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Authors

Haley, John

Jang, Cholsoon

Guertin, David

Publication Date

2023-12-01

DOI

10.1016/j.gde.2023.102112

Peer reviewed

HHS Public Access

Author manuscript

Curr Opin Genet Dev. Author manuscript; available in PMC 2024 December 01.

Published in final edited form as:

Curr Opin Genet Dev. 2023 December ; 83: 102112. doi:10.1016/j.gde.2023.102112.

A New Era of Understanding in vivo Metabolic Flux in Thermogenic Adipocytes

John A. Haley[1], **Cholsoon Jang**[2], **David A. Guertin**[1,*]

[1]Program in Molecular Medicine, UMass Chan Medical School, Worcester, MA, USA.

[2]Department of Biological Chemistry, University of California Irvine, Irvine, CA, USA.

Abstract

Non-shivering thermogenesis by brown adipose tissue (BAT) is an adaptive mechanism for maintaining body temperature in cold environments. BAT is critical in rodents and human infants and has substantial influence on adult human metabolism. Stimulating BAT therapeutically is also being investigated as a strategy against metabolic diseases because of its ability to function as a catabolic sink. Thus, understanding how brown adipocytes and the related brite/beige adipocytes use nutrients to fuel their demanding metabolism has both basic and translational implications. Recent advances in mass spectrometry and isotope tracing are improving the ability to study metabolic flux in vivo. Here, we review how such strategies are advancing our understanding of adipocyte thermogenesis and conclude with key future questions.

Keywords

Brown adipose tissue; Brown Adipocyte; Beige Adipocyte; Adaptive thermogenesis; UCP1; Stable Isotope Tracing; metabolic flux; arteriovenous metabolomics; glucose metabolism; lipid metabolism

Introduction

Brown adipose tissue (BAT) is the major site of adaptive non-shivering thermogenesis in mammals. BAT was first described by Conrad Gessner, who in 1551 identified the tissue in marmots [1]. Its role in thermogenesis was solidified in the mid-1900s [2], but its definitive role in adult human metabolism was not appreciated until the late 2000s [3–7]. Numerous studies since, have associated human BAT with resistance to cardiometabolic diseases and cancer [8–11]. This is attributed to BAT's ability to function as a "catabolic sink" through heat generating energy wasting cycles [10] and has ignited interest in

*Corresponding: David A. Guertin, UMass Chan Medical School, 2 Biotech, 373 Plantation Street, Worcester, MA. 01605 | 508-856-8064 | david.guertin@umassmed.edu.

Disclosures

The authors declare no conflict of interest.

therapeutically stimulating BAT to combat metabolic diseases. However, achieving this goal requires a deeper understanding of BAT fuel utilization and metabolic flux. In this article, we review how recent advances in metabolomics and stable isotope tracing are improving our understanding of thermogenic adipocyte metabolism and conclude with key future questions.

Brown Fat Basics

In rodents, BAT depots are distributed in the cervical, suprascapular, axillary, paravertebral, and perirenal regions, the largest depot being interscapular like in human infants [Figure 1A]. In adult humans, BAT depots are predominately located in the anterior cervical, axillary, supraclavicular, paravertebral and perirenal regions [12]. BAT thermogenesis is stimulated by the sympathetic nervous system in response to cold exposure, high calorie diets, and beta-adrenergic agonists [2,13–18]. Upon stimulation, classical non-shivering thermogenesis is mediated by the inner mitochondrial membrane protein uncoupling protein 1 (UCP1), which dissipates the electrochemical proton gradient, releasing heat [2][Figure 1B]; however, UCP1-indendent energy wasting pathways may also exist in parallel and/or as compensatory pathways [Box 1]. A variety of experimental conditions are used to study BAT thermogenesis, which are summarized in [Box 2].

From a tissue metabolic viewpoint, mitochondrial acetyl-CoA is one of the principal metabolites that drives adaptive thermogenesis [Figure 1B]. The mitochondrial acetyl-CoA pool is derived from pyruvate (the end-product of glycolysis), from fatty acid beta-oxidation, and from catabolism of some amino acids. Acetyl-CoA carbons power the TCA cycle, which generates reducing equivalents (NADH and $FADH_2$) that drive the electron transport chain (ETC) and therefore uncoupling respiration. Uncoupled respiration also demands large amounts of oxygen and thus oxygen consumption is often used to measure thermogenesis [2]. Glucose and lipids have long been considered the major carbon fuels for thermogenesis (i.e. mitochondrial acetyl-CoA), but the preferred mixture and exact fates of each have been a matter of debate [2].

