[Frontiers in Bioscience 16, 1589-1608, January 1, 2011]

Brown adipose tissue growth and development: significance and nutritional regulation

Michael Carey Satterfield, Guoyao Wu

Department of Animal Science, Texas A and M University, College Station, TX 77843-2471

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1. ABSTRACT

The last decade has witnessed a profound resurgence in brown adipose tissue (BAT) research. The need for such a dramatic increase stems from the ever-growing trend toward global obesity. Indeed, it is currently estimated that rates of obesity in developed countries such as the United States exceed 35% of the population (1). The higher incidence of obesity is associated with increased prevalence of the metabolic syndrome including diabetes, hypertension, and coronary heart disease, among others (1, 2). BAT holds great promise in combating obesity given its unprecedented metabolic capacity. Leading the way has been recent studies, which conclusively demonstrate significant quantities of functional BAT in adult humans (3-7). These findings have been complimented by elegant studies elucidating the developmental origin of the brown adipocyte and the transcriptional regulation involved in its differentiation. This review will attempt to meld the wealth of new information regarding BAT development with established literature to provide an up to date synopsis of what is known and thus a framework for future research directions.

2. ADIPOSE TISSUE

2.1 Anatomy of adipose tissue

Adipose tissue is an endocrine organ that plays a central role in regulating a number of physiological processes including energy homeostasis, insulin sensitivity, and thermoregulation. White adipose tissue (WAT) is the primary site of energy storage being comprised of 85% lipids of which 90-99% are triglycerides (8, 9). Only 2-3% of WAT mass is protein with the remainder being water, minerals and other substances (e.g., vitamins). White adipose tissue is distributed throughout the body and shows distinct compositional and functional roles depending on anatomical location (8). In contrast, brown adipose tissue (BAT) plays a central role in basal and inducible energy expenditure (10). It does so via its role in regulating thermogenesis. BAT is distinct from WAT, both anatomically and histologically. While WAT is diffusely distributed throughout the body, BAT exists in discrete lobes in many species. Interestingly, among livestock, the pig does not have functional BAT in any stage of its growth and development. In many mammalian newborns, including humans, sheep and rats, BAT is located in two

major depots, the intrascapular region and the peri-renal region, but can also be found in cervical, supraclavical, paravertebral, pericardial, para-aortic, pancreatic, splenic hilum, lung hilum, surrounding the trachea and esophagus, and surrounding the intercostal and mammary vessels (11). Within the first month of life, depots of BAT are drastically reduced in the infant in response to initiation of thermogenesis at birth. Nonetheless, BAT can be detected in these depots during the first decade of life, after which detection is highly variable among adults (11). Depots of BAT are most consistently observed in the cervical, supraclavical, paravertebral, and peri-renal regions of adult humans (6, 7, 11, 12). One interesting discovery that may alter the view of BAT as existing in depots is the observation that brown adipocytes are interspersed within skeletal muscle in a strain of mice resistant to developing obesity (13) and interspersed within white adipose depots in both rats and humans (14, 15). Future studies are needed to determine if brown adipocytes are present within the skeletal muscle of humans and if so what is the contribution of these cells to whole-body metabolism.

2.2 Histology of adipose tissue

Histologically, WAT is comprised of relatively large cells (50-105 μm) characterized by a unilocular lipid compartment (16). WAT is diffusely vascularized often with a single capillary providing the necessary nutrient and waste transport for the cell. In contrast, BAT is highly vascularized to provide adequate substrates (e.g., fatty acids, glucose, lactate, and amino acids) for oxidation as well as a means to remove heat from these discrete locations to maintain whole-body thermogenic homeostasis. Thus, endothelial cells, which line all blood vessels, represent the most abundant cell type within BAT. Additionally, activation of BAT results in substantial increases in blood flow to this tissue. BAT is also highly innervated by postganglionic sympathetic fibers that mediate BAT activity (17). Individually, brown adipocytes are smaller (25-40 μm) and often contain multiple lipid droplets (18). Interestingly, the number of lipid droplets within BAT is altered depending on the metabolic status of the animal. In a neutral thermal environment, brown adipocytes may contain a single lipid droplet; however, during periods of cold-stress a more multilocular phenotype may develop (19). It is therefore impossible to distinguish small adipose depots solely based on histological evaluation at the light microscope level. Ultrastructural analyses of BAT have identified distinct differences between WAT and BAT, with the most striking difference being the number and structure of the mitochondria present within each cell type. Brown adipocytes contain a much greater number of mitochondria than white adipocytes (20). Mitochondria are also larger in brown versus white adipocytes (>0.5 μm versus <0.3 μm, respectively) (21). Ultrastructurally, the inner mitochondrial membrane has substantially more surface area than the outer mitochondrial membrane and possesses a unique uncoupling protein-1 (UCP1) whose activity results in the generation of large quantities of heat (22). The capacity to generate heat is directly related to the amount of uncoupling protein and the surface area of the inner mitochondrial membrane (23). Interestingly, chronic stimulation of BAT results in increases in both the presence of multilocular cells and the surface area of the inner mitochondrial membrane (24, 25). A detailed discussion of the uncoupling process will follow in a later section.

3. BAT IN THERMOREGULATION

Thermogenesis is the process of heat production in organisms to maintain core body temperature. This process can be achieved by either shivering or non-shivering thermogenesis. Shivering thermogenesis occurs when opposing muscle fibers are activated simultaneously to hydrolyze ATP into ADP plus Pi, resulting in heat production. Such an event is similar to heat production during exercise. Non-shivering thermogenesis is regulated by a unique process in BAT whereby heat is produced when translocation of H^+ through a specific inner membrane protein pore into the mitochondrial matrix bypasses ATP synthesis, effectively uncoupling the proton motive force and releasing energy as heat. Note that the reduced proton motive force is not sufficient to drive ATP synthesis from ADP plus Pi by the ATP synthase complex, therefore resulting in the production of large amounts of heat rather than the conversion of ADP to ATP. Because of this unique process, BAT can produce 150-300 times more heat per kg tissue than non-BAT organs (26). Stock and Rothwell (27) estimated that as little as 80-100 g of BAT generating heat at only half of its maximal capacity could account for roughly 20% of the daily energy expenditure of a 70-kg man. Of particular interest, BAT makes up between 0.05%-0.1% of the total body weight of an adult human (28), corresponding to between 35-70 g of BAT in a 70-kg man. Although slightly less than those used for the energy expenditure calculations by Stock and Rothwell, these estimates are nonetheless promising if ways to increase BAT mass or activity can be developed.

