Cell Metabolism
Review

New Advances in Adaptive Thermogenesis: UCP1 and Beyond

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Brown and beige adipocytes can catabolize stored energy to generate heat, and this distinct capacity for thermogenesis could be leveraged as a therapy for metabolic diseases like obesity and type 2 diabetes. Thermogenic adipocytes drive heat production through close coordination of substrate supply with the mitochondrial oxidative machinery and effectors that control the rate of substrate oxidation. Together, this apparatus affords these adipocytes with tremendous capacity to drive thermogenesis. The best characterized thermogenic effector is uncoupling protein 1 (UCP1). Importantly, additional mechanisms for activating thermogenesis beyond UCP1 have been identified and characterized to varying extents. Acute regulation of these thermogenic pathways has been an active area of study, and numerous regulatory factors have been uncovered in recent years. Here we will review the evidence for regulators of heat production in thermogenic adipocytes in the context of the thermodynamic and kinetic principles that govern their therapeutic utility.

Introduction

Adaptive thermogenesis refers to the generation of heat by the body in response to external stimuli, and this process can be leveraged to counteract the hypercaloric state of obesity. Since the earliest mammals depended on their ability to occupy niches that were cooler than could be easily inhabited by ectotherms, adaptive thermogenesis was critical to their success during evolution (Crompton et al., 1978; Oelkrug et al., 2013). As might be expected, mechanisms to expend stored calories to generate heat also have substantial effects on metabolic disease outcomes. As such, studies of the mechanisms of non-shivering thermogenesis (NST) have been energized by the enormous interest in human metabolic diseases linked to obesity, including type 2 diabetes and fatty liver disease (Pfeifer and Hoffmann, 2015).

Generally, adaptive thermogenesis is separated into shivering and non-shivering forms. This review will focus on NST as this has been the intense focus of recent scientific explorations. Brown adipose tissue (BAT) has been recognized as a major site of NST for decades and is critical for the maintenance of body temperature (Foster and Frydman, 1978; Ma et al., 1986). This developmentally formed depot is abundant in the interscapular and perirenal regions of rodents and human infants. Additionally, brown fat-like cells appear in white fat depots, and these distinct beige adipocytes exhibit substantial capacity for induction of thermogenesis (Wu et al., 2012). In the last decade, several studies have definitively shown that adult humans possess thermogenic adipose tissue, further stimulating research into understanding mechanisms that control heat production in these cells (Cypess et al., 2009; van Marken Lichtenbelt et al., 2009; Virtanen et al., 2009).

Along these lines, essential regulators of thermogenic adipocyte identity have been determined, and these findings have been expertly reviewed elsewhere (Harms and Seale, 2013). The mechanisms by which adipocytes with the thermogenic apparatus become activated to produce heat is a question of equal importance from a therapeutic standpoint, and one on which we focus here. The purpose of this review is to contextualize recent findings that identify effectors of adipose tissue heat production within established principles of thermodynamics. We will also comment on their apparent physiological importance in experimental animal models, and the potential relevance to human physiology.

Principles of Cellular Thermogenesis

Long-standing interest in the molecular basis for brown and beige adipose tissue thermogenesis has its origins in the classical observations that their mitochondria possess an unusual mechanism for uncoupling respiration from ATP synthesis (Smith et al., 1966). Identification of uncoupling protein 1 (UCP1), the protein responsible for this phenomenon, has greatly improved our understanding of how these cells engage in thermogenesis (Nicholls, 2017). More recently, thermogenic processes independent of UCP1 have been demonstrated, both in brown/beige adipocytes and in other tissues. Interpretation of these thermogenic mechanisms first requires (re-)consideration of general thermodynamic and kinetic principles that bound our understanding of cellular heat production (Nicholls and Locke, 1984).

In mammalian cells, the free energy required for life is provided by reduced substrates. Mitochondrial oxidative phosphorylation dominates metabolism in mammalian cells, transducing this free energy into displacement of the [ATP = ADP + P_i] reaction from equilibrium. Importantly, only a fraction of the enthalpy of substrate oxidation is conserved in the free energy of the displaced [ATP = ADP + P_i] equilibrium (ΔG_p); most is lost as heat.

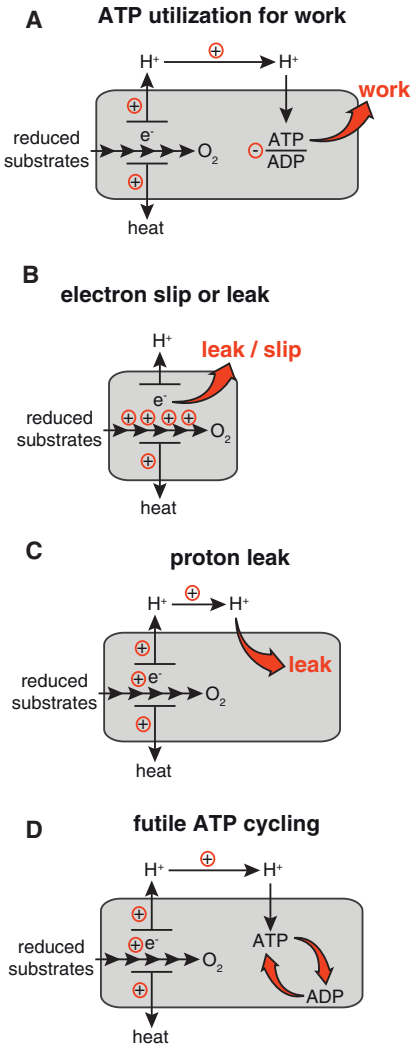

Figure 1. Variables that Affect Thermogenesis

In mammalian cells, mitochondrial oxidative phosphorylation dominates metabolism and as such is the major contributor to cellular heat production. The free energy lost as heat by oxidation of reduced substrates can be elevated by modification of a small number of variables.