Major Nutrients that Support Thermogenesis

Thermogenesis is energetically costly and active BAT must consume copious amounts of fuel. While glucose and lipids are considered the major fuels [Figure 1A], the amount and importance of each depends upon many factors including stored energy, diet, and both the degree and duration of cold. At thermoneutrality, BAT stockpiles energy in lipid droplets (and as glycogen), which provides on-demand (emergency) energy source during acute cold exposure. During prolonged cold exposure, stored energy is depleted, and consequently, prolonged thermogenesis requires much more fuel from circulation. Imported fuel can come from dietary nutrients or from other tissues through metabolite sharing [19,20].

During prolonged thermogenesis, BAT must also maintain tissue homeostasis, i.e. maintain DNA, RNA, and protein integrity, remove excess ROS and metabolic waste, prevent organelle damage from accumulating, and make new brown adipocytes to meet thermogenic demand. This raises important questions about how BAT partitions fuel to support both

uncoupled respiration and maintain tissue homeostasis. BAT is also a rich source of hormones (batokines) and signaling metabolites [21–23], the production of which requires nutrient resources. The use of BAT fuels outside of uncoupling respiration has overall been less studied.

Glucose

BAT's high capacity for consuming glucose has important implications for treating diabetes and cancer and is the basis for detecting human BAT by positron emission tomography (PET) using ^{18}F-fluorodeoxyglucose (^{18}F-FDG). However, ^{18}F-FDG cannot be metabolized in cells and thus the fate of glucose cannot be inferred by these methods. Many studies show that cold increases glucose uptake into BAT [2,4,6,24], and that both glycolysis and pyruvate uptake into mitochondria are required for optimal thermogenesis [25,26]. Yet how much glucose carbons directly contribute to the TCA cycle is unclear with some reports suggesting only a small fraction (~30–35%) at least at room temperature [27]. What, then, happens to the glucose consumed during thermogenesis? The answer likely depends on environmental conditions and diet.

During acute cold exposure, the intracellular lipid reservoir is initially the main source of carbon for oxidation, while glucose uptake increases modestly. Some of the glucose is converted to pyruvate during glycolysis, which can generate mitochondrial acetyl-CoA via pyruvate dehydrogenase perhaps helping drive up ROS production to upregulate UCP1 [28,29]. A massive simultaneous influx of acetyl-CoA from beta-oxidation of the stored lipid pool could also necessitate significant additional anaplerotic pyruvate flux into oxaloacetate to allow the citrate synthase reaction of the TCA cycle to efficiently proceed, especially if some carbons are exiting the TCA cycle prior to its completion, such as for lipid and amino acid synthesis [30]. Alternatively, some pyruvate can be converted to lactate [19,31] or other metabolites derived from glycolysis (discussed below).

In contrast, glucose uptake increases substantially during cold adaptation [2,24]. Interestingly, active BAT is also one of the most lipogenic tissues in the body [24,32–34]. and expression of enzymes in the *de novo* lipogenesis pathway (which converts glucose carbons via into lipids via mitochondrial citrate export) and the pentose phosphate pathway (which provides NADPH for lipid synthesis) markedly increase during cold adaptation [15,24,33]. This phenomenon also occurs after chronic β3-adrenergic receptor agonist treatment, which couples *de novo* lipogenesis, lipolysis, and fatty acid oxidation [32,35–37]. The coupling of fatty acid synthesis and fatty acid oxidation in BAT (FAS-FAO cycling) is a peculiar aspect of adaptive thermogenesis that is not well understood but may function as a UCP1-indepenent heat generating substrate cycle [Box 1].

Recent advances in metabolomics and stable isotope tracing (Reviewed in [38–41]) are shedding new light on the fascinating features of BAT metabolism, substantiating the FAS-FAO cycling model and filling other gaps in understanding BAT glucose utilization [Table 1].

One study orally delivering ^{13}C-Glucose to mice adapted to thermoneutrality, room temperature, and severe cold, and then collecting samples over a time course, found that

BAT glucose fluxes vary markedly with the degree and duration of cold exposure [24]. During cold adaptation, glucose not only provided carbon for the TCA cycle, but also for many auxiliary pathways beyond glycolysis/TCA cycle including rapid flux into *de novo* lipogenesis and acyl-carnitine production (i.e. FAS-FAO cycling). Consistent with high flux into DNL, there was increased flux into the PPP, which is the major source of NADPH for BAT lipid synthesis [42]. There was also high flux into glycerol-3 phosphate (G3P) consistent with an active glycerol-phosphate shuttle [43,44] [Box 1] and/or glycerolipid synthesis. Other glucose carbon fates included nucleotide metabolism, glutamine synthesis (discussed below), and many N-acetylated amino acids, the functions of which are unknown. Another study delivering ^{13}C-glucose by intraperitoneal injection and examining glucose labeling 15 minutes after delivery also found that chronic cold exposure increases glucose labeling of TCA cycle intermediates [26]. Overall, these studies show that increased DNL is a definitive feature of active brown adipocytes, at least when carbohydrates are plentiful, and they reveal the versatility of glucose as a BAT fuel.