Much of what we now know about the process of non-shivering thermogenesis was derived from studies of the neonate. At birth, the fetus is expelled from a temperature-controlled uterine environment into a relatively cool external environment. The ability of the neonate to adapt to this environment (e.g. generate sufficient heat to maintain core body temperature) is essential for survival. Indeed, infant mortality rates are significantly increased with even short periods of hypothermia. Due to similarities in fetal development in relation to birth, the sheep has provided a valuable model to understand factors regulating neonatal thermogenesis. In the fetal lamb, BAT development increases rapidly between Days 70 and 120 of gestation and then slows to term (Day 147) (29). At birth, BAT is responsible for approximately 50% of the heat generated in newborn lambs although BAT constitutes only 2% of birth weight (30-32). Neonatal thermogenesis is controlled by norepinephrine, which acts as a proliferative agent for brown preadipocytes while stimulating differentiation in mature brown adipocytes (33). Following parturition, cooler extra-uterine temperatures results in release of norepinephrine, which binds beta-adrenergic receptors and stimulates nonshivering thermogenesis. An intracellular signaling cascade is initiated by norepinephrine that stimulates the

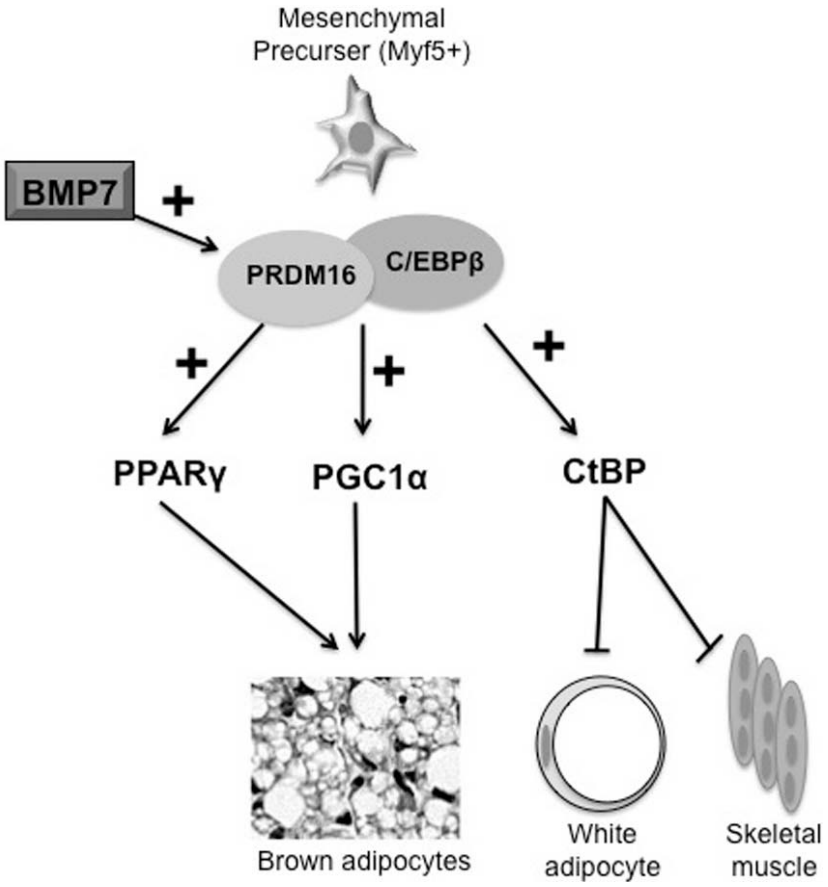

Figure 1. Transcriptional regulation of Myf5^{+} mesenchymal precursers into the brown adipocyte lineage. The transcription factors PRDM16 and C/EBP beta complex to regulate a bi-directional switch to regulate brown adipocyte differentiation. Activation of the PRDM16/CEBP beta complex upregulates expression of PPAR gamma, PGC1 alpha, and CtBP. PPAR gamma and PGC1 alpha subsequently upregulate a number of genes involved in BAT differentiation and function including UCP1. In addition, PGC1 alpha plays a vital role in mediating mitochondrial biogenesis. The upregulation of the potent corepressor CtBP by PRDM16 further prevents differentiation into either white adipocytes or skeletal muscle. The TGF beta family member, BMP7 is a positive regulator of PRDM16 and may be an early differentiation factor regulating fate determination.

hydrolysis of triglycerides to fatty acids and glycerol by hormone-sensitive lipase. Fatty acids are then trafficked to the mitochondria where they undergo beta-oxidation, releasing acetyl CoA moieties for oxidation via the citric acid cycle.

For decades it was believed that BAT was a transient organ in humans that disappeared early in life as the body adapted to the extrauterine environment. A limited number of reports of the existence of BAT in adults were met with skepticism or disregard as these studies only employed gross histological assessment and found what were presumed to be BAT cells in extremely small amounts that were of little consequence. Only recently, were a series of well-designed studies published that not only supported the early observations of BAT in adult humans, but also went one step further to show that these depots were actively responding to environmental stimuli. Given the capacity for metabolism, these small depots were quickly recognized as a potential target for anti-obesity therapy. Early investigations in the neonate elegantly outlined the mechanisms by which BAT functions; however, an understanding of the developmental cascade that regulates cell fate was lacking.

4. BAT CELL LINEAGE

With the discovery of stem cells and the emergence of BAT as a means to combat obesity, understanding the mechanisms driving the differentiation of the brown adipocyte lineage has seen a recent explosion in investigation and knowledge. Brown and white adipocytes, as well as muscle and bone, are believed to be of predominantly mesodermal origin (Figure 1). Mesodermal stem cells can differentiate into a number of different cell types, including adipocytes, osteoblasts, chondrocytes, myoblasts, and connective tissue. Until recently it had been assumed that white and brown adipocytes were derived from a common precursor. The basis for this assumption was that peroxisome proliferator-activated receptor-gamma (PPAR gamma) is required for the development of both brown and white adipocytes. Indeed, chimeric deletion of PPAR gamma in mice indicated that stem cells lacking this transcription factor could not

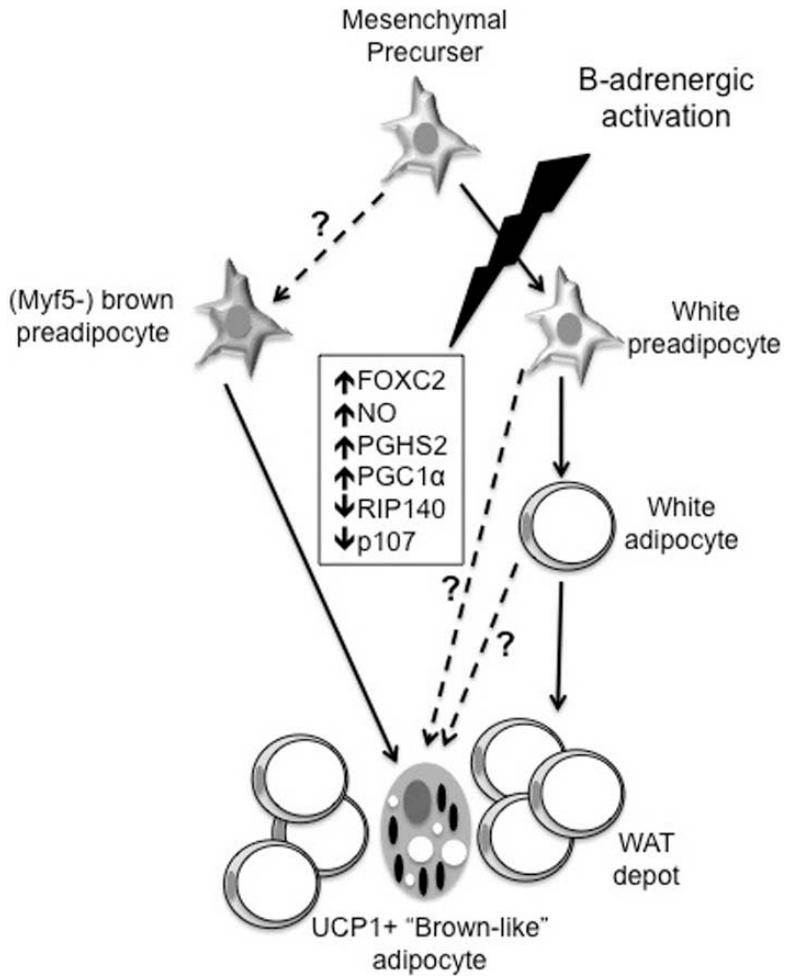

Figure 2. Recruitment of UCP1-positive brown-like adipocytes within WAT depots. In response to adrenergic activation the presence of brown-like adipocytes can be detected within WAT depots. The developmental origin of these cells is unknown although they are not derived from the $Myf5^+$ cells observed for the major BAT depots. Kajimura *et al.* proposed that these cells could arise from 3 sources; 1) a yet undefined $Myf5^-$ brown preadipocyte, 2) a white preadipocyte, or 3) conversion of a mature white adipocyte (15). A number of genes have been associated with the appearance of the brown-like adipocytes within WAT depots. The upregulation of FOXC2, NO, PTGS2, and PGC1 alpha have been associated with an increase in brown-like adipocytes while repression of RIP140 and p107 also results in induction of brown-like adipocyte development within WAT depots. Adapted from Kajimura *et al.*, Cell Metabolism (15).