(A) When ATP is used to drive mechanical or cellular work in the cell, the resultant decrease in the mitochondrial ATP/ADP ratio increases ATP synthase turnover, which elevates mitochondrial oxidative metabolism and heat production.

(B) If electron transfer reactions were somehow modified to allow substrate oxidation to become uncoupled from concomitant proton translocation across the mitochondrial inner membrane, the rate of substrate oxidation and heat production would increase.

(C) Dissipation of the proton electrochemical gradient through the mitochondrial inner membrane, independent of ATP synthesis, uncouples protonmotive force from the ATP/ADP ratio, and increases substrate oxidation and therefore heat production.

(D) Stimulation of ATP hydrolysis without performance of chemical or physical work in the cell increases proton flux through the ATP synthase, substrate oxidation, and heat production.

Therefore, in cells where the mitochondrial respiratory chain dominates oxidative metabolism, the rate of mitochondrial respiration is the major determinant of heat production (Nedergaard et al., 1977). Since brown and beige adipose tissue metabolism is predominantly oxidative, thermogenesis in these cells is effectively controlled by manipulation of rate limiting steps in respiration.

In this context, Peter Mitchell's chemiosmotic theory provides the framework for our understanding of respiratory control (Mitchell, 1976). Substrate oxidation by the mitochondrial respiratory chain drives an electrochemical proton gradient across the mitochondrial inner membrane. The protonmotive force (Δp) generated by mitochondrial respiration drives protons back into the mitochondrial matrix through the ATP synthase, providing energy for the reaction ADP + Pi → ATP. Therefore, substrate oxidation, oxygen consumption, and ADP phosphorylation are intimately coupled. Respiration rate is controlled directly by Δp, which depends on ATP utilization. In turn, an increase in the ATP/ADP ratio and resulting increase in Δp will slow the rate of mitochondrial respiration. Within the constraints of Mitchell's model, there are a small number of variables that can be manipulated to elicit increased thermogenesis, as discussed below.

Most obviously, cellular demand for chemical and mechanical work can increase, which in turn can drive elevated ATP turnover (Figure 1A). The thermic effects of these kinds of physiological perturbations are evident, for example, upon increased muscle ATP turnover during exercise. Alternatively, mechanisms could exist to alter the efficiency of coupling between (1) substrate oxidation and Δp generation, (2) Δp dissipation and phosphorylation of ADP, and (3) dephosphorylation of ATP and ΔG_p-dependent work in the cell. We consider the coupling of these variables individually:

Scenario 1: Inefficient coupling of respiration and Δp generation could arise if electron transfer reactions become modified to allow electron transfer without concomitant proton translocation (Figure 1B).

Scenario 2: Inefficient coupling of Δp and phosphorylation of ADP could arise if proton re-entry into the mitochondrial matrix occurs independently of the ATP synthase (Figure 1C).

Scenario 3: Inefficient coupling of ΔG_p and cellular work could arise if ATP were hydrolyzed to ADP + Pi in the absence of additional product accumulation or a net mechanical result (Figure 1D).

Inefficient coupling in Scenario 1 would require an alteration of the stoichiometry of the electron transfer and proton translocation reactions, so-called electron slip. Alternatively, it could arise from electron escape from the respiratory chain through a pathway uncoupled from proton translocation, resulting in "electron leak." Although models of electron slip have been proposed, they have not been demonstrated to occur under physiological conditions (Murphy, 1989). The major established electron leak pathway in mitochondria occurs through electron escape from mitochondrial oxidoreductases by single electron reduction of oxygen to generate superoxide. This reaction is thermodynamically favorable and its kinetics are based on variables that determine the proportion of mitochondrial electron carriers that are reactive with O_2 to form superoxide. There are numerous protein sites that contain electron carriers capable of engaging in this electron leak reaction, and this subject is expertly reviewed elsewhere (Murphy, 2009). However, it is unlikely that electron leak to superoxide is sufficient to drive substantial thermogenesis per

se. Even the highest estimations of superoxide generated *in vivo* in this way are not plausibly sufficient to substantially increase substrate oxidation rates (Murphy, 2009). As will be discussed in later sections, the role of superoxide and related reactive species that are generated by mitochondrial electron leak appears instead to have substantial thermogenic effects by enhancing other forms of uncoupling, i.e., via post-translational regulation of protein activity by reactive oxygen species (ROS).

The best characterized form of thermogenesis arises from Scenario 2, uncoupling Δp from ATP synthesis. UCP1 is required for this inducible proton leak in brown and beige adipocytes, and the mechanisms responsible for modulating its activity are discussed in the next section. In addition to UCP1, a number of other protein factors have been suggested to initiate uncoupling of Δp from ATP synthesis. We will discuss the evidence for these pathways as well.

In the case of Scenario 3, futile utilization of ATP has been demonstrated as a thermogenic mechanism to uncouple ΔG_p and cellular work. Evidence has been provided for cellular pathways that initiate this process both in brown and beige adipocytes, as well as in skeletal muscle. In the final sections, we will discuss the evidence and relevance of these pathways in cellular thermogenesis.

Thermogenesis by Inducible Proton Leak through UCP1

The unique aspects of BAT thermogenesis originate from classical biochemical studies of their mitochondria, conducted not long after Mitchell's chemiosmotic theory was first proposed. Studies from the Lindberg and Rafael groups demonstrated that BAT mitochondria exhibit a lack of respiratory control (that is, control by the ATP/ADP ratio), which could be regained in the presence of purine nucleotides and through sequestration of free fatty acid from the incubation medium (Hittelman et al., 1969; Rafael et al., 1969).