Other studies have examined BAT glucose use through minimally perturbative stable isotope infusion analysis (i.e. minimally increasing blood glucose levels) [27,38,45]. Unlike short-term bolus tracer treatment, infusions require tracer delivery for a few hours. Thus, the exact route(s) carbons take to the TCA cycle is more difficult to determine, requiring deconvolution of primary and secondary conversions of metabolites by other tissues to measure direct metabolite contribution to a specific tissue of interest. Two studies examining the use of many nutrients across many tissues quantified metabolite contribution to the TCA cycle and glycolysis, respectively [27,45]. These studies individually infused numerous tracers so that linear equations could be used to calculate the direct contributions of each metabolite to tissue metabolism. Both studies used standard conditions (room temperature mice fed a chow diet) and found that ~60% of direct TCA cycle contribution in BAT is from glucose and lactate, while ~50% of direct glycolytic contribution is from glucose. Moreover, in the fasted state, BAT uses more lipids to fuel the TCA cycle and relies more on glycogen, glycerol, and lactate to fuel glycolysis. In contrast, in mice fed a ketogenic diet, BAT predominately uses lipids to fuel the TCA cycle regardless of fed or fasted state [27], suggesting a natural metabolic plasticity in BAT that is heavily condition dependent.

Lipids

A defining feature of brown adipocytes is the dynamics of their intracellular lipid droplets. When thermogenically inert, brown adipocytes have a large unilocular lipid droplet, like a white adipocyte; when stimulated, the lipid droplets become numerous and smaller (multilocular) depending on the thermogenic state [Figure 1C]. These stored lipids are the major fuel for thermogenesis upon acute activation [2] and moreover, free fatty acids bind and activate UCP1 [2,46]. Many models also argue that lipids are the predominant fuel for thermogenesis [2,47,48]. Notably, with chronic cold adaptation, the stored lipids are turned over and depleted yet even in chronically cold adapted mice, lipid droplets persist though they are much smaller and more uniform in size [Figure 1C]. Interestingly, BAT lipid droplets themselves may be dispensable for thermogenesis as deleting DGAT1/2, the major enzymes for TAG synthesis, in BAT ablates lipid droplets but does not affect body temperature maintenance in the cold [49]. It has also been observed that mice with BAT-

specific knockout of ATGL, which regulates the first step in lipolysis, are also cold tolerant, while all fat ATGL knockout mice are acutely cold sensitive when fasting, suggesting lipids from WAT are an important BAT fuel during fasting [50,51]. Nevertheless, if lipids are the primary fuel source for non-shivering thermogenesis, then how are lipids obtained when internal stores are depleted?

As discussed, one source may be *de novo* lipid synthesis. Other studies have demonstrated key roles for lipid uptake, such as liver-derived acylcarnitines that originate from WAT-released free fatty acids [52]. Triglyceride-rich lipoproteins in circulation also provide lipids, with cold-stimulated BAT activity correcting hyperlipidemia and insulin resistance in ApoE knockout mice [48]. In addition, a fatty acid tracer (^{18}F-fluoro-thiaheptadecanoic acid) combined with PET imaging showed that cold stimulated BAT increases fatty acid uptake in adult males [53,54], which was impaired in obesity [54]. Similar imaging was done in male mice comparing glucose and fatty acids [55]; however, this study found that glucose tracers were better at distinguishing BAT depots while fatty acid tracers were better at identifying classic inguinal WAT locations.

Recent work further indicates that BAT lipid uptake is highly dependent on nutrient availability. In one of the isotope tracer infusion studies described above, it was found that in room temperature housed mice, palmitate, oleate, and linoleate account for ~12% of direct TCA cycle contributions [27]. However, in fasted mice, the direct TCA contribution of these fatty acids increases to ~25%. The study went a step further by placing the mice on a ketogenic diet (~75% fat) which dramatically increases BAT fatty acid TCA contribution regardless of fed state. Although BAT function was not examined in these studies, it again illustrates the importance of diet and feeding state.

Amino Acids

Branched-chain amino acids (BCAAs) are also substrate for thermogenesis [56], which may have additional therapeutic implications because elevated circulating BCAAs are associated with insulin resistance [57–60]. While BCAAs constitute ~50% of essential amino acids in diet, they are unlike other essential amino acids in that they are predominantly catabolized to acetyl-CoA and succinyl-CoA by the skeletal muscle and adipose tissue [61]. Interestingly, the anti-obesity drug tirzepatide induces BCAA catabolism in BAT [62]. In addition to BCAAs, both the synthesis and catabolism of glutamine, the most abundant circulating amino acid, may also have unique roles in BAT (discussed below). But for most other amino acids, their exact roles as substrates and/or signaling metabolites are less understood.

