLUT-3 and GLUT-5 (fructose transporter) in human adipocytes cultured in 1% (v/v) O₂ for 24 h. On the other hand, GLUT-4, GLUT-10 and GLUT-12 mRNA levels are unchanged (44).

Western blot analysis has shown that the protein abundance for GLUT-4 is also unchanged (44). However, this outcome may reflect the presence of GLUT-4 mainly as intracellular stores that readily move to the plasma membrane in response to insulin. Hence, determination of total cellular GLUT-4 abundance in adipocyte cultures will not reveal whether GLUT-4 is involved in hypoxia-induced glucose uptake. Studies in muscle cells have shown that hypoxia can induce rapid translocation of GLUT-4 to the cell surface using mechanisms distinct from the insulin signalling pathway but similar to that observed with exercise-induced translocation of GLUT-4 (53,54). However, initial studies in the authors’ laboratory, using subcellular fractionation, have suggested that in human adipocytes GLUT-4 remains within the intracellular stores for between 1 and ≤24 h of hypoxia treatment; furthermore, there is no acute increase in glucose transport following ≤4 h of hypoxia (IS Wood and P Trayhurn, unpublished results).

A further consequence of the switch to anaerobic glycolysis is an increase in lactic acid production, which has to be rapidly exported from the cell; in the case of human adipocytes it has recently been found that hypoxia leads to a stimulation in lactate release (F Pérez de Heredia, IS Wood and P Trayhurn, unpublished results). The monocarboxylate transporters (MCT) are a large gene family comprising fourteen members, four of which (MCT-1–MCT-4) are recognised to transport lactate (55). A recently reported study that used gene promoter analysis has found that MCT-4, but not MCT-1, transcription is increased under hypoxic conditions in HeLa cells (56). Studies have found that several MCT are expressed in human adipocytes and that expression of both MCT-4 and MCT-1 is increased in response to hypoxia and is apparently HIF-1 dependent; interestingly, MCT-2 mRNA levels are decreased (F Pérez de Heredia, IS Wood and P Trayhurn, unpublished results). This finding would indicate that MCT are an important component of the increased glucose utilisation that occurs during the adaptation of adipocytes to a hypoxic environment.

Hypoxia and insulin resistance

The inflammatory state associated with obesity is considered to be a causal event in the development of insulin resistance (57), to which adipose tissue dysfunction is considered to be a major contributor. Two adipokines implicated in the development of insulin resistance are IL-6 and adiponectin, the production of which increases and falls respectively in obesity and in response to hypoxia (40,41,43). This finding has raised the question of whether hypoxia per se may be an underlying cause of insulin resistance. Indeed, recent studies have found that in mouse adipocytes subjected to 1% (v/v) O₂ for 16–24 h insulin-stimulated glucose transport is attenuated (49,50). Moreover, the insulin receptor-β and insulin receptor substrate-1 protein levels are reduced in obese mice and 3T3-L1 cells (50). Phosphorylation of insulin receptor-β and insulin receptor substrate-1 has been shown to be reduced in both mouse and human adipocytes via HIF-1-dependent mechanisms (49). These findings provide strong evidence for a central role for adipocyte tissue hypoxia in the induction of insulin resistance.

A closer examination has been carried out of the role of GLUT-4 in the induction of insulin resistance, based on the primary observations that in obese subjects GLUT-4 protein levels decrease in adipose tissue, although not in skeletal muscle (58), and that ablation of GLUT-4 in adipose tissue-specific knock-out mice results in whole-body insulin resistance (muscle and liver) (59). The effects of prolonged (chronic) exposure of adipocytes in culture to hypoxia have been investigated with the view that this approach would better represent the situation for adipose tissue in obesity. Previous studies have found that adipocyte GLUT-4 mRNA and protein levels are unchanged for ≤24 h exposure to 1% (v/v) O₂ (44). However, by 48 h GLUT-4 mRNA levels fall (56) and initial indications are that GLUT-4 protein abundance is reduced in both mouse and human adipocytes following 2–4 d exposure to 1% (v/v) O₂ (F Pérez de Heredia, IS Wood and P Trayhurn, unpublished results). Further studies are underway to determine whether disturbance of GLUT-4 levels in adipose tissue as a result of hypoxia may contribute to the insulin resistance state associated with obesity.
Hypoxia and non-adipocyte cells

Other cellular components of adipose tissue include resident macrophages and preadipocytes, and these components may both contribute to the inflammatory response observed in adipose tissue. Indeed, preadipocytes have been found to exhibit a substantial response to inflammatory stimuli such as lipopolysaccharide (61), with the increased secretion of inflammatory adipokines (e.g. IL-6, monocyte chemoattractant protein 1). They have also been found to secrete increased levels of IL-1β and TNFα in response to leptin (62). Hypoxia has been shown to inhibit differentiation of mouse preadipocytes, probably as a result of a decrease in PPARγ levels (63–66). Consistent with this outcome are findings in human preadipocytes in which exposure to hypoxic conditions results in a fall in PPARγ gene expression, together with increased HIF-1α protein and GLUT-1 expression (67). The overall response of preadipocytes to hypoxia appears to be blunted compared with that of adipocytes, there being some marked differences such as a lack of response in angiopeitn-like protein 4 and IL-6 expression to low O2 tension.