contribute to the development of adipose tissue (34, 35). Although the development of BAT absolutely requires PPAR gamma, this transcription factor alone is not sufficient to drive mesenchymal stem cells to develop into brown adipocytes, indicating the presence of additional factors responsible for brown fat delineation. Subsequent studies led to the exciting discovery that brown adipocytes can be derived from Myf-5-expressing myogenic precursors (Figure 2). Genetic fate mapping revealed that both brown adipocytes and skeletal muscle, but not white adipocytes, are derived from Myf-5 expressing myogenic precursors (36). Gene profiling studies further indicated that brown but not white adipocyte precursors expressed a gene signature similar to that of skeletal muscle cells (37). Similarly, proteomic analysis supports the notion that mitochondria from brown adipocytes are similar to those from skeletal muscle but not white adipocytes (38).

4.1. PRDM16 and transcriptional control in the Myf 5 lineage

Within the Myf-5-expressing precursors, PRD1-BF1-RIZ1 homologous domain containing protein 16 (PRDM16) was shown to act as a bidirectional switch to drive differentiation of the mesenchymal stem cell into the brown adipocyte. Knockdown of PRDM16 in brown adipocyte precursors results in loss of brown fat characteristics, altered gene expression, and their differentiation into muscle, while ectopic expression of PRDM16 in white preadipocytes or myoblasts results in brown adipocyte differentiation (36, 39). The transcriptional mechanism(s) by which PRDM16 acts as a bidirectional switch are complex. Although PRDM16 had been shown to bind directly to a specific DNA sequence *in vitro*, point mutations within the two zinc finger-binding domains did not substantially alter the induction of brown

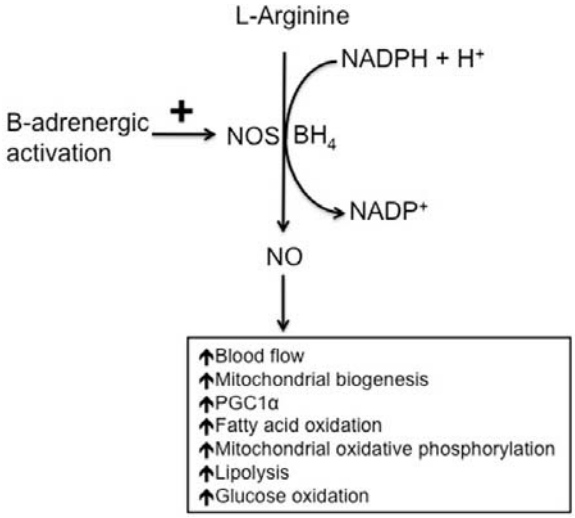

Figure 3. Mechanisms by which L-Arginine can enhance BAT development and function. L-arginine, a conditionally essential amino acid, is converted to nitric oxide by nitric oxide synthase and tetrahydrobiopterin. Nitric oxide can subsequently act on BAT to increase blood flow, stimulate expression of PGC1 alpha, and enhance mitochondrial biogenesis. NO also plays a vital role in regulating metabolism including increasing lipolysis, oxidation of fatty acids and glucose, and increasing mitochondrial oxidative phosphorylation. The actions of L-arginine could be enhanced with simultaneous beta-adrenergic activation. Beta-adrenergic activation has been shown to increase the expression of nitric oxide synthase, which could potentiate the conversion of L-arginine to NO.

adipocyte formation by PRDM16. These data suggest that PRDM16 does not act as a classical DNA-binding transcription factor (15). Interestingly, PRDM16 has been shown to bind to a number of known DNA-binding transcription factors, including PPAR gamma-coactivator 1 alpha and beta (PGC1 alpha and beta), PPAR alpha, PPAR gamma, p53, and several members of the CAAT Enhancer Binding Protein (C/EBP) family (36, 40). Further, deletion of C/EBP beta inhibits the ability of PDRM16 to induce differentiation of myoblasts into the brown adipocyte fate. Supporting the role for the PDRM16-C/EBP beta complex is the observation that PRDM16 and C/EBP beta knockout mice produce a similar phenotype. These mice contain morphologically abnormal BAT with reduced expression of BAT-selective genes and elevated expression of skeletal muscle-selective genes (36, 41). In addition to its function as an activator for genes such as PGC1 alpha, PRDM16 also serves as a repressor with the cooperation of C-terminal binding proteins (CtBP1). PRDM16 binding of CtBP1 in either white preadipocytes or myoblasts results in repression of marker genes specific to white adipocytes and skeletal muscle cells, respectively (42).

4.2. Bone morphogenic proteins (BMPs)

The emergence of PRDM16 as a critical mediator of brown adipocyte differentiation from the myoblast lineage has led to the investigation of the potential upstream regulators of this gene. During development, bone morphogenetic proteins (BMPs) have been described as niche factors that instruct the development of pluripotent stem cells. One reported role of BMPs has been in the determination of the adipose cell fate. Using C3H10T1/2 cells, which are mesenchymal stem cells capable of differentiating into myoblasts, adipocytes, chondrocytes, and osteoblasts (43-45), specific concentrations of BMP2, BMP4, and BMP7 can induce differentiation of adipocytes (44, 46). Interestingly, treatment of C3H10T1/2 cells with BMP4 results in the differentiation of white adipocytes (47, 48). In contrast, BMP7 drives differentiation of these mesenchymal precursors into the brown adipose lineage. Transplanting these BMP7 treated mesenchymal stem cells into the fat pad of nude mice leads to the development of a UCP1 positive brown fat pad. BMP7 knockout mice on E17.5 and E18.5 exhibit minimal brown adipose tissue development and UCP1 expression (49). BMP7 also induces the expression of PRDM16 and PGC1 alpha as well as PPAR gamma and C/EBPs, while increasing expression of UCP1 and enhancing mitochondrial biogenesis (49).

4.3. PPAR gamma coactivator-1 alpha (PGC1 alpha)

PGC1 alpha was first identified in BAT in response to cold activation and was shown to stimulate the expression of PPAR gamma as well as the thyroid hormone receptor. Ectopic expression of PGC1 alpha results in the induction of a number of genes related to mitochondrial development and function as well as thermogenesis, including UCP1 (50-52). Subsequent studies indicated that PGC1 alpha was a master regulator of mitochondrial biogenesis and oxidative metabolism in many cell types (53, 54). Ablation of the PGC1 alpha gene results in an impaired thermogenic response to cold stress and dampened responsiveness to cAMP (53, 55). Despite these functional changes, BAT develops normally in PGC1 alpha-null mice, indicating that although PGC1 alpha is essential for BAT function, it is not involved in specification of the brown fat lineage (at least from Myf5 expressing preadipocytes).

