The Annual Charity Meeting was held at the Royal College of Nursing, London, on 9 September 2019

Gowland Hopkins Award Lecture

Through fat and thin – a journey with the adipose tissues

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The paper is based on the lecture that I gave on receiving the Nutrition Society's inaugural Gowland Hopkins Award for contributions to Cellular and Molecular Nutrition. It reviews studies on the adipose tissues, brown and white, conducted by the groups that I have led since entering nutrition research in 1975. The initial focus was on exploring metabolic factors that underpin the development of obesity using animal models. This resulted in an interest in non-shivering thermogenesis with brown adipose tissue being identified as the key effector of facultative heat production. Brown fat is less thermogenically active in various obese rodents, and major changes in activity are exhibited under physiological conditions such as lactation and fasting consistent with a general role for the tissue in nutritional energetics. My interests moved to white adipose tissue following the cloning of the *Ob* gene. Our initial contributions in this area included demonstrating nutritional regulation of *Ob* gene expression and circulating leptin levels, as well as a regulatory role for the sympathetic nervous system operating through β_3-adrenoceptors. My interests subsequently evolved to a wider concern with the endocrine/signalling role of adipose tissue. Inflammation is a characteristic of white fat in obesity with the release of inflammation-related adipokines, and we proposed that hypoxia underlies this inflammatory state. O_2-deprivation was shown to have substantial effects on gene expression and cellular function in white adipocytes. The hypoxia studies led to the proposition that O_2 should be considered as a critical macronutrient.

Adipokines: Brown adipocyte: Hypoxia: Oxygen: White adipocyte

Background

I am greatly honoured to receive the Gowland Hopkins Award from the Nutrition Society, and indeed to be the first recipient. I never met Sir Frederick Gowland Hopkins OM PRS, who received the Nobel Prize for Physiology or Medicine in 1929 for the discovery of vitamins, being born a year after he died. There is, however, a tangential link in that my entry into nutrition from a basic science background in physiology and biochemistry came through joining the MRC Dunn Nutritional Laboratory in Cambridge in 1975. The Dunn had been founded in 1927 when Gowland Hopkins was Professor of Biochemistry in the University, and the original intent was that he should be directly involved in its research. In practice, because of extensive other commitments, including as President of the Royal Society, his primary role with respect to the Dunn was as an advisor and member of the Management Committee. Gowland Hopkins has, of course, a close association with the Nutrition Society as one of the principal figures behind its foundation.

Fig. 1. (Colour online) 'Engaging' with nutrition at the Dunn: Friday morning group 'coffee and cake'. Eating and drinking in the laboratory is, of course, prohibited now, but was normal in my early years as a scientist.

My research over the past 40+ years since entering nutrition has centred on the adipose tissues; first brown and then white. This began following my initial studies at the Dunn where I had been recruited by Philip James as a member of the then newly formed Energy Group (Fig. 1). The group had been established in recognition of obesity beginning to emerge as a public health problem. At the time, the incidence of obesity was considerably less than now; in 1980, for example, 6 % of adult males and 8 % of adult females in the UK were classified as obese[1] on the basis of a BMI ≥30, while by 2017 the figure was approximately 30 % of all adults (https://www.worldobesitydata.org/country-profiles/). Obesity is, of course, important primarily because of the increased risk of several associated diseases, particularly type-2 diabetes, hypertension, CHD and certain cancers[2,3].

The ethos prevailing when we began in the mid-1970s was that obesity is the product of 'gluttony and sloth', but our focus was on exploring whether there are important metabolic factors which underpin the development of the disorder. My remit was to investigate the fundamentals of the regulation of energy balance using animal models. The animal of choice was the genetically obese ob/ob (Lep^{ob}/Lep^{ob}) mouse, and a colony of the Aston strain of these mutants was set-up. The attraction of ob/ob mice, which at the time were the most widely used animal model in obesity research, was that not only is the obese state extreme with body weight being up to three times that of lean siblings but that it is reducible to a mutation in a single recessively inherited gene[4]. The link to a mutant gene meant that the obesity of ob/ob mice results from a change in just one protein, and that protein must play a critical role in the regulation of energy balance.

The ob/ob mouse is not, of course, the only rodent in which obesity is the result of a single gene mutation,

and we subsequently established colonies of the other major obese mutants: the Zucker fa/fa ($Lepr^{fa}/Lepr^{fa}$) rat and the diabetic-obese db/db ($Lepr^{db}/Lepr^{db}$) mouse[4]. We were later able to house colonies of the adipose mouse (Ad), golden hamsters ($Mesocricetus auratus$) and Djungarian hamsters ($Phodopus sungorus$), each as a specific model within our energy regulation studies. This was based on the August Krogh Principle that 'for a large number of problems there will be some animal of choice or a few such animals on which it can be most conveniently studied'[5]. As I have noted previously, the ability to maintain multiple colonies of experimental animals at the Dunn without direct cost to the investigator was remarkable[6].