Perhaps the most interesting finding from the study on preadipocytes is that hypoxia induces the expression and secretion of leptin (67), these cells are considered not to express leptin, which is a differentiation-dependent gene in adipocytes. However, hypoxia has been shown previously to increase leptin expression in other cell types (26, 68), including adipocytes (27, 40, 43, 46). These data imply that preadipocytes may contribute to a hypoxia-induced increase in leptin production by adipose tissue in obesity.

While the recruitment and infiltration of macrophages into obese adipose tissue have been recognised for several years, the mechanisms involved are still largely unknown. These cells are capable of increasing their inflammatory response in the face of hypoxia (69, 70), and more recently in the specific context of adipose tissue (41). The finding that macrophages are present around areas of hypoxic adipocytes in adipose tissue (71) may suggest that tissue hypoxia could provide a means of macrophage recruitment. The increased production of monocyte chemoattractant protein 1 (chemoattractant) and macrophage migration inhibitory factor-1 (prevention of macrophage release from tissue) have been proposed as key determinants of macrophage infiltration and enhanced inflammatory response (72, 73). Indeed, leptin has also been suggested as a macrophage chemoattractant (21, 74).

In addition to an endocrine role, it is evident that the majority of adipokines produced by adipocytes may also act in a paracrine and/or autocrine manner within adipose tissue (3, 75). Several studies have reported considerable inflammatory responses when cultured adipocytes and/or preadipocytes are treated with conditioned media used to culture macrophages (76–79). It is highly likely that induction of adipose tissue hypoxia may elicit an inflammatory response from each of the different cell types that is amplified and promulgated through cellular cross talk (see Fig. 2).

**Implications and perspectives**

It is evident that hypoxia can induce inflammatory responses in the different cell types contained within adipose tissue and that collectively these responses may underlie tissue dysfunction giving rise to the metabolic complications observed with obesity, consistent with the original hypothesis (3, 75). However, it is recognised that hypoxia may not necessarily be the only factor involved. It should be noted that alternative mechanisms have been proposed linking cellular stress and inflammation, i.e. oxidative stress (mitochondrial reactive oxygen species generation) (80) and endoplasmic reticulum stress (81, 82). Interestingly, hypoxia has also been shown to stimulate these two processes, suggesting that the various postulated mechanisms underlying inflammation are not necessarily mutually exclusive. Indeed, a modest increase in reactive oxygen species production, through mitochondrial leakage within the electron transport chain when O2 levels initially fall below optimal levels, has been suggested as one possible mechanism to inhibit the prolyl hydroxylase domain enzymes and induce HIF-1 stability in order to protect against further rises in reactive oxygen species production (48). Hypoxia has been shown to induce the expression of the CCAAT-enhancer-binding protein homologous protein and glucose-related protein 78 genes; these proteins are two markers of endoplasmic reticulum stress (40).

Obstructive sleep apnoea has received increased interest recently, mostly for its association with systemic inflammation and the development of insulin resistance (particularly in CVD), but also because obesity is recognised as a risk factor (83). The cessation of breathing for brief periods during sleep induces bouts of chronic intermittent hypoxia that has marked mechanistic similarities to that found with continuous hypoxia (as described earlier). The response to intermittent hypoxia appears to be mediated through the transcription factor NF-kB, typically recognised as a major regulator of the inflammatory response, and has been suggested as a link between obstructive sleep apnoea and insulin resistance (83). It is of interest to investigate whether systemic intermittent hypoxia has an influence on adipose tissue hypoxia.
The majority of studies have focused on hypoxia associated with increased fat mass through obesity. It is expected that hypoxia may also occur in other instances in which increased adiposity occurs, such as fat wrapping (associated with areas of mucosal inflammation in patients with Crohn’s disease) and pregnancy. These areas may provide opportunities to explore further the role of hypoxia under conditions not necessarily linked to overnutrition.

Therapeutic intervention in obesity has focused on the direct causes of the positive energy balance. However, targeting the consequences of obesity is important and may be more fruitful. From this perspective inflammation in adipose tissue may be a key target for treating the consequences of obesity. The recognition that hypoxia is found in obese adipose tissue presents new opportunities to reduce the adverse health consequences of a chronic inflammatory status. However, careful dissection of the intricate pathways will be required to provide specific targets to avoid unwanted interference with other essential systems.

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References