A number of genes have been shown to regulate BAT function by altering the expression or activity of PGC1 alpha (10, 56). Of these, FOXC2, CREB, SIRT3, SRC-1, p/CI, and PGHS2 act in a positive manner to stimulate PGC1 alpha activity. Interestingly, PGHS2 did not stimulate PGC1 alpha expression in brown adipocytes of the Myf5 lineage but did increase expression of PGC1 alpha and UCP1 in primary cells isolated from the stromal vascular fraction of white adipose tissue (57). FOXC2 is a member of the forkhead/winged helix transcription factor family that has been reported to induce the development of brown-like adipose cells within WAT depots. FOXC2 transgenic mice exhibit a lean phenotype and are resistant to diet-induced obesity (58). FOXC2 not only upregulates expression of UCP1 and PGC1 alpha, but also angiopoietin-2, which subsequently stimulates vascular development within the adipose tissue (59). In addition to these proteins, the intracellular signaling molecules, p38 MAP kinase and nitric oxide [NO, a product of L-arginine degradation (Figure 3)] stimulate PGC1 alpha activity (10, 56). The potential role for NO as a mediator of

mitochondrial biogenesis will be discussed later. A number of PGC1 alpha repressive factors have also been identified, including pRB, necdin, p107, TIF2, SHP, LXR, and WNT10b (10, 56, 60). Of particular note, transgenic expression of WNT10b in intrascapular tissue results in a lack of functional BAT (60). This tissue also lacks expression of PGC1 alpha and UCP1 but exhibits a histological and genetic fingerprint similar to WAT. In addition to effects on gene transcription, activation of the translational inhibitor 4E-BP1 has been shown to reduce brown adipogenesis via translation repression of PGC1 alpha (10). PGC1 alpha can also be regulated by posttranslational modification by p38 MAP kinase, which phosphorylates the PGC1 alpha protein to enhance its transcriptional activity (10). Collectively, these results highlight a complex regulatory pathway by which PGC1 alpha mediates its effects.

4.4. Brown-like adipose tissue not of Myf5 lineage

Complicating the matter is the observation that a population of brown-like adipose cells that express UCP1 can be found within WAT depots in response to cold or beta-adrenergic stimulation (36). These cells are not descendent from the Myf-5 expressing lineage but a number of genes that regulate PGC1 alpha are involved in the emergence of the brown-like adipose cells (e.g., FOXC2, PGHS2, and NO synthase). Recent evidence suggests that the occurrence of brown-like adipose cells within WAT depots results from transdifferentiation induced by beta 3-adrenergic receptor activation (61). These researchers demonstrated that in response to cold challenge, a population of brown-like adipose cells could be identified and close examination of a portion of these UCP1-expressing cells revealed a mixed morphology of lipid droplet formation and a mixed mitochondrial population, having mitochondria characteristic of both white and brown adipocytes. These effects were similarly observed when a beta 3- but not beta 1-adrenergic receptor agonist was administered. Future studies are needed to determine if a unique precursor population gives rise to these brown-like adipose cells and what factors result in their induction following appropriate environmental stimuli.

4.5. Adipocyte mitogens

Understanding the cellular and molecular mechanisms that drive a precursor cell into a specific lineage is central to developing therapeutic means to combat obesity. In pursuit of this knowledge, however, we must not overlook the importance of factors that stimulate growth of both the undifferentiated as well as differentiated cells, as these represent the functional unit of the tissue of interest. A number of growth factors have been implicated in stimulating the proliferation of both the precursor and mature cell populations. At least four members of the fibroblast growth factor (FGF) family (FGFs 1, 10, 16 and 19) are involved in adipose tissue development. Specifically, FGF16 is abundantly expressed in BAT during fetal development (62) and has been shown to stimulate proliferation of BAT preadipocytes *in vitro*. Transgenic expression of FGF19 results in increased BAT mass while protecting against diet-induced obesity (63). Treatment of fetal rat brown adipocytes with transforming growth factor beta 1 (TGF beta1) enhances proliferation and differentiation of these cells (64, 65). TGF beta 1 also increased the expression of UCP1 as well as a number of genes involved in adipogenesis (64). The importance of insulin and insulin-like growth factor (IGF) signaling has also been implicated in BAT development. Insulin receptor substrates (IRSs 1-4) are a family of proteins that are phosphorylated in response to activation by insulin, IGF-1, growth hormone, and other cytokine receptors (66). Specific knockout of these four family members has revealed their individual functions for regulating BAT development (67). IRS1 appears to be the most critical member of this family in terms of BAT development (67). Knockout of IRS1 in brown preadipocyte cell lines causes a severe defect in BAT differentiation while IRS3 knockout leads to only a moderate defect. An IRS1/IRS3 double knockout completely inhibits BAT differentiation. The actions of IGF2 in brown adipocytes are at least partially controlled by activation of the prolactin receptor. Indeed, prolactin receptor-knockout mice exhibit a marked reduction in expression of PPAR gamma 2, PGC1 alpha, UCP1, beta 3-adrenergic receptor, and IGF2 within BAT (68). These mice possess less BAT, are more susceptible to cold challenge, and their preadipocytes fail to differentiate into mature adipocytes. Importantly, IGF2 treatment induces differentiation of the preadipocytes into mature adipocytes (68).

5. UNCOUPLING PROTEINS

To date five uncoupling proteins (UCP1-5) have been described. However, without question the best understood is UCP1. UCP1 is a member of the mitochondrial anion carrier family and plays a central role in mammalian thermogenesis. Located on the inner mitochondrial membrane of brown adipocytes, UCP1 catalyses a proton leak by partially uncoupling electron transport from ATP synthesis. The activity of UCP1 can be inhibited by purine nucleotides and conversely stimulated by free fatty acids. Following environmental stimulation of the sympathetic nervous system, lipolysis in BAT is initiated. Elevations in circulating levels of free fatty acids stimulates UCP1 activity, resulting in increases in proton leakage and heat production.

5.1. Uncoupling protein 1 (UCP1)

To understand the significance of UCP1 in regulating non-shivering thermogenesis, one must look at data from UCP1 knockout mice. These mice are incapable of initiating nonshivering thermogenesis in response to cold despite having a greater quantity of BAT than wild-type mice, supporting the conclusion that UCP1 is essential to generate heat via the uncoupling process (69). Given that thermogenesis is initiated by lipolysis and is capable of metabolizing large quantities of substrates, it has been proposed that manipulation of this process may be the key to combating obesity. Unexpectedly, UCP1 knockout mice from undefined mixed background (B6 and 129 SvPas strains) did not become obese on either a standard or high fat diet (69). Interestingly, while B6 mice become obese on a high fat diet (70), the 129 strain is protected from the

development of diet-induced obesity (71). Not only do strain differences play a central role in susceptibility to obesity, it was recently shown that genetic background has overwhelming effects on cold-tolerance in UCP1 deficient mice (72). Thus, factors other than UPC1 also play a critical role in fat synthesis, oxidation and storage.

Using congenic lines of C57BL/6J mice, UCP1 knockdown paradoxically resulted in resistance to obesity in a temperature-dependant manner, whereby UCP1-deficient mice were surprisingly protected from developing obesity at 20°C but not at 27°C. That UCP1-null mice were resistant to obesity rather than subject to obesity at any temperature is astounding and requires future studies to investigate alternative mechanisms of body weight regulation in the absence of such a metabolically powerful protein. In contrast to these findings, a recent report suggests that maintaining C57BL/6J UCP1 null mice at 29°C indicates that UCP1 knockdown gives rise to obesity under both normal and high-fat diets (73). These investigators speculate that the ambient temperature under which these and prior studies were conducted has profound effects on the metabolic activity of the mice. In support of this view, maintaining mice at 18-22°C, which is standard for many laboratory animal housing facilities, actually induces chronic thermal stress in mice and results in a 50-60% increase in food intake to combat this stress (74). Despite these variable results from gene ablation studies, a variety of other studies using different approaches highlight the importance of UCP1 in not only regulating thermogenesis but also whole-body metabolism. For example, overexpression of UCP1 in both diet- and genetically-induced mouse strains caused a reduction of subcutaneous WAT. In addition, a number of genetic and pharmacological manipulations that confer resistance to obesity increased UCP1 expression, including a) disruption of the RII beta subunit of protein kinase A (75); b) administration of beta 3 agonists (76, 77) or apelin (78); c) overexpression of forkhead box C2 (58) or apolipoprotein A-1 (79); and d) knockout of nerve growth factor (VGF) (80), nuclear receptor interacting protein 1 (81, 82), activating transcription factor 4 (83), or tumor necrosis factor receptor one (84).