Other Metabolites

The uptake and accumulation of succinate in BAT was recently shown to stimulate BAT thermogenesis in mice independent of adrenergic signaling [63,64]. The mechanism is through succinate oxidation by succinate dehydrogenase, which initiates the production of reactive oxygen species that drive thermogenesis. Follow-up work further revealed that during diet induced obesity, BAT may clear circulating succinate in a UCP1-dependent manner, which protects the liver from inflammation [65].

Measuring Net BAT Fluxes by Arteriovenous Metabolomics

While stable isotope tracing methods can reveal BAT metabolic pathway activity and tissue/circulatory fluxes, another way to quantitatively and more broadly assess metabolite net uptake and release from circulation is by arteriovenous (AV) sampling [66] combined with metabolomics (or AV metabolomics). AV gradient measurements have an important history in BAT research. Using rats in the 1970's, and taking advantage of the large Sulzer's vein that drains blood from the interscapular BAT [67], AV sampling showed BAT almost completely deoxygenates the blood, which was instrumental in identifying BAT as the main site for non-shivering thermogenesis [68–70]. Later rat AV sampling studies in the 1980's measured glucose, lactate, and amino acids utilization [71] by more traditional approaches. However, general interest in AV sampling was recently renewed because of advances in metabolomics instrumentation [72].

AV metabolomics can be used to measure the net consumption (uptake) and production (release) of hundreds of metabolites from an organ provided a sufficient arterial and venous blood route is accessible [Figure 2A]. This can be taken even further by quantifying the metabolite concentrations to obtain net tissue fluxes providing the blood flow rate can be measured through the organ.

AV metabolomics was recently adapted to mice to study nutrient fluxes across BAT [Figure 2B][37]. This study compared BAT metabolite exchange in male mice eating a standard diet in four different thermogenic states: thermoneutral, acute cold, chronic cold, and acute CL-316,243 treatment. By quantifying the uptake and release from blood of the most abundant high tissue flux metabolites, it was found that nearly all major flux-carrying metabolites are net consumed especially in cold adaptation; many other metabolites also showed unexpected uptake or release. However, factoring in blood flow revealed that most of the circulating carbons used by BAT are obtained from glucose or lactate [Figure 2C], while the net uptake of carbon derived from free fatty acids and triglyceride-rich lipoproteins was considerably less and quantitatively similar across conditions. Importantly, this study was performed with fed mice on a diet plentiful in carbohydrates. It is likely that circulating lipids would be utilized to a greater extent in fasted mice [Figure 2D], as recently suggested [27]. The consumption of nitrogen was also greatly increased in cold adapted BAT mainly from circulating amino acids including BCAAs, which results in a substantial nitrogen imbalance. It was also found that the effects of acute CL316,243 on metabolic fluxes markedly differed from physiological acute cold exposure. AV metabolomics was also performed on the leg, revealing unexpected metabolites consumed and released from this site and evidence of organ metabolite sharing.

Another unexpected finding was that, unlike other amino acids that showed a net uptake, the most abundant circulating amino acid glutamine, which is an important fuel source during high energy demand, showed a net zero flux. This was explored using ^{13}C-^{15}N-glutamine and ^{13}C-glucose stable isotope tracers, which showed that glutamine is both catabolized as a fuel source for the TCA cycle, and simultaneously synthesized using glucose as a precursor [24,37] highlighting yet another BAT substrate cycle. Moreover, glutamine synthetase, which condenses ammonia onto glutamate to make glutamine in an ATP-dependent

reaction, is highly upregulated in BAT during cold and its activity was confirmed by ammonia tracing. At least in a cell culture system, glutamine synthetase ablation increased ammonia levels and impaired norepinephrine-stimulated oxygen consumption; the in vivo significance of glutamine cycling remains to be determined. Overall, this study shows that AV metabolomics can be applied in mice and thus used with genetic models and diets to study BAT nutrient fluxes.

Potential Advantages of a Glucose-Based Fuel Economy During Adaptive Thermogenesis

Clearly, BAT metabolism upon acute cold exposure is quite different than during chronic cold adaptation, with one major difference being higher glucose uptake and flux into a variety of auxiliary pathways in cold adaptation [Figure 3A]. An analogy could be drawn to aerobic glycolysis in many tumors [73]. Yet, while glucose is the preferred fuel when the full menu of nutrients is available, BAT can modulate the quantities of glucose versus lipid that it uses in the fed and fasted states, during circadian cycles [74–78], and upon different diets to allow for maximum fuel flexibility. Nevertheless, we can hypothesize as to why BAT might prefer a glucose-based fuel economy during prolonged thermogenesis.