Energy balance and thermogenesis

Hyperphagia is part of the basis for the obesity of the ob/ob mouse, and indeed that of the other obese mutants, food intake being greater than in lean siblings[4]. However, studies where young ob/ob mice were either directly pair-fed to the $ad libitum$ intake of their lean siblings, or otherwise given restricted amounts of food, indicated that obesity still develops without hyperphagia[7,8]. Our own work, in which full energy balance studies were performed, clearly illustrates the point; young ob/ob mice pair-fed to the $ad libitum$ intake of lean siblings at room temperature (23°C) exhibited a rate of energy deposition 2·3 times that of the lean[7]. The study was conducted at four different environmental temperatures: 33 (thermoneutral for the mouse), 28, 23 and 17°C. At each temperature, the energy gain of the obese animals was greater than the lean, but the lower the temperature the higher the excess gain[7].

The capacity for excess energy deposition in the absence of hyperphagia indicated that one or more components of energy expenditure is reduced in the ob/ob mutants. Of the main components of expenditure, facultative (or adaptive) non-shivering thermogenesis (NST) was particularly attractive as the key element. Not only is thermoregulatory thermogenesis a major part of total expenditure in small mammals in order to maintain body temperature, but reduced expenditure on thermogenesis was also being advocated by Miller and Stock as a causal factor in the development of obesity[9,10]. Furthermore, some 25 years earlier impaired homeothermy had been noted in ob/ob mice[11]. This appeared counter-intuitive given the improved insulation provided by the additional body fat of the obese animals, and was suggestive of a reduced capacity to generate heat. In our own studies, core temperature fell rapidly, to as low as 15°C, just 3 h after exposure of ob/ob mice to 4°C, whereas lean siblings maintained their temperature above 35°C[12].

Direct measurements of NST from the peak increase in the RMR at thermoneutrality following the administration of noradrenaline indicated that the capacity for this form of heat production was 2-fold lower in ob/ob mice than that in lean siblings[12]. Furthermore, RMR expressed 'per animal', as should be done in energetic

studies, was reduced in the obese mice relative to the lean at every temperature examined below thermoneutrality, indicating a lower expenditure on NST[12].

Brown adipose tissue

An immediate question raised by these physiological studies was the nature of the molecular and cellular mechanisms of NST. Several possibilities were under consideration at the time, including protein turnover, the α-glycerophosphate shuttle, Na$^+$ transport across the plasma membrane mediated by Na$^+$-K$^+$ATPase, and futile/substrate cycles such as that between fructose-6-phosphate and fructose-1,6-bisphosphate[13–19]; in practice, most are in effect a form of energy-consuming substrate cycle. There were, however, substantial reservations with each of these mechanisms[20] in that it seemed unlikely that they had the potential to generate sufficient quantities of heat and to do so acutely without disrupting normal metabolic control. In addition, there was a central issue of tissue localisation; several of the mechanisms, particularly protein turnover and Na$^+$ transport, being essentially universal rather than restricted to a specific tissue site.

The question of the tissue basis for NST subsequently centred on brown adipose tissue (BAT, or brown fat), which had first been described by Conrad Gessner in 1551. Although different roles had been proposed for this tissue, including as an endocrine organ, the principal function was resolved in the early 1960s, as a thermogenic organ with heat as the primary product[21]. The tissue is prominent in hibernating species, in the newborn of many mammals (including human subjects) and in rodents acclimated to the cold[22,23]. The quantitative importance of BAT in adaptive NST in rodents was demonstrated in influential studies by Foster and Frydman[24–26]. These authors mapped regional blood flow to different tissues using radioactively-labelled microspheres in rats in which NST was maximally stimulated following either cold-acclimation or the administration of noradrenaline. From the measurements of regional blood flow, together with the cardiac output and the oxygen extraction across the interscapular depot, BAT was estimated to account for 60 % of NST in cold-acclimated rats[25]. Our own studies on mice using the same approach suggested a broadly similar figure[7].
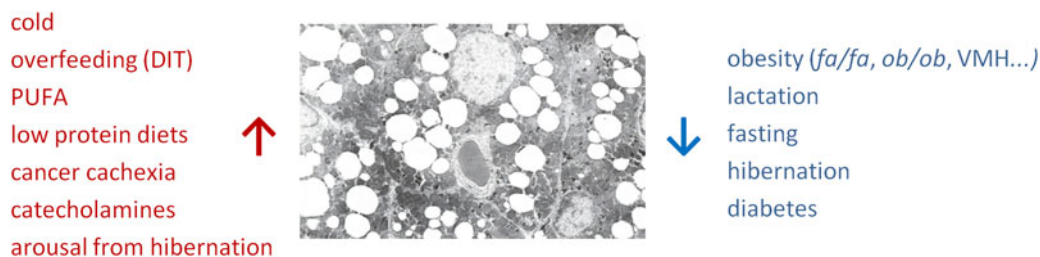
In parallel with the identification of BAT as the principal locus for NST, the unique bioenergetic properties of the tissue's mitochondria were being elucidated. Heat was shown by Nicholls to be generated by a regulated uncoupling of oxidative phosphorylation, the energy inherent in the proton gradient across the inner mitochondrial membrane being dissipated as heat rather than coupled to ATP synthesis[22]. This process is controlled by the $32\,000\text{-}M_r$ mitochondrial uncoupling protein-1 (UCP1) discovered by Ricquier[22,27]. Acute stimulation of BAT thermogenesis leads to an activation of UCP1, while chronic stimulation results in an increase in the amount of the protein, through a combination of a higher concentration in the mitochondria and through mitochondriogenesis[23]. These changes, both acute and chronic, are primarily driven by the release of noradrenaline from the extensive sympathetic innervation of BAT, acting mainly via β₃-adrenoceptors[28,29].