The rodent has a relatively large amount of BAT and is an exceptional model for the study of BAT development. However, employing a comparative biology approach can also provide valuable insight into the relative function and/or significance of a tissue. Pigs, *sus scrofa*, are highly efficient metabolically, being able to deposit considerable amounts of fat following birth, but exhibit poor thermoregulation. At birth, piglets contain very small quantities of WAT (~1% of body weight) but no BAT and do not express UCP1 in tissues (85). In keeping with the absence of BAT in the body, neonatal piglets generate the vast majority of heat by shivering. Shortly after birth, piglets begin to deposit adipose and by 2-3 months of age are comprised of nearly 15% fat primarily in the subcutaneous depot (86). Recently, Berg and colleagues discovered that the UCP1 gene was inactivated in pigs and their wild ancestors due to three distinct mutations some 20 million years ago (87). Thus, like the UCP1-knockout mice, piglets exhibit high rates of mortality in response to cold stress.

Although available evidence is compelling, a conclusive role for UCP1 as a mediator of resistance to obesity has not been defined using available rodent models. A more compelling argument exists in the analysis of the human genome. In humans, UCP1 is located on chromosome 4 and contains six exons. Sequencing has identified several single nucleotide polymorphisms (SNPs) in the UCP1 gene. Interestingly, these polymorphisms have been associated with increased body fat accumulation, body weight gain, body mass index, obesity, diabetes mellitus, and lipid/lipoprotein-related disease (88-95). Also of interest was the observation that UCP1 mRNA levels in biopsied adipose tissue of morbidly obese subjects were lower than those from lean controls (96). One polymorphism that has gained considerable interest is the A-3826G polymorphism located in the UCP1 promoter, in which a point mutation from A to G occurs at position -3826 (88). This mutation results in reduced expression of UCP1 and, therefore, is functionally significant (96). When obese subjects were placed on a low-calorie diet and exercise regimen for 3 months, weight loss for subjects containing the G allele at position -3826 was less than those containing the A allele (97). In addition, carriers of the GG allele exhibit reduced thermogenesis in response to acute cold exposure (98). These results implicate BAT as a potentially important target for prevention and treatment of obesity in humans.

5.2. Other uncoupling proteins

In contrast to the well-established role for UCP1, the significance of UCP2 and UCP3 remains elusive. Studies involving overexpression of UCP2 and UCP3 genes have shown that they are capable of increasing mitochondrial proton leakage (99,100). However, the supra-physiological levels of expression required to observe this leakage suggest that these effects may be an artifact rather than establish a physiological role for these family members. Indeed, UCP2 and UCP3 protein levels are present at less than 1% of the abundance of UCP1, contributing to the difficulty in studying the function of the proteins (99, 100). In addition, UCP2- and UCP3-knockout mice exhibit normal non-shivering thermogenesis in response to cold challenge while UCP1-knockout mice that still express UCP2 and UCP3 are incapable of non-shivering thermogenesis, providing additional evidence that these two proteins are not essential for the thermogenic process (101-104). There is evidence that UCP-2 in pancreatic beta-cells may have a regulatory role in insulin secretion (105). UCP4 and UCP5 are recently described "uncoupling proteins" present in the brain, but they appear to have very little in common with UCP1 (105). In fact, there is no phylogenetic data supporting the classification of UCP4 and UCP5 in the UCP family (106).

6. ADRENERGIC CONTROL OF BAT ACTIVATION

6.1. Effects of catecholamines on lipolysis and proliferation of brown adipocytes

The catecholamines, epinephrine and norepinephrine are produced by the adrenal glands and their release into the blood circulation is enhanced in response to imminent danger (e.g. "fight or flight"

response), cold stress, or intensive exercise. Through the cAMP-dependent signaling pathway, catecholamines stimulate the mobilization of triglycerides to provide free fatty acids and glycerol to combat the impending "danger". Interestingly, homeothermic species of birds and mammals have employed the same mechanism of sympathetic response to low environmental temperatures via sympathetic activation of BAT.

Adrenergic stimulation of BAT by norepinephrine initiates a cascade of effects that mediate multiple facets of BAT development and function. These facets include the acute thermogenic response, stimulation of cell proliferation and differentiation, as well as inhibiting apoptosis. Norepinephrine acts through beta-adrenergic receptors, of which three sibling family members (beta 1, beta 2, and beta 3) have been identified. Importantly, these three receptor subtypes have unique functions within BAT to mediate the effects of norepinephrine. In addition to activation of beta adrenergic receptors, norepinephrine also stimulates alpha 1 and alpha 2 receptor subtypes. However, the relative importance of these receptors in BAT function appears to be limited. In fact, the alpha 2-receptor subtype is stimulated by norepinephrine and leads to an inhibition of adenylyl cyclase in response to beta-adrenergic receptor activation (33). Activation of the alpha 1–receptor subtype, which is present at high levels in BAT, also occurs in response to norepinephrine, but alpha 1- stimulation accounts for less than 20% of the total thermogenic response (107-110). Classically, beta-adrenergic receptors signal primarily through the phosphorylation of adenylyl cyclase and PKA. Additionally, norepinephrine activates the extracellular regulated kinase 1/2 (ERK1/2) signaling cascade in multiple cell types, including brown adipocytes from newborn rats and adult mice (111-114). Within BAT, ERK activation mediates IGF-1/insulin-induced proliferation while playing a negative role in differentiation (115). Recent evidence shows that the norepinephrine-induced proliferation of brown adipocytes is independent of cAMP/PKA but is mediated by ERK activation (113).

6.2. Functional significance of beta-adrengergic receptors

Beta 1-adrenergic receptors are expressed in both mature brown adipocytes and their precursors. Within mature brown adipocytes, Beta 1-adrenergic receptors do not appear to be coupled to any major signaling pathways involved in the acute thermogenic response. However, beta 1-adrenergic receptors play a central role in expansion and proliferation of the brown adipocyte population. Activation of beta 1-adrenergic receptors stimulates the production of cAMP from the preadipocytes. Increased levels of intracellular cAMP are required for expansion and proliferation of the preadipocyte population.

Beta 2-adrenergic receptors are expressed in brown adipocyte precursors but are not expressed or are weakly expressed in mature brown adipocytes (33). Beta 2-adrenergic receptors are, however, expressed in BAT, suggesting that this receptor subtype is localized to the extensive vasculature for supporting the brown adipocytes (33). Interestingly, within human skeletal muscle, infusion of beta 2- but not beta 1- or beta 3- agonists increased tissue blood flow (116). It is clear that induction of the thermogenic process augments blood flow to BAT as a means to deliver substrates and remove heat. To date, the contribution of specific adrenergic receptors for mediating blood flow to BAT has not been elucidated. However, selective agonists for beta 1, beta 2 and beta 3 adrenergic receptors can promote NO synthesis by rat brown adipocytes (117).