Following this, work by Nicholls, Lindberg, Cannon, Rafael, Kozak, and others led to elucidation of UCP1 as a molecular basis for protonophoric activity through the mitochondrial inner membrane (Jacobsson et al., 1985), and identification of the now best-characterized regulators of UCP1: fatty acids and purine nucleotides (Hittelman et al., 1969; Locke et al., 1982; Rafael et al., 1969). The precise mechanisms and interactions of these species with UCP1 are a developing area that has been expertly reviewed recently (Bertholet and Kirichok, 2017). Here we will discuss the aspects of these interactions that inform our current understanding of the role of inducible proton leak through UCP1, and its integration with cellular thermogenesis.

Experiments in isolated BAT mitochondria have demonstrated that increasing concentration of fatty acids drives increased proton conductance through UCP1. Earlier functional studies also suggested that following depletion of endogenous fatty acids from the incubation medium using bovine serum albumin (BSA), UCP1-dependent leak may persist (Parker et al., 2009). These findings suggested that while fatty acids can drive UCP1-dependent uncoupling, they are not required. In contrast, more recent investigations that applied careful titrations of albumin to remove endogenous fatty acids concluded that in the absence of fatty acids, UCP1-dependent leak respiration is absent (Shabalina et al., 2010b). Similar conclusions were made from functional examination of UCP1 function when ectopically expressed in HEK mitochondria (Jastroch et al., 2012). More recently, a seminal study by Kirichok and colleagues used mitochondrial patch-clamp methods to examine fatty acid-dependent UCP1 proton current directly. By applying more stringent methods for removal of endogenous fatty acids, they found that UCP1 proton conductance was completely lost in their absence (Fedorenko et al., 2012). Therefore, current evidence suggests that fatty acids are sufficient to initiate proton leak through UCP1.

Purine nucleotides are capable of inhibiting UCP1 proton translocation at physiologic concentrations. Although all purine nucleotides have similar inhibitory characteristics, because ATP is the dominant species in cells, it is likely to be the most physiologically relevant. Purine nucleotides bind to the cytosolic facing side of UCP1 and appear to occlude the putative proton translocation pathway. Experiments in isolated systems have demonstrated that purine nucleotide inhibition can be overcome to an extent by increasing the levels of long-chain fatty acids. Therefore, purine nucleotide concentrations that exist in the cell are sufficient to substantially inhibit UCP1 proton current. Additionally, elevated local concentrations of long-chain fatty acids overcome purine nucleotide inhibition and drive H^+ leak through a fatty acid/H^+ symport mechanism (Fedorenko et al., 2012). As such, any interpretation of additional factors regulating UCP1 function must consider these variables.

Examination of mechanisms of UCP1 function in *in vitro* systems (for example, isolated mitochondria or proteoliposome reconstitution models) led to identification of these key regulatory factors. However, a major limitation of isolated modeling approaches is that mechanisms are examined when removed from the cellular or organismal context, which necessarily obviates regulatory factors that operate at this level. For this purpose, intact thermogenic adipocytes and genetically engineered mouse models have proven to be highly informative experimental systems that recapitulate many of the basic aspects of UCP1 regulation while allowing for assessment of adipocyte thermogenesis in a physiologically relevant context.

Regulation of UCP1-Dependent Thermogenesis in Brown/Beige Adipocytes

Evidence for the opposing interaction between fatty acids and purine nucleotides is supported by examination of leak respiration in intact brown adipocytes, which, as discussed above, is a reliable assessment of thermogenesis. Remarkably, under unstimulated conditions, brown adipocytes lacking *Ucp1* (UCP1-KO) exhibit a rate of respiration that is indistinguishable from wild-type cells in culture. It is also the case that animals with constitutive inactivation of *Ucp1* exhibit metabolic rates comparable to wild-type animals, again under non-stimulated conditions. This would support the model proposed above whereby under basal conditions in a cell, purine nucleotide concentrations (and perhaps hitherto undiscovered regulatory factors) are sufficient to fully inhibit UCP1-dependent leak.

However, external stimulation reveals the thermogenic capacity of these cells, both in culture and *in vivo*. In experimental mammals and humans, environmental cold is a powerful trigger of brown/beige fat respiration. This stimulus engages cold-sensitive thermoreceptors, which transmit afferent signals to the hypothalamus and brain stem (Morrison et al., 2014; Nautiyal

et al., 2008), leading to the release of norepinephrine from postganglionic sympathetic nerves that innervate brown adipocytes (Hsieh and Carlson, 1957). Norepinephrine acts on adrenoreceptors on the adipocyte plasma membrane, which ultimately results in the release of free fatty acids from stored triglycerides (Harms and Seale, 2013). Upon adrenergic stimulus that results in activation of the brown (and white) adipocyte lipolytic cascade, leak respiration increases in a UCP1-dependent manner (Li et al., 2014). Therefore, it seems as though the opposing effects of purine nucleotides and fatty acids are recapitulated in brown adipocytes *in vivo*.

The relevance of the adrenergic cascade is not limited to cold-induced NST. In fact, the capacity for BAT-mediated NST is increased by exposure to high-calorie diets (diet-induced thermogenesis), suggesting that BAT is a central effector of energy balance (Bachman et al., 2002; Himms-Hagen et al., 1986; Lowell et al., 1993; Rothwell and Stock, 1979). Indeed, genetically engineered mouse models that are predisposed to obesity exhibit reduced adrenergic activation of metabolic rate (Cohen et al., 2014; Lowell and Bachman, 2003). Moreover, individuals who are predisposed to obesity exhibit decreased adrenergic-dependent thermogenic capacity (Jung et al., 1979), suggesting that this impairment may be relevant in the context of human weight gain. Although components of this regulatory system have been identified (Bachman et al., 2002; Huszar et al., 1997; Krude et al., 1998; Lee et al., 1994), the molecular mechanisms that integrate adipocyte thermogenesis with feeding remain incompletely understood.

