Mitochondrial reactive oxygen species and adipose tissue thermogenesis: Bridging physiology and mechanisms

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Brown and beige adipose tissues can catabolize stored energy to generate heat, relying on the principal effector of thermogenesis: uncoupling protein 1 (UCP1). This unique capability could be leveraged as a therapy for metabolic disease. Numerous animal and cellular models have now demonstrated that mitochondrial reactive oxygen species (ROS) signal to support adipocyte thermogenic identity and function. Herein, we contextualize these findings within the established principles of redox signaling and mechanistic studies of UCP1 function. We provide a framework for understanding the role of mitochondrial ROS signaling in thermogenesis together with testable hypotheses for understanding mechanisms and developing therapies.

Brown adipose tissue (BAT) has long been recognized as a critical thermogenic organ, exemplified by its importance in maintenance of thermal homeostasis in cold environments (1). More recently, clusters of distinct adipocytes with thermogenic capacity have been characterized in white adipose tissue—beige adipocytes—arising in response to various physiological stimuli (2, 3). The thermogenic endowment of these tissues has prompted the reasonable hypothesis that they might be leveraged to catabolize lipid and glucose as an anti-obesity and anti-diabetic therapy. As such, recent decades have seen vigorous research into the mechanisms controlling brown and beige adipose tissue identity and function. Thermogenesis in BAT and beige adipose tissue relies on the key effector protein uncoupling protein 1 (UCP1), which can dissipate the electrochemical \( \Delta H^+ \) gradient across the inner mitochondrial membrane (4). This UCP1-mediated inducible proton “leak” uncouples protons from ATP synthesis, leading to increased mitochondrial respiration and heat generation. Additionally, UCP1-independent effectors of thermogenesis have been uncovered in recent years, including utilization of creatine-dependent substrate cycling in beige adipose tissue (5). Of course, expression of UCP1 (or other effectors) per se is not sufficient to drive thermogenesis; these are gas pedals that need a foot, fuel, and an engine. Indeed, thermogenic adipocytes require exceptionally high mitochondrial content and oxidative capacity to support elevated respiration. Moreover, thermogenic respiration is regulated acutely by upstream physiological signals that are required to activate UCP1-mediated uncoupling. So, elucidating the mechanisms that activate thermogenically-poised adipocytes is critical from a therapeutic standpoint.

The purpose of this review is to contextualize recent findings that provide evidence for an important signaling role by reactive oxygen species (ROS) in adipose tissue thermogenesis. Remarkably, the importance of ROS signaling in this context has been demonstrated primarily through investigation of redox metabolism using \textit{in vivo} mouse and intact adipocyte models of thermogenesis. In contrast, studies of ROS and thermogenesis using \textit{in vitro} and reconstituted systems have provided a more confusing picture. This juxtaposition exemplifies an important general concept in the investigation of ROS biology, which we will detail herein. In doing so, we hope to provide a framework for examining mechanisms of ROS signaling in thermogenesis, toward bridging the striking physiological observations and their mechanistic underpinnings.

Examination of mitochondrial ROS signaling in adipose tissue physiology

Perhaps more than any other signaling process, the physiological causes and consequences of ROS production are extremely difficult to faithfully model using \textit{in vitro} systems. Although ROS are often described and studied as a monolithic entity, they are in fact a diverse class of redox-active molecules each of which exhibit distinct dynamics, reactivity, and biological consequences (6–9). Moreover, physiological ROS production exhibits extraordinary spatiotemporal regulation (10–15). These principles are well-reviewed elsewhere (10–15) and underscore the framework required for molecular characterization of physiological ROS processes.