First, during acute thermogenesis, when most of the fuel is provided by intracellular lipid droplets, fatty acid oxidation would generate vast amounts of mitochondrial acetyl-CoA to power the TCA cycle into action (and increase ROS production). Glucose uptake begins to rise under these conditions, contributing to acetyl-CoA production, possibly ROS, and possibly to the anaplerotic production of oxaloacetate to sustain TCA cycle flux [30] or other pathways. Perhaps this acute thermogenic state is largely UCP1-dependent requiring high TCA flux, ETC activity, and oxygen consumption [Figure 3A]—like a car engine when ignition and acceleration expend large amounts of fuel to increase rpms (TCA cycle flux) while getting up to speed.

However, perpetually running a high rate of uncoupled metabolism may come at a cost—e.g. oxygen depletion, ATP depletion (from uncoupling), excessive ROS, fewer substrates for homeostatic processes, etc. Perhaps, switching to a more glucose-based economy improves long-term efficiency. Notably, many of the glucose pathways increasing during cold adaptation include an ATP hydrolysis step, and several in the FAS-FAO cycling pathway, which are balanced by glycolytic ATP production [Figure 3A]. Thus, potential advantages of using glucose may include (1) ATP synthesis from glycolysis; (2) a more balanced use of UCP1-dependent and UCP1-independent (ATP hydrolyzing) thermogenic pathways; (3) less demand for oxygen and less ROS production; (4) increased antioxidant power via cytosolic NADPH synthesis; (5) and increased availability of signaling metabolites and macromolecule building blocks, especially those generated in mitochondria. Overall, fueling with glucose may just be better for the cellular environment.

Notably, this model does not argue against lipids as a major BAT fuel. Rather, it suggests that it may be advantageous to synthesize at least some lipids first, before catabolizing them, at least when glucose is readily available. But if glucose is not plentiful, fatty acids can become more predominant fuels. Lipids may also provide critical signals that help

maintain active UCP1 and/or promote the thermogenic transcriptional program [10,79,80]. Moreover, as maintaining body temperature is critical for survival, BAT must be capable of fuel flexibility. Thus, what BAT can do and what it prefers to do when the full menu is available, need not be the same. What remains unclear is what liabilities might present if metabolic flexibility is impaired. Moreover, several studies have argued that glucose uptake and glycolysis is essential for thermogenesis (Reviewed in [81]); thus, for reasons that still need clarification, glucose may not completely dispensable. Understanding these aspects of adipocyte thermogenesis is important for developing potential translational strategies.

Burning Questions

As advances in metabolomics and stable isotope tracing techniques usher in a new age of understanding adipocyte thermogenesis, many questions arise in parallel. One is the role of tissue heterogeneity. While it is important to gauge activity of whole BAT tissue, as all its parts function together, there are three levels of heterogeneity that cannot be resolved by techniques described above; the contribution of non-brown adipocyte populations (e.g. blood cells, endothelial cells, neurons, immune cells, preadipocytes); heterogeneity within the brown adipocyte population [82,83]; and heterogeneity between pools of brown adipocyte mitochondria [84,85]. Understanding how these layers of heterogeneity affect BAT metabolism is an exciting focus area.

Second is the role of nutrient-sensing pathways. In cool conditions, insulin signaling (based on AKT phosphorylation) is high in BAT and drops with cold adaptation coinciding with increased transcription of glycolysis regulators [15,24,33]. Thus, in cool conditions (i.e. mild BAT activation), insulin/AKT signaling and beta-adrenergic/PKA signaling may intimately cooperate. In more severe cold, glucose uptake may primarily be driven by insulin-independent mechanisms. This may be an advantage in severe cold since obtaining fuel is critical for survival even in conditions where insulin may be low. Understanding how insulin and nutrient-sensing pathways converge with thermogenic signaling pathways is fundamental not only to understanding BAT fuel flexibility, but how to therapeutically target BAT metabolism in humans that typically live in a thermoneutral environment.

A related question is how BAT adapts its fuel usage in different nutrient states? Since maintaining body temperature is essential for survival especially in rodents, it makes sense that BAT should be able to maintain thermogenesis regardless of nutrient availability. BAT metabolism also shows a circadian rhythm for reasons not yet clear [78,86]. Which fuels are preferred when, and why? How does BAT fuel demand affect nutrient use across the body? Are there liabilities to using predominantly lipids, or to only UCP1-mediated thermogenesis? What are the advantages of using glucose? Stable isotope tracing is helping fill these gaps for glucose; but for other metabolites, such as the complex pool of lipids, or the metabolites shared between organs, tracing experiments are more difficult.