In the context of adrenergically stimulated thermogenesis, the standard view is that the lipid stores in brown adipocytes function as essential proximal reservoirs for fuel and for activation of UCP1 protonophoric activity. Indeed, the rapid activation of BAT-mediated NST is understood to be facilitated by the proximity of lipid droplets to mitochondria within the brown adipocyte (Boutant et al., 2017; Pidoux et al., 2011). These triglyceride stores are released upon adrenergic stimulus to drive rapid mitochondrial oxidation. Brown adipocyte mitochondria have high respiratory chain abundance, and a high capacity for fatty acid oxidation and TCA activity. As noted above, the released free fatty acids undergo β-oxidation to provide reducing equivalents to maintain Δp and drive respiration, as they simultaneously activate UCP1 (Bertholet and Kirichok, 2017). Paradoxically, recent studies have provided genetic evidence that brown adipocyte lipolysis is dispensable for BAT NST. BAT-selective deletion of ATGL or the ATGL-activating protein comparative gene identification-58 (CGI-58) does not alter BAT NST *in vivo* (Schreiber et al., 2017; Shin et al., 2017). These provocative findings suggest that alternative activating cascades for thermoregulation must exist, and are perhaps compensated for in part by white adipose tissue lipolysis. Along these lines, examination of UCP1-dependent thermogenesis in intact brown adipocytes has revealed numerous additional regulatory elements that operate at the cellular level. We discuss these now individually.

Redox Regulation

Recent studies by several laboratories have demonstrated an important signaling role by ROS in adipose tissue thermogenesis. The importance of ROS signaling in this context has been demonstrated primarily through investigation of redox metabolism using *in vivo* mouse and intact adipocyte models of thermogenesis. This subject was reviewed recently in detail elsewhere (Chouchani et al., 2017), so we will only summarize the state of knowledge here. Genetic or pharmacological elevation of adipocyte ROS levels, or resulting oxidation of cellular thiol status, is sufficient to drive elevated adipocyte thermogenesis (Chouchani et al., 2016; Han et al., 2016; Lee et al., 2016b; Mills et al., 2018; Schneider et al., 2016). The physiological consequences of these manipulations are remarkably consistent: adipocytes exhibit chronically elevated rates of mitochondrial respiration, and mice display elevated whole-body energy expenditure and resistance to weight gain upon high-fat feeding.

Moreover, activation of thermogenesis in mouse BAT by applying either thermal stress (4°C) or β-adrenergic stimulus results in elevated levels of mitochondrial superoxide, mitochondrial hydrogen peroxide, and lipid hydroperoxides (Barja de Quiroga et al., 1991; Chouchani et al., 2016; Mailloux et al., 2012; Stier et al., 2014). This elevated production of mitochondrial ROS, both in cells and *in vivo*, is paralleled by a shift in cysteine thiol redox status. Both protein thiols and glutathione pools became substantially oxidized during acute thermogenic respiration in BAT. Importantly, this change in redox status upon cold exposure is adaptive and reversible, and does not result in higher-order oxidation events that are known to be deleterious to cells (Shabalina et al., 2006). Moreover, pharmacological depletion of mitochondrial lipid peroxides and superoxide *in vivo* impairs the capacity for BAT-mediated thermogenic respiration. The fact that ROS production contributes to physiological regulation of BAT thermogenesis suggests that these species are subject to upstream regulation. A recent study from our group identified one such mechanism whereby substantial and selective accumulation of the mitochondrial metabolite succinate can act as a potent molecular source for thermogenic ROS in brown and beige fat (Mills et al., 2018). The newfound thermogenic activity of succinate in these cells is independent of the lipolytic cascade and relies on its oxidation by mitochondrial succinate dehydrogenase and consequent ROS production. Interestingly, it was shown in this study that thermogenic adipocytes have the capacity to sequester succinate from the circulation, indicating that this mode of regulation is systemically integrated and can be manipulated through elevation of systemic succinate levels.

A major thermogenic action of mitochondrial ROS is likely mediated through protein cysteine modification (Chouchani et al., 2016). Recent application of high-resolution proteomic methodologies has begun to characterize the protein targets of thermogenic ROS in BAT (Chouchani et al., 2016). One functional residue identified was UCP1 Cys253, which is sensitive to oxidative modification, while mutagenesis of this site to alanine retains UCP1 functionality but decreases sensitivity to adrenergic activation. This suggests that UCP1 Cys253 may be an allosteric site that is sensitive to redox modification during thermogenesis and sensitizes UCP1 to fatty acid activation that occurs upon adrenergic stimulus. More generally, the role of reversible thiol oxidation as an effector of thermogenic ROS signaling appears important in the context of triggering thermogenic gene expression and mitochondrial biogenesis, as well as in the acute control of thermogenic respiration (Chouchani et al., 2016; Han et al., 2016; Ro et al., 2014). It is clear from initial findings that functional

protein cysteine targets of ROS during thermogenesis are not limited to UCP1 (Chouchani et al., 2016). The profound effects of manipulating redox status in adipocytes presumably involve modification of many functional targets involved in regulating thermogenic respiration as well as thermogenic gene expression. Future studies characterizing the functional targets of thermogenic ROS, as well as the metabolic pathways controlling thermogenic ROS, can in this way lead to a new class of molecular targets that may be manipulated to enhance the function of thermogenic adipose tissue.