My group at the Dunn, in parallel with several other groups, began to explore the potential role of BAT in energy balance and the development of obesity. Two seminal observations were pivotal; in the first, Himms-Hagen and Desautels in Ottawa demonstrated reduced GDP binding to BAT mitochondria in *ob/ob* mice relative to lean siblings, this reflecting a reduction in the thermogenic proton conductance pathway of the tissue[30]. Our own blood flow studies indicated that the reduced NST and consequent lower energy expenditure of the obese mutant is entirely due to decreased metabolic activity in BAT[31]. In the second key study, Rothwell and Stock in London proposed that BAT is the locus of the diet-induced thermogenesis that they were observing in rats overfed through the provision of a cafeteria diet[32]. In follow-up studies with our group, key molecular indices of BAT thermogenic activity were demonstrated in the cafeteria-fed animals; increased mitochondrial mass and GDP binding, as well as GDP-sensitive respiration[33].

These initial reports were followed by a series of studies in which the thermogenic activity of BAT was shown to be reduced in a variety of obese rodents. They included other single gene mutants, *fa/fa* rat and *db/db* mouse, and rodents with experimentally-induced obesity such as that following lesioning of the ventromedial hypothalamus, the administration of gold thioglucose, and treatment with corticosteroids[20,34,35]. Along with the studies on obese animals, the role of BAT in nutritional energetics was further explored in a range of physiological and pathophysiological situations in which body fat and energy flux change (Fig. 2). These included the reproductive cycle (pregnancy and lactation) hibernation, photoperiod, cancer cachexia and nutritional manipulations such as fasting/refeeding and the provision of a low protein diet[34,35].

Lactation was a physiological stress of particular interest to us at the Dunn. The energy cost of lactation is high in small mammals, and energy intake is increased approximately 3-fold in lactating rats, for example, compared to virgin animals[36,37]. Our studies in mice showed that BAT thermogenesis is suppressed in lactation, the suppression being maximal in late lactation when milk production peaks[38–40]. Mitochondrial mass and GDP binding are both markedly reduced in BAT of lactating mice, the latter to the same level as virgin animals at thermoneutrality[38,39]. The concentration of UCP1 in the mitochondria is also reduced relative to that of virgin mice, with the total UCP1 content of the interscapular pad at late lactation being <10 % of the virgin animals (Fig. 3)[39]. These changes in BAT activity effectively lead to a substantial energy saving, helping to meet the high energy cost of milk production. However, this adaptation essentially reflects the limited scope for heat dissipation in the face of the high metabolic heat generation associated with milk synthesis rather than a specific

cold
overfeeding (DIT)
PUFA
low protein diets
cancer cachexia
catecholamines
arousal from hibernation

obesity (*fa/fa, ob/ob,* VMH...)
lactation
fasting
hibernation
diabetes

Fig. 2. (Colour online) Schematic of different physiological and pathological conditions in experimental animals in which energy flux and/or balance are altered where increases, or decreases, in brown adipose tissue thermogenesis have been demonstrated. Examples of key situations in which brown fat thermogenesis changes are shown. DIT, diet-induced thermogenesis; VMH, ventromedial hypothalamus.
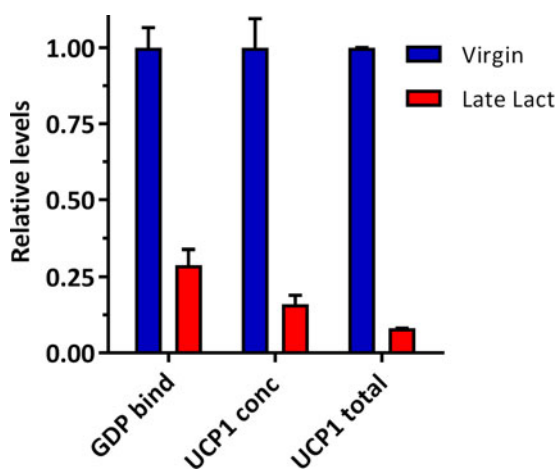
Fig. 3. (Colour online) The thermogenic activity and capacity of brown adipose tissue (BAT) is decreased in lactation (Lact). The changes in mitochondrial GDP binding, the mitochondrial concentration of uncoupling protein-1 (UCP1; UCP1 conc) and the total UCP1 content of the interscapular BAT depot are shown for mice at late lactation (when milk production is close to maximal) relative to virgin mice (virgin = 1)[39].

energy saving mechanism as such, as convincingly argued in a recent review on BAT in lactation[40].