What is the role of UCP1 in fuel utilization? There is contradicting literature on this question, and interesting sex differences in both UCP1 and glucose dependency have been reported [87,88]. Moreover, there is a growing appreciation for UCP1-independent

mechanisms of thermogenesis [Figure 3B][Box 1][10,89,90] and understanding their interplay with UCP1 is another key focus area.

As thermogenic metabolism is better defined, it is also critical to functionally interrogate the key nodes. This is best addressed using genetic models, and the development of CRISPR-Cas9-based methods will significantly advance this [91,92]. Much like vehicles on the streets, there is often more than one metabolic route to a destination, with some being less efficient than others. Thus, for any genetic model, phenotypic differences at the molecular level may not show whole tissue or whole-body effects yet may still introduce metabolic liabilities that require nuanced analyses to detect. Many metabolites also have critical signaling functions [93]. As technologies to find metabolite-protein interactions become more available, it will be important to define thermogenic signaling metabolites.

The final, and most clinically relevant point is to determine the relevance of key metabolic pathways discovered in rodents to human brown fat biology. The cliche “mice are not humans” is often used, however, mice are often not studied in human-relevant conditions [2,16,94]. The use of environmental parameters (e.g. thermoneutrality) or diverse mouse strains that better humanize mouse metabolism could allow for better comparison to human thermogenic fat. Unless human BAT samples or other methods to interrogate human BAT function become available, there must be a continued emphasis on developing “humanized” mouse models.

Concluding Remarks

BAT is a marvel in its metabolic properties, role in evolution, and translational potential. As technologies in metabolomics, stable isotope tracing, and genomics are rapidly advancing, we are dawning a new age in understanding the exquisite metabolic features of thermogenic adipocytes. And with so many exciting and translationally relevant hypotheses to test, the thermogenic adipocyte field will be on fire for many years to come.

Acknowledgments

We thank Qingbo Chen and Marie Isidor for critically reading the manuscript. This work was funded by R01DK116005, R01DK127175 and R01DK094004 to D.A.G.; the AASLD Foundation Pinnacle Research Award in Liver Disease, the Edward Mallinckrodt, Jr. Foundation Award, and NIH/NIAAA R01 AA029124 to C.J; F31DK129018 to J.A.H. Cartoon illustrations were all created in BioRender.com.

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Box 1:

UCP1-independent mechanisms of thermogenesis.

Recent evidence suggests that UCP1-independent mechanisms of thermogenesis may also contribute to overall heat production under certain conditions. Some of the more recently described pathways include futile creatine cycling, triglyceride-free fatty acid cycling, SERCA2b-mediated calcium cycling, and fatty acid synthesis-oxidation (FAS-FAO) cycling [10,24,32,34,89,90,95]. However, the idea of UCP1-independent thermogenesis acting in parallel to UCP1 is not new. In 1981, Trayhurn recognized the importance of BAT as a major site of lipid synthesis, further arguing that "*fatty acid synthesis and breakdown constitutes a significant heat-dissipating 'cycle' in brown adipose tissue of cold-acclimated mice*" [34]. Another pathway discussed many years ago by Kozak is the glycerol-phosphate shuttle. BAT has robust capacity to oxidize G3P relative to other tissues, but because the glycerol-3-phosphate shuttle is inefficient compared to glycolysis--generating 2 rather than 3 ATPs per mol of NADH--it was proposed to be heat generating pathway parallel to UCP1 [44,96–98]. Kozak also made links between UCP1-indepenent thermogenesis and SERCA pathway activity in the white adipose tissue of cold acclimated *UCP1−/−* mice [99]. The role of these substrate cycles in brown vs. brite/beige adipocytes and the metabolic demands that differentiate them from UCP1-dependent thermogenesis is an area of great (renewed) interest.

Box 2:

Common laboratory conditions used to stimulate BAT.

Room Temperature:

Mice adapted to living in a standard vivarium temperature (room temperature, 21–22°C) are cold stressed, which we refer to as a "cool" environment. Under cool environmental conditions relative to thermoneutrality, BAT has elevated UCP1 expression, multi-locular lipid droplets, and an expanded mitochondria network—the typical characteristics of active BAT [Figure 1C]. Thus, even in standard laboratory conditions, BAT exerts a substantial influence on systemic metabolism [2,94].

Acute Cold:

Typically, cool adapted mice (room temperature) are acutely transferred to an even colder environmental temperature, typically 4–10°C for several hours. An improved variation is to start with mice adapted to thermoneutrality (28–30°C) in which there is no thermal stress, and where the BAT is unstimulated and morphologically more like WAT [Figure 1C]. Acute cold exposure experiments are usually done under fasted conditions to avoid the thermogenic effects of eating. Importantly, during acute cold exposure, both non-shivering thermogenesis by BAT and shivering thermogenesis by skeletal muscle contribute to body temperature maintenance [2,100], a factor that must be considered when interpreting acute cold exposure results.