The close interplay between ROS metabolism and UCP1-dependent thermogenesis is further illustrated by the phenotype exhibited by UCP1-KO mice (Enerbäck et al., 1997). This mouse is typically used as a model for the specific physiological relevance of UCP1. However, recent data have demonstrated that UCP1-KO animals acquire molecular changes in BAT that extend well beyond the deletion of UCP1 itself (Kazak et al., 2017b). Importantly, mitochondria from UCP1-KO BAT are extraordinarily sensitive to ROS-induced dysfunction and possess an impaired ability to buffer calcium. This dysfunction is associated with immune infiltration and markers of cell death *in vivo* (Kazak et al., 2017b). These data suggest that UCP1 activity is critical for buffering redox metabolism in the highly oxidizing environment of brown and beige adipocyte mitochondria, while absence of this factor leads to substantial macromolecular dysfunction. In the absence of UCP1, the abundance of the respiratory chain of BAT mitochondria from UCP1-KO animals is dramatically reduced compared to BAT from wild-type mice (Kazak et al., 2017b). Critically, these mitochondrial alterations are present in BAT of UCP1-KO mice at thermoneutrality and become much more prominent following exposure to decreased environmental temperature (Bal et al., 2017; Kazak et al., 2017b; Keipert et al., 2017). A large reduction in respiratory chain abundance of UCP1-KO mice also occurs in beige fat (Shabalina et al., 2013), suggesting that the acquired molecular features following UCP1 deletion are not limited to BAT. It is clear from these findings that the constitutive UCP1-KO mouse is not appropriate for the study of UCP1 function *in vivo*, but is better suited as a model of global BAT and beige fat dysfunction more generally. Thus, prior work using this model must be reinterpreted to consider these new findings.

Regulation of UCP1 Activity through Purine Nucleotide Breakdown

Classical examinations of UCP1 in isolation demonstrated that in the absence of fatty acids, purine nucleotides effectively inhibit UCP1-mediated uncoupled respiration. Based on the mM abundance of purine nucleotides in the cytosol, and their <1 μM dissociation constants with UCP1 determined in proteoliposome reconstitution experiments (Klingenberg and Winkler, 1985), it has been suggested that these species are constitutive inhibitors of UCP1 activity *in vivo*. However, recent examinations of this framework suggest greater complexity. First, the distinct biophysical properties of the brown adipocyte mitochondrial inner membrane (i.e., high cardiolipin content) are likely to substantially increase the dissociation constant of purine nucleotides and UCP1 by at least an order of magnitude (Klingenberg, 2009). Moreover, the cytosolic pH of thermogenic adipocytes is dynamic, for example, increasing substantially upon adrenergic stimulation, which can further increase the K_D of purine nucleotides and UCP1 (Chinet et al., 1978). Finally, inhibitory binding of UCP1 by purine nucleotides only occurs in a state where they are not coordinated by divalent cations. The pools of cytosolic calcium and magnesium are similarly dynamic, and they are therefore likely to influence the total amount of free purine nucleotide available for UCP1 inhibition (Klingenberg, 1988). A recent study of purine nucleotide metabolism in brown adipocytes has indicated that the total purine nucleotide pool size in brown adipocytes is relatively small, and ATP and ADP pools are depleted upon β-adrenergic stimulation (Fromme et al., 2018). Critically, this depletion results in a substantial decrease of the total pool of purine nucleotides. Interestingly, pharmacological inhibitors of the purine nucleotide breakdown pathway significantly inhibit β-adrenergically induced respiration in brown adipocytes, while overexpression of guanosine monophosphate reductase is sufficient to deplete guanosine triphosphase pools and potentiate fatty acid-dependent respiration through UCP1. These findings suggest that modulation of purine nucleotide pool size in thermogenic adipocytes is a mode of regulation of UCP1-dependent thermogenesis.

Regulation of Mitochondrial Architecture

Examination of mitochondrial morphology upon acute activation of brown adipocyte thermogenesis has also revealed the critical importance of structural regulation of these organelles in coordinating heat production. Recent studies have demonstrated that adrenergic stimulation of brown adipocyte thermogenesis results in acute fission of the mitochondrial network, which apparently precedes mitochondrial depolarization (Wikstrom et al., 2014). This acute regulation of mitochondrial architecture involves PKA-dependent phosphorylation on Ser660 of the fission protein DRP1. Interestingly, it has been shown that fatty acid-mediated uncoupling is potentiated by mitochondrial fission (Wikstrom et al., 2014), while genetically driven hyperfusion of adipocyte mitochondria inhibits thermogenesis and increases the propensity for obesity. However, specific inhibition of outer membrane fusion does not recapitulate the effects of mitochondrial inner membrane remodeling. Genetic ablation of MFN2, a factor mediating fusion of the outer mitochondrial membrane, elicits a paradoxical impairment of BAT thermogenesis and protection from diet-induced obesity (Boutant et al., 2017). While at this stage, the mechanisms linking mitochondrial morphology to thermogenic function are unclear, it is reasonable to suppose that modification of the mitochondrial inner membrane architecture could alter relative efficiencies of leak pathways either directly or indirectly. We expect that future studies will examine the mechanisms through which mitochondrial architecture regulates thermogenic modalities described above (Figure 1).

Alternative Effectors of Adipocyte Thermogenesis: UCP1 Is Completely Dispensable

The findings summarized above indicate the complexity of regulatory pathways that converge to activate adipose tissue thermogenesis. Along different lines, substantial work has focused on whether presence of the UCP1 protein alone is necessary to drive adipocyte thermogenesis, with many of the seminal findings originating from the Kozak group. Mice with targeted deletion of *Ucp1* are incapable of regulating body temperature

upon acute exposure to cold (Enerbäck et al., 1997). However, an important (and not often discussed) finding is that the acute cold sensitivity of UCP1-KO mice is particular to the inbred C57BL/6J and 129/SvImJ backgrounds (Ukropec et al., 2006). Of note, the acute cold sensitivity occurs with varying penetrance between these two congenic lines, and is completely lost in the F1 hybrid strain (129/SvImJ × C57BL/6J) (Hofmann et al., 2001; Ukropec et al., 2006). Together, these findings strongly indicate that UCP1-independent thermogenic mechanisms are constitutively present in UCP1-KO mice on a hybrid background. Furthermore, UCP1-KO mice on an inbred background can be adapted to tolerate cold by gradually decreasing environmental temperature, a phenotype that was first demonstrated by the Kozak group (Hofmann et al., 2001) and later confirmed by numerous studies (Golozoubova et al., 2006; Kazak et al., 2015, 2017b; Keipert et al., 2015, 2017; Ukropec et al., 2006). Recently, it was demonstrated that the cold sensitivity of inbred UCP1-KO mice can be completely rescued by crossing this strain to mice expressing transgenic PRDM16 driven by a Fabp4 promoter (Ikeda et al., 2017). Together, these data illustrate the importance of mechanisms independent of UCP1 in the regulation of NST in adipocytes.