In short, to move on from the inexact concept of “oxidative stress,” precise examination of ROS signaling in physiology requires assessment of several variables. Important questions include the following. What reactive species are involved in driving a physiological process? Where are they being produced? What are their molecular targets? What are the consequences of ROS modification of these targets? The extent to which these questions can be answered relies on tools that allow...
us to examine ROS processes in intact organisms. Examining ROS-dependent signaling using *in vitro* systems (for example isolated mitochondria or reconstituted models) necessarily introduces many caveats that may confound interpretation. First, these studies are carried out at atmospheric oxygen tension, and as such ROS-dependent processes almost certainly will not faithfully recapitulate *in vivo* dynamics (6). Second, examining ROS signaling removed from the cellular or organismal context necessarily obviates regulatory factors that both initiate and respond to redox signals (16). Obvious physiological parameters likely to be confounded along these lines include local maintenance of redox status, control of electron supply through physiological sites of ROS production, and redox signaling cascades maintained at the cellular and organismal level.

The clearest evidence for the role of ROS signaling—both in thermogenesis and in physiology generally—has emerged from experimental systems wherein ROS and redox status are manipulated in a physiologically relevant context, *i.e.* intact organisms and cells. Moreover, application of these methods has allowed for differentiation of the role of ROS in supporting thermogenic adipocyte function from the contrasting roles these same molecules play in other cell types during thermogenesis (for example in the context of hypothalamic integration with the thermogenic response (17)). We will begin by providing an overview of the current understanding of mitochondrial ROS metabolism in thermogenic adipose tissue.

**Redox metabolism during thermogenesis *in vivo***

To our knowledge, our recent study was the first detailed assessment of *in vivo* brown adipose tissue ROS metabolism upon acute activation of thermogenesis (18). Established *in vivo* mouse models of acute activation of UCP1-dependent thermogenesis in BAT were examined by applying either thermal stress (4°C) or β3-adrenergic stimulus. Acute activation of BAT thermogenesis was paralleled by elevated levels of mitochondrial superoxide, mitochondrial hydrogen peroxide, and lipid hydroperoxides in BAT. Notably, this phenomenon was recapitulated in isolated brown adipocytes; upon β3-adrenergic stimulus with norepinephrine, these cells exhibited increased levels of ROS, which rose concomitantly with UCP1-dependent respiration. This elevated production of mitochondrial ROS, both in cells and *in vivo*, was paralleled by a shift in cysteine thiol redox status. Both protein thiols and glutathione pools became substantially oxidized during acute thermogenic respiration in BAT. Interestingly, measures of lipid peroxidation are consistently elevated in BAT following several weeks of cold exposure (19). Moreover, oxidation of BAT cellular thiols appears to persist following chronic activation of thermogenesis (20, 21). However, the quantitative effects on redox status appear to be less following chronic BAT stimulation, most likely due to compensatory elevation in expression of endogenous thiol antioxidant systems (19, 21). Very importantly, changes in ROS levels and thiol redox status in these systems are not paralleled by elevation in irreversible oxidative adducts on proteins (22). So, it appears as though the redox changes that occur in BAT upon thermogenesis are dynamic, reversible, and adapted to by endogenous thiol antioxidant pathways.

**Elevated mitochondrial ROS levels support adipose tissue thermogenesis *in vivo***

Elevation of mitochondrial ROS during BAT thermogenesis raises the question of whether these species are regulators of thermogenic function or merely epiphenomena. A remarkable number of recent studies have clarified this question by applying pharmacological and genetic manipulations of mitochondrial redox metabolism using both animal and cellular models.

Boudina and co-workers (23) recently characterized mice with adipocyte-specific knock-out of SOD2 (AdSOD2KO), the primary enzyme responsible for dismutation of mitochondrial superoxide to hydrogen peroxide. These mice exhibit elevated superoxide levels in adipose tissues, no evidence of irreversible oxidative adducts, and are extraordinarily resistant to weight gain on a high-fat diet. Moreover, this effect is entirely attributable to chronically elevated thermogenic energy expenditure in adipose tissues upon high-fat feeding. Analysis of adipose tissues and adipocytes further clarified the effects of KO of SOD2 on thermogenesis, which were 2-fold.