Beta 3-adrenergic receptors are often considered the most functionally relevant receptor subtype for BAT-mediated thermogenesis. These receptors are abundantly expressed in mature brown adipocytes but are absent from brown adipocyte precursors (33). It should be noted that a functional beta 3-adrenergic receptor has not been identified in the guinea pig yet these animals have fully functional BAT (118, 119). In addition, beta 3-receptor knockout mice possess fully functional brown adipocytes, with the beta 1-receptor subtype mediating the effects of norepinephrine, but these mice develop obesity, thus highlighting the importance of the beta 3-receptor subtype in regulating lipolysis (120, 121). In this instance, the beta 1-receptor subtype likely acts in a compensatory fashion, as these animals have elevated expression of this subtype. Indeed, the beta 1 gene is under adrenergic control. Not surprisingly, gene-ablation of all three beta-subunit adrenergic receptors results in severe obesity (122). Of further interest, a number of obese animal models including the C57BL/6J *Lep*^{ob} mouse and the Zucker fatty (*fa/fa*) rat exhibit reduced expression of beta-adrenergic receptors (123-125). These findings have been extended to obese non-mutant mouse models induced by feeding a high-fat diet (126). Further, administration of beta 3 receptor agonists has promise in preventing obesity in a variety of animal models (127).

7. THYROID HORMONE ACTIONS AND THERMOREGULATION

The coordinated actions of the adrenergic system and thyroid hormone mediate the physiological responses to a number of environmental stimuli, including the regulation of thermogenesis. As discussed in the previous section, the effects of catecholamines are exerted rapidly in response to environmental stimuli and can be amplified by thyroid hormones (128). Catecholamines can also stimulate the conversion of type-2 thyroxine (T_4) to triiodothyronine (T_3) and may increase expression and activity of the thyroid hormone receptors by phosphorylation (129, 130). Thus, there are the coordinated actions of norepinephrine and T_3 within BAT. Treatment with either norepinephrine or T_3 alone increased UCP1 gene expression two to three-fold in rat BAT, and, when administered simultaneously, a 20-fold induction was observed (131). Interestingly, inactivation of the type 2 5'-deiodinase, the enzyme that converts T_4 to T_3, results in defective thermogenesis despite normal circulating T_3 (132) These findings highlight the need for locally produced T_3 in BAT function. In the rat, adrenergic activation of BAT results in a dramatic increase in local T_3 production, capable of almost complete saturation of the T_3

receptor population (133). In comparison, a ten-fold increase in circulating T_3 concentrations would be required to attain this level of receptor activation (134). The actions of T_3 on UCP1 gene expression and adrenergic responsiveness of BAT appear to specifically require the thyroid hormone receptor (TR) beta-isoform as evidenced by knockout mouse models (135). However, the TR-alpha isoform may also play a role in thermogenesis, as a dominant negative mutation in TR alpha results in dampened adaptive thermogenesis despite normal UCP1 expression (136). Analysis of the UCP1 gene led to the identification of two thyroid hormone response elements in immediate vicinity to several cAMP response elements (137). Although thyroid hormone alone does not induce a thermogenic response, its additional targets can be beneficial for combating obesity. More research is needed to investigate the interrelationship between the adrenergic and thyroid systems to regulate energy expenditure in response to stimuli.

8. NUTRITIONAL REGULATION OF BAT GROWTH AND DEVELOPMENT

8.1. Diet-induced thermogenesis

The deposition of adipose tissue occurs when the number of calories consumed exceeds the number of calories expended. Energy expenditure can be broken down into three components: 1) basal energy expenditure required for cellular and organ function; 2) adaptive thermogenesis induced by cold exposure or diet; and 3) physical exertion (138). A precise balance of forces regulating intake and expenditure exist to maintain an optimal weight; however, even a slight chronic imbalance can result in obesity. This balance of forces has been well described in rodents. Indeed, dietary manipulation can be a means to up-regulate BAT growth and development, thereby oxidizing excess energy rather than depositing white fat. Evidence from the majority of rodent obesity models indicates the existence of an impaired thermogenic system as a major factor contributing to the obese phenotype. Interestingly, as rodents become more and more inbred, strain differences in energy expenditure have been noted. For example, in rats, feeding a high fat "cafeteria" diet results in variable changes in body composition depending on the strain.

Evidence for a role of diet-induced thermogenesis in humans has been difficult to determine and less well accepted. A number of factors contribute to these shortcomings, including the composition of the diet, level of compliance to dietary regimen, misreporting or underestimation, and population heterogeneity. Nonetheless, the notion that nutrition plays a central role in BAT function is supported by the observation that blood flow to BAT is greater after a high-carbohydrate meal than a high-fat meal (139). Similar to results in rats, a wide range in weight gain in response to a fixed level of total excess energy intake has been reported. In one such study, people were given the same amount of excess energy for 100 days but the resultant weight gain ranged from 4.3 to 13.3 kg (140). Results of these studies led to the conclusion that the differences in weight gain resulted from individual differences in diet-induced thermogenesis (141). In addition, low energy expenditure, normalized for lean body mass, was effective in predicting future weight gain over a two-year period (142). In contrast, the use of double labeled water to assess energy expenditure over time suggests that increases in total energy expenditure during overfeeding are small while physical activity accounts for the greatest amount of variability (143).

Despite contradictory reports in human clinical and epidemiological studies, there is circumstantial evidence that BAT in humans plays a role in the prevention of obesity. In humans, the activity of BAT is reduced in overweight and obese individuals (4, 7). Further, BAT activity was positively correlated with resting metabolic rate. These data were supported by the work of Saito *et al.* (5) who demonstrated an inverse correlation between BAT activity and BMI, total fat, and visceral fat. These authors also found that BAT activity was reduced in elderly (aged 38-65 years) versus younger (aged 23-35 years) subjects, which is consistent with the earlier histological assessment of BAT in humans from the first to eighth decade of life (11). The question of how to increase BAT recruitment and thus energy expenditure without increasing energy intake (negative energy balance) remains the central question in fighting obesity.

8.2. Fetal adiposity

Susceptibility to obesity can be programmed during fetal development in mammals. Thus, understanding how maternal nutrition alters fetal BAT development will likely be beneficial in combating the development of obesity in childhood and later in life in response to excessive nutrient intake. Studies in humans have indicated that the number of fat cells in the body is set during childhood and adolescence and change little into adulthood in both lean and obese subjects (144). Thus, adipocyte number set during fetal and neonatal development is a major determinant of fat mass in adulthood. Interestingly, adipocytes are prematurely upregulated in obese children, some 3.6 years earlier than in children which remain lean. Little is known about the effect of the uterine environment on adipocyte stem cell fate determination; however, in species such as the human and sheep, which have a similar pattern of fetal development adipocytes first appear during mid gestation (145, 146). Importantly, development of these fat stores is essential for adaptation to the extrauterine environment, with BAT being necessary for the production of heat from fatty acids as the major substrates.