Interestingly, UCP1-KO mice are resistant to diet-induced obesity at sub-thermoneutral temperatures, presumably via activation of poorly defined alternate routes of energy loss in the absence of UCP1 (Enerbäck et al., 1997; Liu et al., 2003). These findings contrast with mice lacking β-adrenoreceptors, or models of BAT ablation, both of which become obese at room temperature (Bachman et al., 2002; Lowell et al., 1993). Absent thermal stress UCP1-KO animals succumb to obesity and have altered whole-body energy expenditure in response to exogenous application of adrenergic stimuli (Feldmann et al., 2009; Rowland et al., 2016; von Essen et al., 2017), while the protection against obesity observed in UCP1KO mice at sub-thermoneutral temperatures is reversed by transition to thermoneutrality (Anunciado-Koza et al., 2008; Liu et al., 2003). Of course, interpretation of these data must consider the above-discussed acquired macromolecular dysfunction of UCP1-KO adipose tissues, which substantially extend beyond deletion of the protein itself (Kazak et al., 2017b). However, it is noteworthy that other genetically engineered mouse models that exhibit substantial loss of UCP1 protein in BAT do not succumb to obesity at thermoneutrality, suggesting either UCP1-dependent compensation in other tissues or compensation by alternative thermogenic processes in BAT (Harms et al., 2014; Lee et al., 2016a).

Skeletal muscle has been posited to support Ucp1-independent thermogenesis through enhanced capacity for shivering thermogenesis based on a "training" effect due to chronic contractile activity (Golozoubova et al., 2001), but the evidence for this is indirect and inconsistent. Taken together, the major thrust of reported data show that shivering-induced adaptations to skeletal muscle occur in mice, but the evidence in support of enhanced skeletal muscle metabolism in mice with depleted BAT UCP1, akin to training, is conflicting (Golozoubova et al., 2001; Meyer et al., 2010; Mineo et al., 2012; Monemdjou et al., 2000; Shabalina et al., 2010a; Ukropec et al., 2006). It appears unlikely that enhanced muscle shivering is a sufficient thermogenic pathway in the absence of UCP1 since shivering is quantitatively and qualitatively indistinguishable in UCP1-KO mice compared to wild-type animals whether exposed to cold acutely or gradually (Golozoubova et al., 2001). Next, we discuss alternative mechanisms of NST that have been uncovered in recent years.

Creatine-Dependent Substrate Cycling

Reductions in creatine levels have been linked to deregulated thermal homeostasis in rodent models (Wakatsuki et al., 1996; Yamashita et al., 1995), through unknown mechanisms. The known metabolic utilization of creatine acts in a 1:1 stoichiometric relationship with ATP, such that addition of a quantity of creatine to coupled mitochondria results in a molar equivalent production of ADP and phosphocreatine through CK-mediated phosphotransferase activity: [ATP + creatine → ADP + phosphocreatine] (Jacobus and Lehninger, 1973). This relationship can be observed by monitoring mitochondrial respiration rate, which through the mitochondrial P/O ratio provides an index of the ADP phosphorylation rate in coupled mitochondria. Through this analysis, we recently demonstrated that the respiratory response of thermogenic adipocyte mitochondria to creatine suggests the production of a molar *excess* of ADP with respect to creatine, which leads to enhanced stimulation of mitochondrial respiration under ADP-limited conditions (Bertholet et al., 2017; Kazak et al., 2015). In this case, the relationship of creatine to ADP liberation is substoichiometric. These findings suggest the presence of a mitochondrial substrate cycle that is regulated by creatine to drive thermogenic respiration. Phosphocreatine may be hydrolyzed directly or creatine may control the turnover of ATP indirectly. Importantly, the thermogenic action of creatine seems to occur only when ADP is limiting, which is the expected parameter of the physiological cellular state. This phenomenon was initially identified as selective for mitochondria isolated from beige fat of cold-exposed animals. However, it appears likely that creatine energetics is a key effector of NST in all adipose depots.

Fat-selective deletion of the rate-limiting enzyme of creatine synthesis, glycine amidinotransferase, *Gatm* (Adipo-Gatm KO), reduces creatine in BAT and results in cold intolerance without a change in BAT UCP1 protein abundance (Kazak et al., 2017a). Of note, UCP1 protein levels are markedly higher in purified subcutaneous (SubQ) adipocytes from cold-exposed Adipo-Gatm KO animals (Kazak et al., 2017a), likely a consequence secondary to BAT dysfunction. Along with impaired thermoregulation, Adipo-Gatm KO animals exhibit suppressed thermogenesis following β3-adrenergic agonism, and creatine supplementation rescues this thermogenic defect to wild-type levels. The ability to activate diet-induced thermogenesis is also severely blunted in Adipo-Gatm KO mice, and this is associated with increased metabolic efficiency and susceptibility to rapid-onset diet-induced obesity (Kazak et al., 2017a). Consistent with the work from our groups, global creatine transporter (*Slc6a8*) knockout mice exhibit similar levels of creatine depletion as Adipo-Gatm KO animals, and have increased body fat stores compared to controls (Perna et al., 2016). Moreover, global *Ckmt1*/*Ckb* double knockout animals are cold-sensitive and have impaired capacity to activate thermogenic respiration in response to norepinephrine administration (Streijger et al., 2009). Together, these data provide several lines of genetic evidence in support of a role for creatine energetics in adaptive