In inguinal white adipose tissue (iWAT), the KO of SOD2 was sufficient to increase abundance of UCP1-expressing adipocytes with elevated mitochondrial content, respiratory capacity, and thermogenic potential, suggesting that beige adipocyte differentiation can be driven by mitochondrial superoxide-dependent signaling. Additionally, SOD2KO in BAT drove enhanced basal mitochondrial uncoupling, fatty acid oxidation, and increased cell-autonomous adipocyte leak respiration. Thus, elevated mitochondrial superoxide in adipose tissue appears sufficient to both drive browning in iWAT, as well as to drive activation of UCP1-dependent leak respiration in thermogenically competent adipocytes. In interpreting these findings, it is important to note that although UCP1 facilitates uncoupled respiration, in the cellular milieu it is maintained in a purine-nucleotide-bound state, which renders it inactive (4, 24). Current evidence suggests that in the native adipocyte environment, UCP1 expression is not sufficient to drive uncoupled respiration basally, instead it requires activation (the details of factors affecting UCP1 activation state are described in the final section of this review). Therefore, diet-induced initiation of mitochondrial uncoupling in SOD2KO adipocytes involves cell-autonomous modulation in the activation status of UCP1-dependent respiration. This is supported by the observation that, in SOD2KO adipocytes, leak respiration specifically is enhanced, while chemically uncoupled maximal respiration is unaffected. So, enhancement of respiration is attributable to a specific effect on the UCP1-dependent “leak” component of mitochondrial respiration as opposed to increased mitochondrial content or oxidative capacity. It merits noting that despite chronically elevated energy expenditure upon high-fat feeding and upon mild cold stimulus (15°C), the AdSOD2KO mice are sensitive to acute 4°C exposure. This discrepancy suggests that lack of lipid stores in BAT (which are key for supporting acute thermal stress) in the SOD2KO model is also a key physiological consequence of elevated superoxide-dependent signaling that merits further investigation.
The role of superoxide in supporting thermogenesis has also been examined through generation of mice overexpressing SOD2 in all tissues (SOD2OE) (25). Interestingly, these mice did not exhibit alterations in body weight (although mice were not subjected to high-fat feeding) or in acute activation of adipose thermogenesis with norepinephrine. However, in this study redox status and ROS levels were not assessed in BAT or intact adipocytes during thermogenic respiration, so it is unclear to what extent SOD2OE modified mitochondrial redox status in BAT in vivo. Superoxide levels were lower in isolated BAT mitochondria treated with rotenone and respiring on glycerol 3-phosphate, although it is unlikely that these in vitro conditions recapitulate ROS and redox status in intact cells or the intact organism.

In comparing the effects of SOD2KO and SODOE on thermogenesis, it is worth considering in detail the likely consequences of these manipulations on ROS metabolism. SOD2 very rapidly catalyzes dismutation of superoxide to hydrogen peroxide ($k \approx 10^9 \text{ M}^{-1}\text{s}^{-1}$) by a process that shows first-order kinetics with respect to superoxide, as SOD2 concentrations in mitochondria are orders of magnitude higher than superoxide (26). Therefore, basal levels of SOD2 act as a highly effective superoxide sink that favors reaction of oxygen with electron donors in effect increasing flux to hydrogen peroxide in the mitochondrial matrix (10). Moreover, for SOD2OE to affect this rate of dismutation, basal SOD2 levels would have to be limiting in BAT, which may not be the case in the above model. However, SOD2KO elevates steady-state superoxide and would promote alternative downstream metabolism normally diminished by the endogenous SOD2 superoxide sink. Spontaneous dismutation of superoxide to hydrogen peroxide is only $k \approx 10^6 \text{ M}^{-1}\text{s}^{-1}$, and it requires two superoxide molecules for the reaction thus making it far less favorable kinetically by comparison. SOD2KO would therefore promote generation of alternative ROS species such as hydroperoxide (protonated superoxide) and downstream lipid hydroperoxides, at the expense of hydrogen peroxide generation. Indeed, it will be informative to determine how ablation of SOD2 in adipocytes affects the levels of different types of ROS metabolites and the overall redox status in adipocyte mitochondria.