Perhaps the strongest illustration of the link between energy expenditure and BAT thermogenesis comes from the changes in the tissue that occur when small rodents are acclimated to the cold. In mice, energy expenditure and food intake are increased 3-fold between thermoneutrality and 4°C, reflecting the energy cost of generating heat for homoeothermy[12,41]. In rats acclimated at 4°C, the mitochondrial content, mitochondrial GDP binding and UCP1 concentration were each substantially higher than in rats acclimated to thermoneutrality (29°C), while the total UCP1 content of the interscapular BAT depot was increased >100-fold[42].

Brown adipose tissue in human subjects

By the beginning of the 1990s, the importance of BAT in nutritional energetics had been firmly established across a range of obesity models and under other conditions in experimental animals in which energy flux is altered. In the case of human subjects, interest in the tissue had been driven to a considerable extent by the concept that reduced thermogenesis is a key factor in the development of obesity in human subjects and that BAT is a potential therapeutic target for the treatment of the disorder.

Although brown fat was widely recognised to be an important locus of heat production in the human neonate, the tissue appeared, on the basis of histological appearance, to be absent after the first few years of life. The presence of BAT in adult human subjects was confirmed, however, by immunological studies identifying UCP1 in fat depots, including in some elderly subjects[43–45]. In addition, expression of the *UCP1* gene was evident through detection of the encoded mRNA[46]. Activation of the tissue in patients with phaeochromocytoma was also demonstrated[47,48].

Despite the clear evidence for the presence of BAT in adults, with the capacity for adaptive changes, the prevailing view was that the tissue was of little, or no, significance in human energetics other than in neonates and during the first years of life. Interest in BAT then declined markedly, with the notable exception of those groups (particularly that of Cannon and Nedergaard in Stockholm[23]) whose principal focus was on understanding the fundamental biology of the tissue. Since 2009 there has, however, been renewed interest in BAT in human subjects following the application of fluorodeoxyglucose positron emission tomography[49–51]. This has firmly demonstrated active BAT in adults, activity being reduced in obesity and with ageing, for example, while being stimulated on cold exposure and by the administration of a selective β_3-adrenoceptor agonist[49–55].

White adipose tissue: the discovery of leptin

As interest in BAT declined, my own research focus changed and abruptly so following the cloning of the *Ob* (*Lep^ob*) gene and the identification of the encoded protein[56]. Within days of the report in *Nature* on 1 December 1994, my group at the Rowett in Aberdeen (where I had relocated in 1988) had designed and validated oligonucleotide probes to examine *Ob* gene expression. This move reflected the fact that some 2 years earlier a consortium of us in the UK, which included
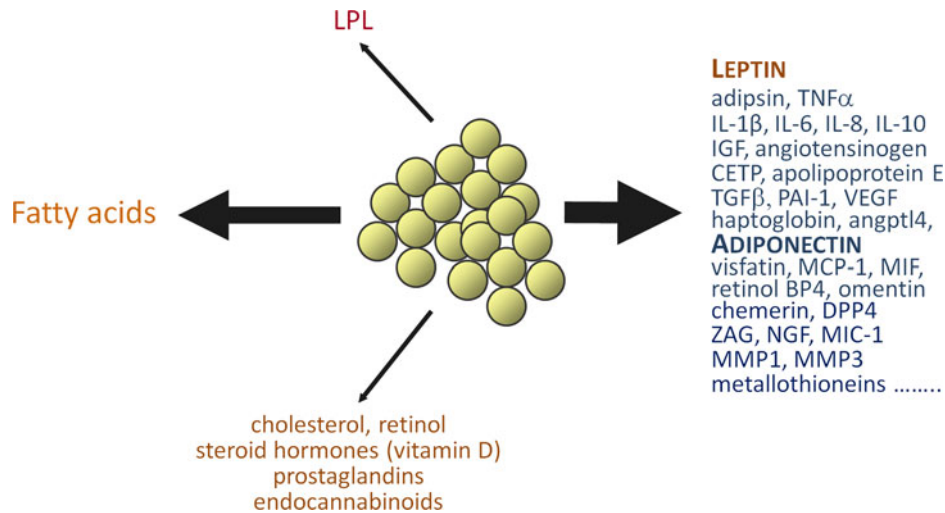
Fig. 4. (Colour online) The secretome of white adipocytes. Fatty acids and other lipids are secreted, together with a multiplicity of adipokines (proteins); examples of some of the lipids and key adipokines are shown. The major adipocyte hormones, leptin and adiponectin, are highlighted. angptl4, angiopoietin-like protein-4; CETP, cholesteryl ester transfer protein; DPP4, dipeptidyl peptidase-4; IGF, insulin-like growth factor-1; LPL, lipoprotein lipase; MCP-1, monocyte chemoattractant protein-1; MIC-1, macrophage inhibitory cytokine-1; MIF, macrophage migration inhibitory factor; MMP, matrix metalloproteinase; NGF, nerve growth factor; PAI-1, plasminogen activator inhibitor-1; RBP4, retinol binding protein-4; TGFβ, transforming growth factor-β; VEGF, vascular endothelial growth factor; ZAG, zinc-α_2-glycoprotein.

Michael Stock and John Stirling, had sought funding to identify the defective genes in the obese mouse mutants. We were, however, unsuccessful since it was argued (correctly) that at least one group in the United States was well-advanced in the goal and that it was unlikely that we could be competitive. My response was that once the *Ob* gene had been identified, our strategy would be to explore the physiology of the protein product.