thermogenesis and suppression of obesity. It is important to note that, while the above studies are consistent with creatine-dependent substrate cycling occurring *in vivo*, it is possible that adipocyte creatine energetics also support thermogenic respiration via alternative mechanisms. Future work will focus on identifying these potential additional processes that are supported by creatine metabolism in thermogenic fat. Areas of particular importance for future study include the use of stable isotope tracing to determine whether the effects of creatine, both in mitochondria and *in vivo*, are in fact due to futile cycling of ATP and ADP. Identification of the small molecules and proteins involved in these phosphotransfer reactions will be important to understand the mechanisms of the thermogenic effects of creatine. In addition, it will be critical to identify the creatine kinases and related proteins that engage in atypical oxidative metabolism in thermogenic fat.

These questions are particularly clinically relevant as genes and proteins regulating creatine metabolism are highly selective for BAT compared to white fat in humans (Müller et al., 2016; Svensson et al., 2011), while clonal human brown adipocytes also exhibit substantial respiratory impairment following modulation of creatine metabolism (Kazak et al., 2015). Finally, a recent analysis of ~2,850 ^{18}F-FDG PET/CT scans from 1,644 human subjects demonstrated that renal creatinine clearance was a significant predictor of total activated human BAT (Gerngroß et al., 2017). Since creatinine is a direct product of phosphocreatine metabolism, these results are consistent with activation of creatine-dependent thermogenesis in human BAT and suggest that creatinine may be used as a biomarker of human BAT activity.

Calcium-Dependent ATP Hydrolysis

A role for calcium transport in NST has been proposed in both BAT and muscle and posited to support thermogenic respiration through modulation of the SERCA ATPase activity (de Meis, 2003; de Meis et al., 2006; Periasamy et al., 2017). Typically, SERCA-dependent ATPase activity is coupled to calcium sequestration in the SR/ER, so calcium accumulation feeds back to prevent excessive ATP turnover that would preclude futile cycling and thermogenesis. However, there is precedence for "calcium leak" pathways involving SERCA that would be predicted to drive thermogenesis, and have been demonstrated in the extraocular heater muscle cells of certain fish (Block, 1994; Rosenberg et al., 2015), and in conditions of malignant hyperthermia. Of course, for such a mechanism to be relevant for NST more generally would require some inducible element that uncouples SERCA ATPase activity from calcium translocation into the ER/SR. In this context, Sarcolipin (Sln), a small peptide localized to the sarcoplasmic reticulum (SR) of skeletal muscle, has been proposed as a key protein to control elevated SERCA-mediated ATP turnover in muscle via calcium cycling (Smith et al., 2002). Sln has been shown to bind SERCA directly and to modulate its capacity to transport calcium across the SR membrane without affecting ATPase activity. So Sln appears to uncouple calcium transport from ATP hydrolysis by SERCA, which would be predicted to elevate thermogenesis (Sahoo et al., 2013). The relevance of Sln for modulating thermogenesis in this way is supported by substantial *in vivo* data. Mice with surgically removed interscapular BAT (iBAT ablation) maintain body temperature in response to acute cold challenge, while *Sln* knockout ($Sln^{-/-}$) animals are mildly cold intolerant (Bal et al., 2012). Importantly, when exposed to 4°C for a prolonged period, iBAT-ablated $Sln^{-/-}$ mice succumb to hypothermia, despite the maintenance of skeletal muscle shivering (Bal et al., 2012). The role of Sln-dependent thermogenesis in diet-induced obesity has further implicated its role as a key regulator of heat production. $Sln^{-/-}$ mice become obese on a high-fat diet, while muscle-specific Sln transgenic animals (Sln^{OE}) are resistant to diet-induced obesity (Rowland et al., 2016; Maurya et al., 2015). The capacity to activate skeletal muscle metabolism following administration of a β2-adrenergic receptor agonist, Formoterol, is impaired in $Sln^{-/-}$ mice, while it is enhanced in Sln^{OE} animals (Maurya et al., 2015). These findings indicate the importance of SLN in modulating whole-body energy expenditure, and a plausible calcium uncoupling phenomenon has been demonstrated *in vitro* (Bal et al., 2012). It remains to be determined whether Sln-mediated SERCA uncoupling drives sufficient ATP turnover rates in cells and *in vivo* to result in physiologically meaningful levels of thermogenesis (Campbell and Dicke, 2018). In addition to SLN, it is possible that other factors modulate the coupling of SERCA ATP hydrolysis to calcium transport. It will be interesting to explore in future work the extent to which these cycles are operational in adipose tissues and muscle, and upon which SERCA isoforms and regulatory factors they depend. A recent study has demonstrated that Fabp4 promoter-controlled PRDM16 overexpression overcomes acute cold intolerance of UCP1-KO animals, which coincides with *Serca2b* mRNA induction (Ikeda et al., 2017), suggesting that SERCA-dependent ATP turnover could play an important role in BAT and beige fat, even in the absence of UCP1. Addressing the thermogenic role of SLN and SERCA metabolism will require direct monitoring of SLN and SERCA-dependent ATP turnover rates and respiration in cellular and mouse models. In this way, direct thermogenic effects can be differentiated from indirect consequences of genetic manipulation, which, as described in the case of the UCP1-KO model, can be misleading on its own.