We examined the causal relationship between mitochondrial ROS and thermogenesis using the mitochondria-targeted antioxidant MitoQ, which is well-established to efficiently deplete mitochondrial lipid peroxides and superoxide upon acute administration in vivo (27–29). Indeed, acute depletion of these species was observed in BAT upon MitoQ administration prior to cold exposure. Under these conditions, capacity for BAT-mediated thermogenic respiration was substantially inhibited. In this study, the importance of mitochondrial ROS in supporting UCP1-dependent thermogenesis was assessed using UCP1 knock-out (UCP1-KO) mice. UCP1-KO mice rely exclusively on alternative mechanisms of thermogenesis (5, 30) and did not exhibit inhibition of thermogenesis following depletion of mitochondrial ROS with MitoQ. This result provides strong genetic evidence that mitochondrial ROS appear key for supporting UCP1-dependent thermogenesis in vivo.

**Modification of thiol redox status**

Effects on adipose tissue thermogenesis by modulation of mitochondrial ROS levels are strikingly similar to those generated by manipulation of adipocyte thiol redox status. Indeed, several genetic manipulations that drive elevated adipocyte steady-state ROS levels and oxidation of adipocyte thiol redox status result in strikingly similar metabolic phenotypes. A prominent example is found when examining mice lacking nuclear factor-erythroid 2-related factor 2 (NRF2), a transcription factor controlling a broad range of ROS- and thiol-targeted antioxidant enzymes. NRF2KO mice are resistant to weight gain on a high-fat diet (31, 32), and these effects are attributable to chronically elevated energy expenditure (33). Like AdSOD2KO mice, this model exhibits increased browning of iWAT as well as elevated basal “leak” respiration (but not elevated maximal respiration) in isolated adipocytes. Importantly, the striking effects in NRF2KO adipocytes are attributable to chronic oxidation of cellular thiol redox status, whereas supplementation with N-acetylcysteine (NAC) to shift cellular thiol redox status is sufficient to reverse effects on cellular respiration (33).

Isocitrate dehydrogenase 2 (IDH2) is an important regulator of the NADPH/NADP$^+$ reduction state in the mitochondrial matrix specifically, and therefore it is critical for controlling mitochondrial thiol redox status (34). Mice genetically lacking IDH2 exhibit oxidation of adipose tissue thiols and the NADPH/NADP$^+$ couple in iWAT, as well as elevated levels of mitochondrial thiol oxidation in brown adipocytes (35). This manipulation drives substantial protection from obesity, which is attributable to elevated energy expenditure based on chronically elevated core body temperature despite food consumption levels comparable with wild type (35). Similarly, ablation of glutathione peroxidase 1 (GPx1), a key enzyme responsible for degradation of mitochondrial hydrogen peroxide, results in resistance to weight gain attributable to elevated energy expenditure, as well as enhanced insulin sensitivity (36). However, effects of GPx1 ablation on adipocyte autonomous processes and thermogenesis have yet to be directly tested. Mice deficient in glutathione due to ablation of the glutamate–cysteine ligase modifier subunit exhibit highly oxidized cellular thiol pools systemically, chronically elevated energy expenditure, and resistance to obesity (37). Also, modulation of the thiol antioxidant function of Sestrin2 is sufficient for controlling UCP1 expression in iWAT and BAT, activation of leak respiration, and thermogenic function in vivo (38).

Similarly, acute chemical depletion of glutathione pools is sufficient to drive mitochondrial biogenesis and UCP1 expression in adipocytes (39) and elevated energy expenditure in mice (40). Moreover, acute activation of UCP1-dependent thermogenesis in vivo is substantially inhibited by pharmacologically increasing reduced cellular thiol content using NAC. NAC-mediated inhibition of cellular thiol oxidation blunts activation of UCP1-dependent thermogenesis upon cold exposure or following β3-adrenergic stimulus (18). Additionally, acute activation of thermogenesis by methamphetamine, which requires functional BAT (41) and UCP1 (42), is substantially inhibited by NAC (43).
Molecular targets of thermogenic ROS

There is strong evidence for the importance of cysteine thiol oxidation as a functional mediator of thermogenic mitochondrial ROS. These findings are in line with the prevailing view that ROS act as biological messengers principally through reversible modification of sensitive surface cysteine residues on proteins (7, 12). Recently developed methodologies now allow for the identification and quantification of redox-modified protein cysteine residues in intact cells and living tissue (44 – 46). These methods rely on strategies to selectively derivatize protein cysteine residues based on their post-translational status followed by assessment by mass spectrometry (MS). This field of cysteine-targeted chemistry is essential for molecular characterization of redox signaling in physiology, because cysteine redox status is fundamentally disrupted upon cell lysis and is not trivial to faithfully model in vitro as described in above sections.