The *Ob* gene was reported to be expressed in white adipose tissue (WAT) and the protein, initially termed 'OB', and then leptin, to act as a lipostatic signal[56–58]. Subsequently, the hormone was found to be produced by several tissues, including BAT[59] and the placenta[60], although WAT is the major source. Similarly, the functions attributed to leptin quickly expanded and it became regarded as a pleiotropic factor[61]. The early studies of my group at the Rowett demonstrated that expression of the *Ob* gene is nutritionally regulated, the mRNA level in WAT of lean rodents rapidly decreasing on fasting with a restoration on refeeding[62]. The circulating levels of the hormone change in parallel with the alterations in gene expression[63].

We then showed that acute exposure of mice to cold led to a strong inhibition of *Ob* expression, and a fall in the circulating leptin level, both of which are rapidly reversed on return to a warm environment[64,65]. The cold-induced reduction in the *Ob* mRNA level was mimicked by the administration of noradrenaline and by the β-adrenoceptor agonist isoprenaline. From these observations we proposed that the sympathetic system plays a key role in the regulation of *Ob* gene expression[64]. Subsequent observations indicated that this operates primarily through β3-adrenoceptors[65,66]. Further studies

on leptin at the Rowett included the demonstration by *in situ* hybridisation that the receptor, and particularly the long form responsible for signalling, is strongly expressed in the regions of the hypothalamus, consistent with being an adipocyte-derived signal for appetite[67].

Adipokines and the secretory function of white adipocytes

Leptin quickly became a major area in research on obesity and its associated disorders. One of the key outcomes of the discovery of the hormone was a radical change in perspective on the functions of white adipocytes and therefore of WAT itself. Adipocytes were recognised as endocrine cells with WAT as a major signalling organ[68–71]. Although secreted protein factors had been identified previously, this had not led to the conceptualisation of white adipocytes as endocrine and signalling cells. The secreted proteins known prior to leptin were adipsin (complement factor D)[72], which is a serine protease, the cytokine TNFα[73], and lipoprotein lipase. Lipoprotein lipase is, of course, released from adipocytes to catalyse the breakdown of circulating TAG to enable the uptake of fatty acids into adipocytes; it was not, however, regarded as a fat cell secretory protein as such.

The secretome of adipocytes, and of WAT as a whole, is extensive (Fig. 4). Quantitatively, fatty acids are the largest secretory product, but there are several other lipid groups released from the cells. Some, such as specific prostaglandins and the endocannabinoid anandamide, are synthesised *de novo* within adipocytes, while others,

including cholesterol and vitamin A, are taken up, stored and subsequently released[61]. A question raised by the discovery of leptin was whether there are a range of protein hormones and signals synthesised and secreted by fat cells. The answer is very much in the affirmative and one of the earliest of these adipokines, as they are termed, identified was another major adipocyte hormone, adiponectin, whose functions encompass insulin sensitising, angiogenic and anti-inflammatory actions (Fig. 3)[74–78].

The search for novel adipokines became a core focus of my group, both at the Rowett and later at the University of Liverpool to where I moved in 2002. Among the several adipokines that we discovered were: the neurotrophic signal nerve growth factor[79]; specific metallothioneins[80], these having metal binding actions, and the lipolytic/cachectic factor zinc-α_2-glycoprotein[81,82]. Nerve growth factor was found to be linked to the inflammatory response in WAT, secretion of the protein being strongly stimulated by TNFα[79]. As in mice, zinc-α_2-glycoprotein expression increased substantially in WAT of patients with cachexia associated with gastrointestinal cancer[83].

An extensive range of individual adipokines has now been identified, and proteomic studies and in silico analysis suggest that there are several hundred in total[84–86]. The wide-ranging secretory function of white adipocytes established over the past two decades has in part served as a model for other cell types which were not previously regarded as having a significant endocrine or signalling function. Myocytes, for example, are now known to release a range of protein signals, myokines[87,88], while another example is hepatocytes which secrete multiple hepatokines[89].

The identification of a multiplicity of protein signals and factors from adipocytes indicated that WAT is involved in a range of physiological and regulatory processes[61,70,90–92]. While some adipokines are endocrine in function, signalling to tissues and organs distant to the adipose depots, others have local paracrine and/or autocrine actions. The processes in which various adipokines play a role include appetite and energy balance, lipid metabolism, vascular haemostasis, blood pressure, angiogenesis and insulin sensitivity (see[61,91]). A number of adipokines are linked to immunity and inflammation, these including classical cytokines and chemokines such as IL-1β, IL-6, IL-10 and monocyte chemoattractant protein-1; they also include inflammation-related factors, examples being vascular endothelial growth factor (VEGF), serum amyloid A and adiponectin (see[61,70,91]).

In obesity, WAT exhibits chronic mild inflammation with increased production and release of inflammatory adipokines. There is a notable exception to this in that the synthesis and release of adiponectin, with its anti-inflammatory action, falls[93,94]. Inflammation in expanded WAT is augmented by the infiltration and activation of macrophages in particular, but also of other immune cells[92,95–97].