Lipid Cycling

Resynthesis of tricylglycerols following lipolysis has been proposed as a thermogenic process based on the ATP demand of tricylglycerol synthesis. Fatty acid synthesis and oxidation are tightly coupled in adipocytes in response to adrenergic activation. Adipocyte triglyceride content remains constant during sustained adrenergic activation, suggesting that adipocytes might upregulate fatty acid synthesis to compensate for elevated fat oxidation (Granneman et al., 2003). Consistently, cold simultaneously increases the expression of genes involved in fatty acid synthesis and oxidation in BAT (Yu et al., 2002). Importantly, this process has been measured directly using deuterium-labeled water. Monitoring lipid cycling in this way *in vivo* demonstrated that β3-adrenergic agonism and cold exposure trigger fatty acid re-esterification, as indicated by deuterium labeling of glycerol in triglycerides (Mottillo et al., 2014; Flachs et al., 2017). This compensation demonstrates that maintenance of triglyceride stores in adipocytes is under strict metabolic regulation. However, the extent to which re-synthesis of triacylglycerols is sufficiently energetically costly

to drive substantial increases in energy expenditure remains to be determined.

UCP1-Independent Proton Leak

In addition to inducible proton leak via UCP1, the capacity for mitochondria to leak protons independent of UCP1 has been the subject of long-standing investigation (Jastroch et al., 2010). Through examination of oxygen consumption rate and membrane potential in isolated mitochondria, evidence has been provided for proton leak that is initiated at high membrane potential and appears to be a feature common to most, if not all, mitochondria. This phenomenon has been characterized indirectly (through examination of oxygen consumption rate, which will increase in a non-linear proportion to elevated proton leak) and the nature of the factor(s) responsible are still unclear. UCP family members have been proposed to play a role; however, direct evidence supporting their activity as leak proteins is absent. Examination of UCP3 function *in vivo* has demonstrated a clear effect on thermal homeostasis (Riley et al., 2016), suggesting it plays a role; however, whether this is via direct activation of proton leak remains to be established. The ATP/ADP carrier (AAC) has also been proposed to play an important role in regulating proton leak, and the ubiquitous presence of AACs in mitochondria supports the observations suggesting proton leak in most/all mitochondrial populations. Moreover, it has been demonstrated that AAC1 controls membrane-potential-dependent increases in mitochondrial oxygen consumption under ATP-synthase-inhibited conditions (Brand et al., 2005). Although direct evidence that AACs translocate protons across the mitochondrial inner membrane is still lacking, the suggestive evidence certainly merits investigation on this point. For example, recently applied methods for determination of protonophoric activity across the mitochondrial inner membrane (Fedorenko et al., 2012) could be applied to determine whether, and under what conditions, AACs and UCP family members are bona fide proton leak proteins.

Modern Therapeutics Focused on Adaptive Thermogenesis

Obesity occurs when there is a chronic imbalance between assimilated energy and energy expenditure. How and why many disorders like type 2 diabetes and cardiovascular disorders arise from obesity are hotly researched topics, but it is clear that obesity may be reduced only by affecting either side of the energy balance equation. Proof that increases in metabolic rate can ameliorate obesity in humans comes from clinical experience with the compound dinitrophenol (DNP) in the era of the 1930s (Tainter et al., 1934). Exposure to this compound first occurred in munitions factories, where people experienced weight loss linked to elevations in metabolic rates. In fact, DNP causes an uncoupling of respiration from ATP synthesis by facilitating proton leak across most or all mitochondrial inner membranes. As predicted from classical bioenergetics, patients felt warm and lost weight. Unfortunately, they also suffered from heart problems and cataracts; several patients died because of DNP ingestion. Obviously, uncoupling in all tissues is potentially problematic as not all tissues have ''spare capacity'' when it comes to mitochondrial function within cells. Similar symptoms are observed in the hypermetabolic condition of Luft's disease due to inefficient coupling of mitochondrial Δp and phosphorylation of ADP, as well as apparent elevations in basal cellular ATPase activity (DiMauro et al., 1976). Nevertheless, the DNP episode is ''proof of concept'' that increased respiration through mitochondrial activity can treat human obesity.

With expanding knowledge about brown and beige fat, interest in these tissues as targets for safely increasing mitochondrial respiration and energy expenditure has re-emerged. Animal models with greatly increased brown and beige fat amounts and activities have shown powerful metabolic improvements with no detectable pathologies. So what are the prospects for seeing new drugs emerge using pathways in these adipocytes? To analyze this question critically, we must first consider the biomedical landscape around the world. There are currently many treatments available to treat type 2 diabetes; while these treatments are not ideal, they represent a formidable barrier for the development of new anti-diabetic drugs. Furthermore, the recent requirement by the Food and Drug Administration for anti-diabetic drugs to demonstrate improvement in cardiovascular outcomes means that the development of these drugs will be costly and take many years. In contrast, there are no effective medical treatments for obesity, so if efficacy is shown in humans for a significant fraction of obese patients, this could represent a ''first in class'' pharmaceutical breakthrough. Similarly, an anti-diabetic action of a ''browning'' agent would be more attractive as a clinical candidate if it were accompanied by strong weight loss, even if the latter was not quite enough to be marketed as an anti-obesity drug per se.

Finally, fatty liver disease has emerged as an important health problem linked to obesity. This condition leads, in a significant number of cases, to cirrhosis and liver tumors. It is notable that mice lacking beige fat cells due to an adipose deletion of PRDM16 develop a fatty liver and profound hepatic insulin resistance (Cohen et al., 2014). Why this phenotype is prominent in beige-less mice is not completely clear, but it does suggest that activation of beige fat cells in humans might well serve a hepato-protective role. Notably, clinical trials in humans targeting brown and beige fat have begun (Cypess et al., 2015), and we will likely see many more in the coming years.

DECLARATION OF INTERESTS

B.M.S. is a consultant for Calico LLC. E.T.C. has filed a patent on his work relating to succinate and thermogenesis.

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