We recently applied these methods to identify BAT protein cysteine residues that become substantially modified by thermogenic ROS (18). These analyses identified mitochondrial metabolic proteins to be a highly enriched target group, such as enzymes involved in fatty acid utilization and citric acid cycle metabolism. The functional importance of these modifications will be a critical avenue of future study, as it is likely from these initial findings that ROS signals act synergistically on several pathways impacting on acute regulation of thermogenic respiration. One modification that has been further characterized was on UCP1 itself. Indeed, one of the seven cysteine residues on UCP1-Cys-253—becomes modified substantially upon activation of thermogenesis in BAT (18). Using well-established methods for selective derivatization based on dimedone chemistry (47, 48), the chemical nature of this modification was demonstrated to be sulfenylation. However, it is important to note that other oxidative modifications are also likely to occur at this site, and this is something well worth investigating. To further explore the functional relevance of this site, a cysteine to alanine point mutant (C253A) of UCP1 was generated. This allowed an examination of UCP1 function in the context of its native environment and in response to physiologically relevant stimuli. Interestingly, C253A UCP1 was functional but required a significantly higher level of adrenergic stimulus to achieve the same level of leak respiration as wild-type UCP1. This finding implies that Cys-253 is not required for UCP1-mediated uncoupled respiration, but it sensitizes the protein to activation by adrenergic stimulus.

In summary, based on the numerous studies described above, it is reasonable to propose that the thermogenic action of mitochondrial ROS are likely mediated in large part through protein cysteine modification of target proteins. The role of reversible thiol oxidation as an effector of thermogenic ROS signaling appears important in the context of triggering thermogenic gene expression, as well as in the acute control of thermogenic respiration.

Modeling the relationship between ROS and UCP1-mediated respiration in vitro

The recent discoveries highlighting the role of mitochondrial ROS and redox signaling in adipose tissue thermogenesis described above (Fig. 1) were preceded by numerous studies investigating the role of ROS and related species as direct activators of UCP1 using in vitro systems. To interpret in vitro findings in the context of the above-mentioned work first requires an outline of our current understanding of acute regulation of UCP1. The precise mechanisms of the UCP1-medi-
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Revised by Rafael et al. (50), Cannon and co-workers (51), and Nicholls and co-workers (52) led to identification of the best-characterized regulators of UCP1 protonophoric activity: fatty acids and purine nucleotides. More recently, a seminal study by Kirichok and co-workers (24) used mitochondrial patch-clamp methods and added further detail on these key regulators of UCP1 function. Although the precise mechanisms and interactions of these species with UCP1 is a developing area (53), many of these findings inform the evolving understanding of physiological regulation of UCP1. First, purine nucleotide concentrations that exist in the cell are sufficient to substantially inhibit UCP1 proton current. Second, elevated local concentrations of long-chain fatty acids overcome purine nucleotide inhibition and drive H+ leak through a fatty acid/H+ symport mechanism (24). As such, any interpretation of additional factors regulating UCP1 function must take into account these variables (4).