## Hypoxia and the metabolic response to oxygen deprivation in adipocytes

Inflammation in WAT has been considered a key factor in the development of the major obesity-associated disorders, particularly insulin resistance and the other components of the metabolic syndrome[3,70,98,99]. The question that intrigued me in the early 2000s was why does inflammation develop as adipose tissue mass expands? A 'News' article in Science on how cells endure low oxygen[100] encouraged me to consider the possibility that hypoxia might be a key. This was presented as a hypothesis in a Horizons article in the British Journal of Nutrition in 2004[90]. I am particularly proud of this paper: not only does it describe the hypoxia hypothesis, but it is my most highly cited publication (>1400 citations in the Web of Science; >2450 citations in Google Scholar) as well as being the fourth most highly cited article in the Nutrition Society's flagship journal (or indeed in all of its journals).

The hypothesis proposed that as adipose tissue mass expands with the development of obesity, areas within the tissue become relatively hypoxic as the enlarging adipocytes become more distant from the vasculature, this leading to major adaptive changes involving the hypoxia-inducible transcription factor-1 (HIF-1). The recruitment of HIF-1 was hypothesised to lead to increased expression of a series of hypoxia-sensitive genes linked to inflammation and the inflammatory response in WAT. The proposition was based on the following: (i) hypoxia occurs in situations such as ischaemic injury, wound healing and solid tumours leading to extensive metabolic changes[90], (ii) blood flow to WAT is not increased in obese subjects, despite the higher mass of the tissue[101–104], (iii) in contrast to lean subjects, blood flow to WAT does not increase post-prandially in the obese[104–106], (iv) large adipocytes (which may be up to 200 μm diameter[107]) are further from the vasculature than the normal diffusion distance for O_2 (100 μm)[108]. These observations refer to local hypoxia, but the provision of O_2 on a whole-body level is reduced in specific environmental and pathological situations, such as high altitude, deep sea dives, lung diseases and obstructive sleep apnoea[61,108].

In 2007, studies using two separate techniques reported that WAT depots in different types of obese mice are hypoxic, with the O_2 tension being 2- to 3-fold lower than in lean mice[109,110]. Subsequent studies on mice were consistent with these observations[111]. In contrast, although some human studies have indicated that WAT depots are relatively hypoxic[103,112] others have reported either the same or an increase in pO_2 (partial pressure of oxygen)[106,113]. The issue remains unresolved, but there is evidence that differences in the way in which O_2 is delivered in terms of vascularisation and utilisation may occur[106,113,114].

From 2004 the focus of my group in Liverpool was on examining the direct effects of hypoxia on gene expression and cellular function in adipocytes. Almost all of our studies were conducted on human adipocytes, differentiated in culture from fibroblastic preadipocytes. The initial priority was to examine whether incubation under a low O_2 tension leads to increased expression and release of inflammation-related adipokines consistent with our initial hypothesis. A candidate gene approach was employed and increased production of several adipokines
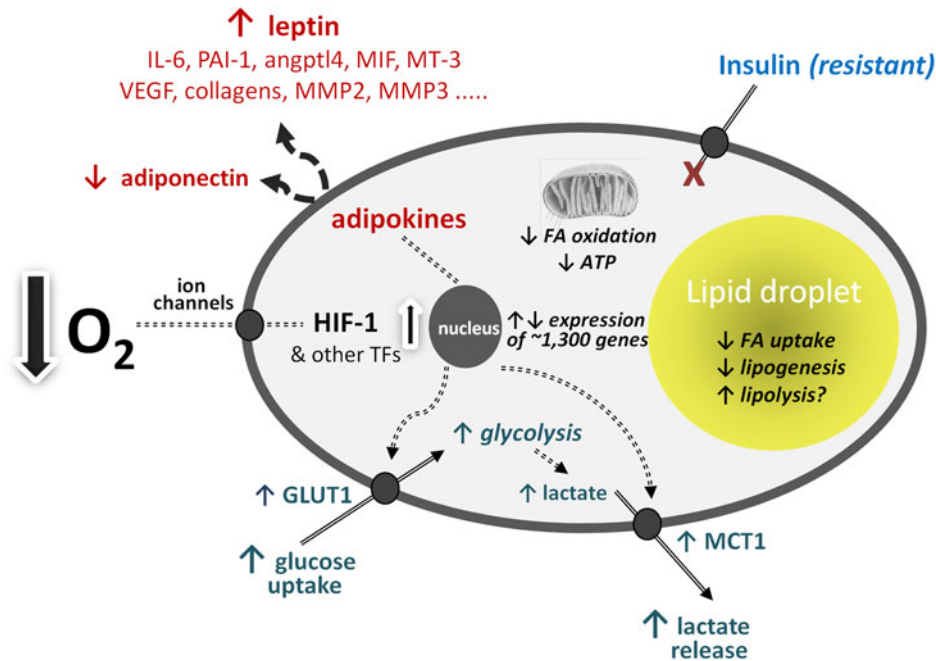
Fig. 5. (Colour online) Schematic representation of the central cellular responses to hypoxia in white adipocytes. The effect of low partial pressure of O_2 on gene expression, glucose uptake and utilisation, and the production of selected key adipokines is shown. angptl4, angiopoietin-like protein-4; FA, fatty acid; GLUT1, facilitative glucose transporter 1; HIF-1, hypoxia-inducible factor-1; MCT1, monocarboxylate transporter-1; MIF, macrophage migration inhibitory factor; MMP, matrix metalloproteinase; MT-3, metallothionein-3; PAI-1, plasminogen activator inhibitor-1; TF, transcription factors (other than HIF-1); VEGF, vascular endothelial growth factor. Modified from[131].