The sheep has provided a valuable model for understanding the development of adiposity in the fetus. Although fetal WAT is markedly affected by prolonged manipulation of maternal feed intake (147, 148), the effect of maternal nutrition on fetal BAT development and function is largely unknown. Observations in the sheep indicate that maternal obesity results in increased fetal and neonatal adiposity as well as an increase in leptin expression in peri-renal and subcutaneous adipose tissue depots (149, 150). If an excessive number of white adipocytes or reduced number of brown adipocytes is

Table 1. Effects of L-arginine supplementation on white adipose tissue loss

Species	Treatment	Duration	White Adipose Tissue Loss	Ref
ZDF[1] Rats	1.51% L-arginine-HCl in drinking water	10 weeks	28% loss in epididymal fat 46% loss in retroperitoneal fat	159
DIO[2] Rats	1.51% L-arginine-HCl in drinking water	12 weeks	30% reduction in adiposity of major fat depots	161
Obese Pregnant Sheep	I.V. infusion of L-arginine-HCl (81 mg L-arginine/kg body weight per day	25 days	16% reduction in whole-body carcass lipid content	158
Growing-Finishing Pigs	1% L-arginine in a corn- and soybean meal-based diet	60 days	11% reduction in whole-body fat content	163
Obese Humans	Oral administration of L-arginine (80 mg/kg body weight per day)	21 days	43% increase in fat loss compared with placebo	164

ZDF[1], Zucker diabetic fatty; DIO[2], diet-induced obese.

recruited during fetal development, the potential risk for later obesity may be enhanced. Interestingly, maternal nutrient restriction in sheep from Day 115 of gestation to term reduced mRNA levels for UCP1 in fetal peri-renal brown fat without affecting BAT mass (151). Further, lambs born to sheep fed 1.5 times their requirements of nutrients (including energy) had lower BAT per kg fetal weight, compared to control-fed sheep (152). It is noteworthy that maternal undernutrition is associated with the development of obesity later in life.

8.3. Dietary arginine supplementation

To date, nutritional strategies have had limited success in increasing fetal BAT growth *in utero*, perinatal thermogenesis, or survival of newborn neonates (152-157). For example, lambs from sheep fed a high-fat (8%) diet containing both saturated and monounsaturated fatty acids during late gestation had reduced thermogenesis in response to cold challenge (153). In contrast, we have found that maternal arginine supplementation to both undernourished and overnourished, obese pregnant sheep from Days 100-125 of gestation (term = 147 days) resulted in a 62% increase in fetal peri-renal BAT mass (158). Future studies are needed to determine if the increase in peri-renal BAT mass confers a protective effect to cold-stress at birth, as well as the development of excessive adolescent and adult adiposity. Interestingly, arginine treatment did not alter the morphology of fetal BAT or the amount of immunostaining for Ki-67, a marker for brown adipocyte proliferation. It is possible that arginine stimulates the proliferation or differentiation of a brown adipocyte precursor during fetal growth.

Recently, dietary supplementation with arginine (a conditionally-essential amino acid) was shown to reduce white fat mass while increasing brown fat mass in Zucker diabetic fatty rats (159, 160), diet-induced obese rats (161), and cold-acclimated rats (162). In addition to the previously mentioned increase in fetal BAT mass in response to maternal arginine administration (158), we also found that this short period of supplementation resulted in decreased maternal carcass lipid content and a corresponding decrease in maternal concentrations of plasma leptin (Satterfield MC, unpublished data). However, to date the presence of BAT in the adult sheep has not been reported and therefore the potential role of BAT as the mediator of maternal weight loss in the sheep is unknown. Similarly, arginine administration to growing pigs results in an 11% reduction in whole-body fat content while increasing muscle content by 5.5% (163); however, the existence of functional BAT in either the neonatal or adult pig has not been described. Only one study in humans has evaluated the effect of dietary arginine supplementation in hospitalized obese subjects. In this study, arginine administration (80 mg/kg body weight per day) resulted in significant reductions in fat mass and waist circumference (164). While subjects receiving both arginine and placebo lost body weight during the course of the three week study, 100% of weight loss in arginine-treated subjects was attributed to loss of fat mass while only 43% was attributed to loss of fat mass in placebo treated subjects. Collectively, these data provide valuable evidence that dietary supplementation with arginine can promote reduction in white fat mass while increasing brown fat mass in at least postnatal rats and fetal sheep (Table 1). Clearly, more studies are needed to assess the affect of arginine supplementation on adult human brown fat activity using fluoro-deoxyglucose-positron emission tomography.

The mechanisms by which arginine increases BAT in rats and neonatal lambs remain largely unknown (165). However several lines of investigation provide evidence for a link between arginine supplementation [an effective means to enhance arginine availability in tissues (166)] and BAT development (Figure 4). For example, via gaseous signaling (167), arginine-derived NO has been shown to play a central role in heat production and thermoregulation in mammals (168). It was recently reported that BAT differentiation and mitochondrial biogenesis is under the control of the NO target protein, protein kinase G (169). These results are supported by analysis of the endothelial NO synthase knockout mouse, which has a reduced metabolic rate and accelerated weight gain (170). These mice also exhibited reduced mitochondrial biogenesis within BAT in response to cold-exposure. Further, inhibition of NO synthesis results in the reduction of BAT blood flow, BAT development, and cold-induced thermogenesis in rats. In line with these findings, cold exposure results in the upregulation of endothelial NO synthase (171) coordinate with increased BAT blood flow. Inhibition of NO production by the NO synthase inhibitor N-omega-nitro-L-arginine methyl ester (L-NAME), which results in reduced BAT blood flow, can be overcome by administration of L-arginine. Interestingly, increased BAT temperature in response to norepinephrine was inhibited by L-NAME, indicating that NO mediates the norepinephrine-induced increase in BAT blood flow (172). Furthermore, an increase in NO production or PGC1 alpha has been shown to convert white adipocytes into UCP1 expressing