The possibility that ROS could be direct activators of UCP1 was first proposed by Brand and co-workers (54), who examined proton leak in BAT mitochondria in the presence of a xanthine oxidase superoxide-generating system. Addition of xanthine and xanthine oxidase resulted in an increase in protonophoric activity, which was completely abolished by addition of 0.5 mM of the purine nucleotide GDP. Similar results were found when examining the effects of 4-HNE, a cysteine-reactive aldehyde product generated by a superoxide reaction with polyunsaturated fatty acyl groups (55). Subsequent examination of 4-HNE effects on both GDP-inhibited and GDP-uninhibited UCP1 function in BAT mitochondria found no stimulating effects attributable to UCP1 (22). More recently, patch-clamping methods have shown that 4-HNE is not sufficient to stimulate UCP1 protonophoric activity directly (24). 4-HNE was later found to act synergistically with fatty acids to stimulate UCP1 proton leak in yeast and BAT mitochondria suggesting it may instead be an allosteric regulator that potentiates UCP1 activation by long-chain fatty acids (56). This synergistic effect on UCP1 activity was also found in studies of UCP1 in planar lipid bilayers. Again, 4-HNE alone had no effect on UCP1 activation but strongly potentiated membrane conductance mediated by fatty acids (57). Interestingly, these effects coincided with cysteine modification of UCP1 by 4-HNE, whereas pre-treatment of UCP1 with cysteine-alkylating agents could inhibit 4-HNE-dependent potentiation (57).

Taken together and in the context of studies of ROS and UCP1-dependent thermogenesis from in vivo and cellular models, some tentative conclusions can be made regarding the somewhat confusing and sometimes contradictory findings from in vitro experiments. (i) Superoxide or 4-HNE effects on proton leak that are inhibited by purine nucleotides would be unlikely to extrapolate to a cellular context in which this inhibition is likely to be persistent. However, additional levels of regulation of purine nucleotide/UCP1 interactions may exist at the cellular level and are not recapitulated in experiments on isolated mitochondria. (ii) 4-HNE alone is not sufficient to directly activate UCP1 protonophoric activity. (iii) 4-HNE may be a relevant modifying species by allosterically potentiating fatty acid-dependent leak through UCP1. Although 4-HNE may potentiate UCP1 activation by fatty acids in vitro, other cysteine-reactive species may be more relevant in the context of in vivo thermogenesis because 4-HNE levels do not appear to change following chronic cold exposure in BAT (22) or in models of ROS-dependent thermogenesis in vivo (23).

It is informative to consider these findings in light of our recent investigation of UCP1 Cys-253 and the extensive evidence for acute regulation of UCP1-dependent respiration in cells and in vivo by ROS and redox status (Fig. 1). We found UCP1 Cys-253 to be sensitive to oxidative modification, whereas mutagenesis to alanine retained UCP1 functionality but decreased sensitivity to activation by adrenergic stimuli. Taken together, it is reasonable to propose that UCP1 Cys-253 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. This model reconciles the fact that cysteines are not required for UCP1 activity (18, 58) and that any potential effect of the small number of ROS species tested in vitro is limited to potentiation of fatty acid-driven activation.

Of course, to further clarify this model—or indeed any model of redox signaling—requires acknowledgment of the complex variables outlined in the first section of this review. Indeed, a prerequisite to faithful modeling of ROS interactions with UCP1 function in simple model systems will require identification of the type(s) of ROS that modify thermogenic function in vivo in the models described above. Additionally, it will be important to understand the breadth of types of cysteine-centered modifications on functional targets such as UCP1 Cys-253. Because precise and stable in vitro modeling of modifications such as sulfenylation is non-trivial, it may be that development of selective cysteine-reactive electrophiles to stably modify functional sites such as UCP1 Cys-253 would provide more insight using in vitro systems.

More generally, the profound effects of manipulating redox status in adipocytes presumably involves 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—could lead to a new class of molecular targets that may be manipulated to enhance the function of thermogenic adipose tissue. The recently developed genetic and pharmacological models described herein now allow for examination of the molecular mechanisms of ROS-mediated activation of thermogenic gene programs and thermogenic respiration on a cellular and organismal level. By studying ROS-dependent signaling in physiologically relevant model systems, substantial added clarity can be achieved and testable hypotheses generated. Moreover, the extent to which mechanisms of signaling can be determined will allow for examination of these processes in the context of human adipose tissue thermogenesis. In applying these approaches, the complex regulatory networks involved in the relationship between ROS/redox status and thermogenesis are maintained, and regulatory factors that are not amenable to in vitro study can now be examined.
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