was observed, including IL-6, VEGF and leptin[115]. Raised production of VEGF and leptin, as well as of specific matrix metalloproteinases, had been reported earlier in 3T3-F442A adipocytes (a mouse cell line), reflecting a pro-angiogenic response to hypoxia[116].

The key cellular adaptation to O_2 deficiency is a switch from aerobic to anaerobic metabolism. Mitochondrial oxidative phosphorylation cannot, of course, continue when O_2 is severely limited, and there is instead increased anaerobic glycolysis. As expected, adipocytes exhibit greater glucose uptake under hypoxic conditions, as demonstrated by 2-deoxy-D-glucose uptake studies[117] and by measurement of glucose in the culture medium[116]. This is mediated through increased synthesis of the GLUT1 facilitative glucose transporter, driven by a marked stimulation of GLUT1 gene expression[117]. The expression of several genes encoding glycolytic enzymes is also raised, glucose-6-phosphate isomerase and phosphofructokinase, for example[118,119]. Lactate release is augmented in hypoxic adipocytes[116,120], reflecting the increased glucose utilisation and glycolytic flux, this being mediated by increases in the synthesis of the monocarboxylate transporter, MCT1[120].

While our initial exploration of the effects of hypoxia on gene expression in human adipocytes probed selective candidate genes, in subsequent studies more comprehensive approaches were taken. In the first of these, PCR arrays for eighty-four genes linked to the hypoxia-signalling pathway were employed. The expression of a

number of the genes changed, with one particular gene exhibiting dramatically increased expression[121]. The gene in question was MT3, which encodes a member of the metallothionein family, metallothionein-3 (also known as growth inhibitory factor). This protein binds zinc and copper, and linked to its marked induction by O_2-deprivation has been implicated as an angiogenic factor and to protect against hypoxic damage[122,123].

PCR arrays are themselves limited in terms of the number of genes whose expression can be screened and a specific pathway or metabolic system needs to be selected. DNA microarrays offer an unbiased approach in which all, or almost all, the genes expressed in a tissue or cell can be probed simultaneously. Our microarray studies at Liverpool, in collaboration with colleagues at Unilever, indicated that the expression of >1300 genes was altered in human adipocytes cultured under hypoxic conditions, stringent criteria being used to evaluate changes[119]. Of these genes, the expressions of approximately half were up-regulated and half down-regulated under low pO_2. Bioinformatic analysis showed that a number of metabolic pathways and functions are altered in human adipocytes by hypoxia, these including lipolysis, lipid oxidation, glucose utilisation, cell to cell signalling and cell death[119].

It is evident from these and other studies that hypoxia results in extensive changes in gene expression in adipocytes. Several important functional changes have been described, in addition to increased anaerobic glycolysis (Fig. 5). These include the rapid induction of insulin

resistance through the direct inhibition of insulin signalling[124,125], and the disruption of the extracellular matrix within WAT that characterises fibrosis[126,127]. With respect to fibrosis, hypoxia leads to changes in the expression of collagens released as components of the extracellular matrix, as well as matrix metalloproteinases[116,126] involved in tissue remodelling. The overall cellular response to low O_2 is regulated by a series of hypoxia-responsive transcription factors of which HIF-1, consisting of two subunits (HIF-1α and HIF-1β), is the best characterised[108,128,129].

Our studies, like most that examine the response of cells to hypoxia, were undertaken by comparing 1 to 20 % O_2 (95 % air/5 % CO_2; 'normoxia'). However, this is an extreme, effectively representing a comparison between ambient air (higher than arterial pO_2) and marked hypoxia. A question that interested me was whether there is a critical point at which adaptation to reduced O_2 is initiated in adipocytes, or if there is a gradual response to falling O_2 tension. Experiments in which human adipocytes were incubated with a range of O_2 levels between 20 and 1 % clearly demonstrated a dose-dependent response to lowering O_2 tension with differences being observed between 21 and 15 % and between 15 and 10 % O_2[130]. This was true for the expression and secretion of several adipokines, including leptin and VEGF, as well as 2-deoxy-D-glucose uptake and *GLUT1* gene expression. Nevertheless, changes tended to be more marked between 10 and 5 % O_2. Since the pO_2 in WAT of lean mice is equivalent to about 7 % O_2 while in obese mice it is about 2 %, it is evident that there are responses to O_2-deprivation over physiologically relevant differences in tissue oxygenation between the phenotypes[91].