Chronic Cold Adaptation:

To employ chronic cold adaptation, fed mice are maintained in either a cool (21–22°C) or cold (4–10°C) environment for days to weeks, typically using thermoneutral adapted mice as controls [Figure 1C]. During chronic cold adaptation, muscle shivering subsides, and non-shivering thermogenesis predominates as the main defense of body temperature [2,100]. In chronic cold mice, BAT lipid droplets are also much smaller and more uniform in size than in the other conditions, and BAT has the highest level of UCP1 expression [Figure 1C]. Cold exposure and cool/cold adaptation remain the gold standard techniques for naturally activating BAT thermogenesis.

Adrenergic Agonists:

Administration of adrenergic agonists are used to mimic some of the effects of cold and noradrenaline. The beta-adrenergic agonist CL-316,243 is one such agonist commonly used. CL316,243 targets β3 adrenergic receptors that are highly expressed in adipose tissues, and it triggers a marked thermogenic response [2,32]. However, recent work shows that adipocyte thermogenesis requires the coordinated action of α1 and β3 AR signaling to fully induce thermogenic genes including those in the futile creatine cycle [101]. Thus, caution should be used when interpreting results using βAR agonists, a point revisited below.

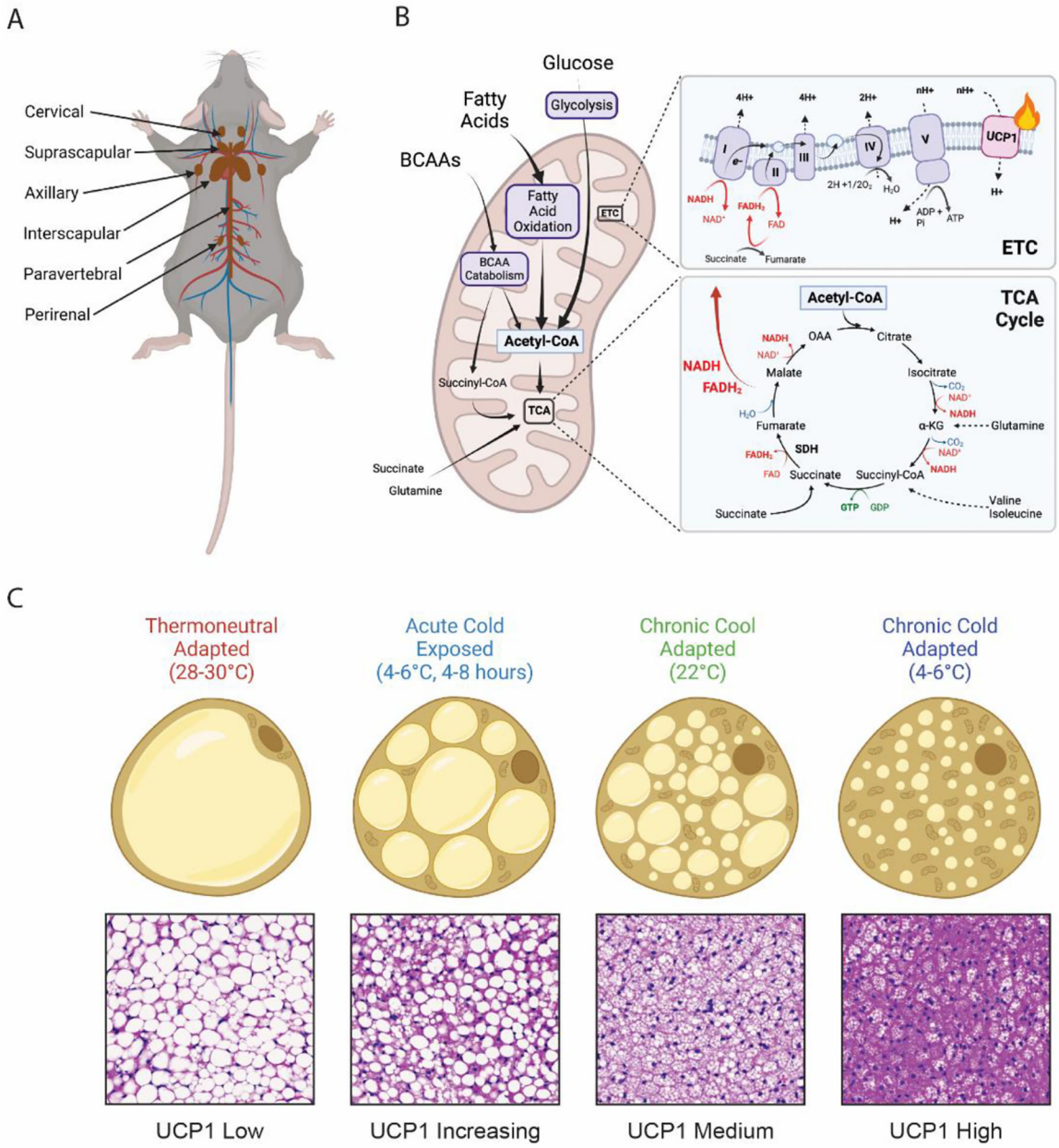

Figure 1. Overview of Brown Fat Anatomy and Thermogenesis