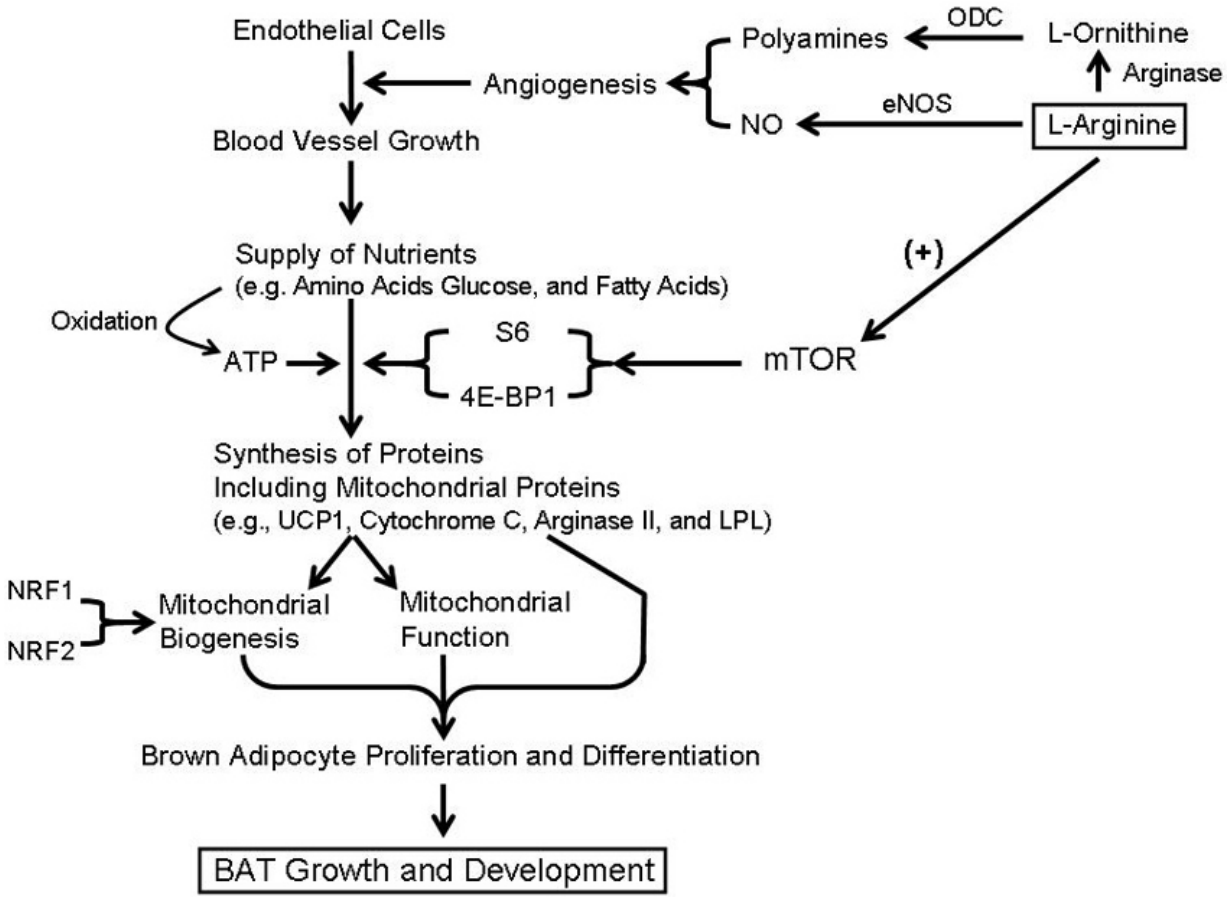

Figure 4. Arginine stimulates growth and development of fetal brown adipose tissue (BAT) via NO-dependent and –independent mechanisms. Arginine is the common substrate for the synthesis of NO and polyamines that are essential for angiogenesis (the formation of new blood vessels from existing vessels). Blood vessel growth increases the supply of nutrients (including amino acids, glucose, and fatty acids) to BAT via the circulation. The macronutrients are oxidized to provide energy (in the form of both ATP and heat), and arginine may activate FRAP1 resulting in S6 and 4E-BP1 phosphorylation. These signaling events can enhance synthesis of proteins including mitochondrial proteins (e.g., UCP1, cytochrome C, arginase-II, and lipoprotein lipase), which is critical for mitochondrial biogenesis and function that are also augmented by physiological levels of NO. Additionally, mitochondrial biogenesis is controlled by NRF1 and NRF2 (transcription factors-1 and -2). Collectively, increases in protein synthesis and mitochondrial biogenesis are expected to promote proliferation and differentiation of brown adipocytes, ultimately leading to enhancement of BAT growth and development. Abbreviations: BAT, brown adipose tissue; Cyt C, cytochrome C; eNOS, endothelial NO synthase; 4E-BP1, eukaryotic initiation factor 4E-binding protein-1; LPL, lipoprotein lipase; NRF, a nuclear respiration factor; ODC, ornithine decarboxylase; S6, ribosomal protein S6; UCP1, uncoupling protein-1.

brown adipocytes (170). PGC1 alpha is a master regulator of mitochondrial biogenesis whose expression is increased 5-fold in abdominal fat of arginine-treated ZDF rats (159) and can be regulated by dietary protein intake (173). Finally, arginine can stimulate the oxidation of long-chain fatty acids and glucose in insulin-sensitive tissues (59,174) as well as expression of key genes in this biochemical event (175-178), thereby reducing lipid storage in WAT.

9. CONCLUSIONS AND PERSPECTIVES

BAT is essential for neonatal thermogenesis. Functional BAT persists in adult humans and is activated in response to cold. Multiple observations in humans and rodents suggest that possessing more functionally active brown adipocytes is helpful in combating obesity. First, PET/CT scans of normal and obese humans indicate that obese subjects contain less active BAT than lean subjects. Second, single nucleotide polymorphisms in the human UCP1 gene are associated with an increased incidence of obesity. Third, the majority of mouse models of obesity exhibit a defect in some component of BAT development and/or function. Fourth, treatment of multiple species with dietary arginine, the precursor for the production of NO, results in increased BAT development and WAT loss. Conclusive evidence for functional BAT in adults has been complemented by a number of elegant studies highlighting the transcriptional control of brown adipocytes from their mesenchymal stem cells. This knowledge will no doubt play a vital role in understanding the genetic regulation of obesity. These exciting new findings have spurned many new questions to be answered over the next decade. What

contribution to whole-body energy metabolism does BAT have in lean versus obese subjects? At what point during development is the number of brown fat precursors determined? What is the developmental lineage of the brown-like adipose cell that can be recruited in WAT and skeletal muscle depots? Following recruitment and activation of these cells, what is their metabolic contribution? What is the mechanism by which dietary arginine increases BAT development and is the loss in WAT mass a consequence of increased BAT or from a secondary mechanisms of arginine action? Answering these questions will be pivotal for developing strategies to combat obesity and the myriad of health issues associated with this disease. It must be noted that obesity is a complex disease resulting from a variety of genetic, developmental, and environmental factors. Therefore, not every obese person will need or respond equally to a given therapy. Additionally, studies evaluating the efficacy of any therapy to combat obesity will need to consider and account for (a) skeletal muscle mass and function; (b) existence of genetic mutations; (c) variation in the fetal environment that may epigenetically program subsequent metabolic function; and (d) lifestyle. Unfortunately, given the current abundance of food and increased sedentary lifestyle in much of the developed world, the trend toward obesity will likely continue. That obese people give birth to offspring that are epigenetically programmed toward obesity themselves, will further fuel this epidemic. Without question, intervention is needed, and harnessing the metabolic power of BAT may be a viable option.

10. ACKNOWLEDGMENTS

This work was supported by the National Institutes of Health (1R21 HD049449), National Research Initiative Competitive Grants from the Animal Reproduction Program (2008-35203-19120) and Animal Growth & Nutrient Utilization Program (2008-35206-18764 and 2009-35206-05211) of the USDA National Institute of Food and Agriculture, American Heart Association (0755024Y), and Texas AgriLife Research (H-82000). We thank our colleagues (Drs. Fuller Bazer, Stephen Smith, Thomas Spencer, and Malcolm Watford) for helpful discussion, all the personnel in our laboratories for their technical support, and F. Mutscher for office support.

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Abbreviations: BAT, brown adipose tissue; WAT, white adipose tissue; PPAR, peroxisome proliferator-activated receptor; PRDM16, PRD1-BF1-RIZ1 homologous domain containing protein 16, PGC1, PPAR gamma coactivator-1; C/EBP, CAAT enhancer binding protein; CtBP, C-terminal binding protein; BMP, bone morphogenetic protein; UCP, uncoupling protein; FOXC2, forkhead/winged helix transcription factor; CREB, cAMP response element binding protein; SIRT3, sirtuin 3; SRC1, steroid nuclear receptor coregulator 1; PTGS2, prostaglandin-endoperoxide sythase-2; pRB, ; p107, retinoblastoma-like 107; TIF2, nuclear receptor coactivator 2; SHP, nuclear receptor subfamily 0, group B, member 2; LXR, nuclear receptor subfamily 1, member H; WNT, wingless-type MMTV integration site family; MAP, mitogen-activated protein kinase; 4E-BP1, eukaryotic translation initiation factor 4E binding protein 1; NO, nitric oxide; FGF, fibroblast growth factor; TGF, transforming growth factor; IRS, insulin receptor substrate; IGF, insulin-like growth factor; VGF, nerve growth factor; SNP, single nucleotide polymorphism; ERK, mitogen activated protein kinase 1/2; PKA, protein kinase A; T_4, type-2 thyroxine; T_3, triiodothyronine; TR, thyroid hormone receptor.

Key Words: Obesity, Brown Adipose Tissue, Metabolism, Uncoupling Protein, Adipocyte, Arginine, Nitric Oxide, Thermogenesis, Review

Send correspondance to: Michael Carey Satterfield, Department of Animal Science, Texas A and M University, College Station, TX 77843-2471, Tel: 979-845-6448, Fax: 979-862-2662, E-mail: csatterfield@tamu.edu

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