These experiments on the effects of a range of O_2 levels demonstrate that while the customarily employed protocols in hypoxia studies offer proof of principle, they lead to an exaggerated view of the scale of the cellular response to relative O_2 lack under normal physiological conditions. This raises a question of the extent to which our understanding of cellular processes has been conditioned, or even distorted, by the routine use of 20 % O_2 as the gas phase in cell culture and other *in vitro* experiments. It is intriguing that careful attention is paid to the pH (7·4), temperature (37°C) and the concentration of glucose and other nutrients in cell culture to ensure physiological conditions' (except for when they are the parameters under investigation), but the O_2 tension employed is quite unphysiological and indeed reflects overt hyperoxia.

Oxygen: an overlooked macronutrient

A corollary of our studies on hypoxia is that they underscore that O_2 is a key nutrient at the cellular level. Indeed, investigation of hypoxia is in effect exploration of the molecular and metabolic consequences of the deficiency of a nutrient. However, O_2 is not considered as a nutrient as such in the context of nutritional science. Textbooks of nutrition do not contain sections on O_2, and reference to it is generally restricted to discussion of metabolic rate and respiratory quotient. I have argued recently that O_2 should be included alongside the other elements/molecules/macromolecules that are defined as nutrients[131,132].

O_2 undoubtedly meets dictionary definitions of a nutrient; for example, 'as a substance that provides nourishment for the maintenance of life and for growth' (*Oxford English Dictionary*). The central reason why O_2 is not considered as part of nutritional science is because of the route of entry; the nose/lungs in higher terrestrial animals, rather than the mouth/gastrointestinal tract. However, I argue that the route of entry should not be the critical determinant of whether O_2 is, or is not, considered a nutrient, but rather its function and essentiality[132]. O_2 is, of course, critical to all aerobic species without which mitochondrial oxidative phosphorylation cannot take place.

Early organisms developed under anoxic conditions, the level of O_2 in the atmosphere being just one part in a million soon after the Earth was formed some 4·54 billion years ago[133,134]. It was only after the initiation of the 'Great Oxidation Event' some 2·45 billion years ago that considerable amounts of O_2 began to appear in the atmosphere[134–136], the present level of 21 % being essentially stable over the past 600 million years[134]. The availability of O_2 in abundance in the atmosphere was critical to the evolution of life as we know it.

Conclusions

An odyssey with the adipose tissues that began for me over 40 years ago has provided much by way of riches and changed perspectives. The unique bioenergetic properties of BAT mitochondria, through the presence of the cell-specific UCP1, were initially thought to generate heat only in relation to temperature regulation. Subsequently, the link to energy balance was established and the tissue has provided a theoretical target for the treatment of obesity. BAT is also implicated in metabolic regulation more broadly than was originally envisaged, through roles in glucose homoeostasis and TAG clearance[55,137–140]. Whether it is a realistic target for the treatment of obesity and the metabolic syndrome, as many propose[55,137–140], remains a matter of continuing debate; my own view, as noted recently, is that there are formidable barriers to this concept[141].

Perspectives on the physiological role of WAT have changed radically since the discovery of leptin. An organ that appeared confined to fuel storage, a view reinforced by the histological structure with a single lipid droplet taking up most of the volume of mature white adipocytes, has emerged as having major endocrine and signalling functions. For specific adipose tissue depots there is good evidence of local impact in relation to the organs and tissues with which they abut[142]; examples are the epicardial fat, postulated to play a role in CVD[143,144], and dermal adipose tissue which is implicated in hair cycling and wound healing[145,146]. A specific role in relation to cancer and tumour microenvironment is also evident for some depots[147,148].

My research has centred throughout on what are traditionally considered to be BAT and WAT, both of which are defined by their respective signature cells. However, a third type of adipocyte is now recognised, namely the beige or brite cell[149,150]. Beige adipocytes have some, though not all, the characteristics of brown fat cells, and in particular are thermogenic through the presence of UCP1. Beige adipocytes are found predominantly within what are regarded as WAT depots and a number of factors lead to their recruitment, particularly cold exposure and β-adrenergic stimulation[151,152]. The complexity and diversity of fat cells may be even greater with a recent study reporting four distinct human adipocyte subtypes[153].

Although work on hypoxia has focused on WAT, with substantial changes in gene expression and function being demonstrated in white adipocytes, a deficiency in O_2 availability can also occur with BAT. BAT has an exceptionally high O_2 demand in order to fuel thermogenesis and hypoxia has been noted in the tissue of normal mice exposed to cold[154]. Hypoxia is not evident, however, in mice acclimated to a warm environment (30°C), and studies on *Ucp1* knockout animals indicate that it occurs only with thermogenesis[154]. Obese mice exhibit vascular rarefaction and a substantial reduction in pO_2 in BAT compared with lean mice, leading to a 'whitening' of the tissue together with mitochondrial dysfunction and loss[155].

From the effects on hypoxia on white adipocytes, it was stressed earlier that as cells are customarily incubated under hyperoxic conditions (20 % O_2) we may have obtained a somewhat distorted view of cellular processes. This may be true for many types of cells, including brown adipocytes. Finally, one of the implications with the response of white adipocytes to graded levels of O_2 is that cells carefully titrate small changes in the concentration of this critical nutrient and this results, as with other nutrients, in the continuous modulation of cellular function.

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