(A) Model depicting major brown adipose tissue (BAT) depots in mice. (B) UCP1-dependent Thermogenesis occurs in mitochondria. Nutrients such as glucose and lipids are mainly converted to acetyl-CoA, which powers the TCA cycle and the electron transport chain. Brown adipocyte mitochondria contain UCP1 on the inner mitochondrial membrane, which dissipates the electrochemical proton gradient to generate heat. Emerging roles for BCAAs, succinate, and glutamine as thermogenic fuels have been reported but are less understood. (C) Brown adipocyte dynamics. Brown adipocyte morphology dramatically changes with temperature. At thermoneutrality, brown adipocyte lipid droplets coalesce and UCP1 is low. Upon acute cold, lipid droplets start to breakdown as UCP1 rises. Most mice are maintained in cool-adapted (room temperature) conditions, in which BAT is active, has

medium to high levels of UCP1, and lipid droplets multi-locular and of variable sizes. With more severe cold adaptation, lipid droplets persist but are much smaller and more uniform in size, and UCP1 is at its highest expression level.

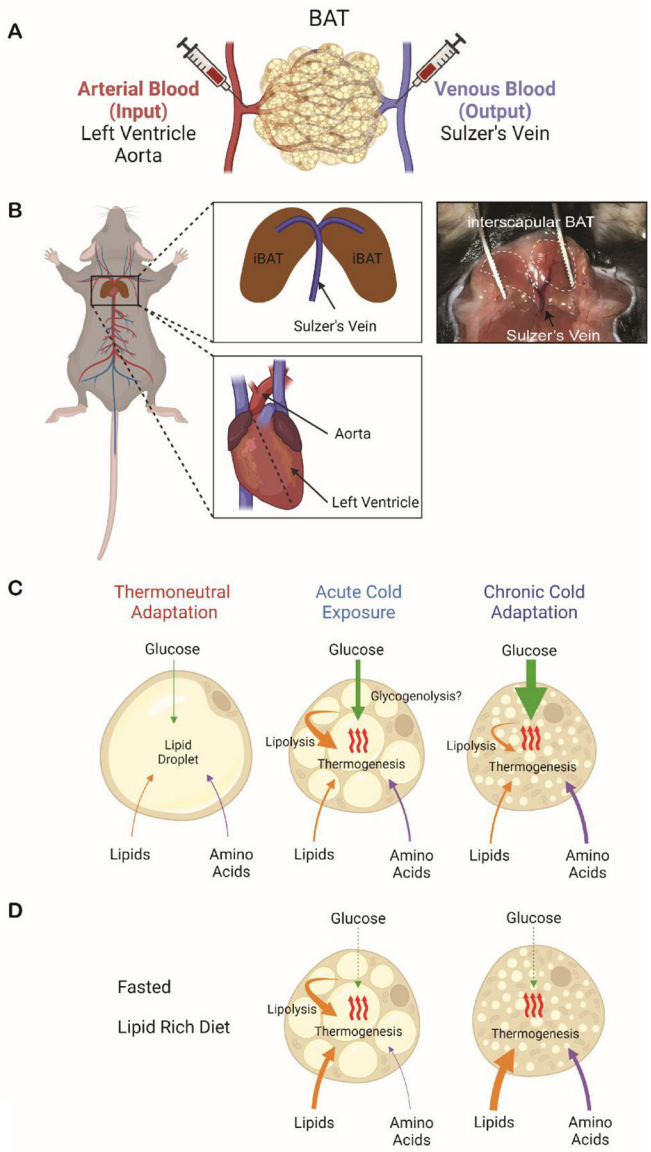

Figure 2. Overview of arteriovenous (AV) sampling.

(A) AV metabolomics blood sampling strategy. Blood is collected from both the artery nourishing a tissue and a major vein draining the tissue. Subtracting a metabolite's venous concentration from arterial concentration can be used to determine the net uptake and release of a metabolite, and when corrected for blood flow rate, metabolic flux of a metabolite across a tissue can be inferred. By performing AV metabolomics across many tissues, evidence of inter-organ metabolite exchange can also be obtained. (B) In mice, BAT venous blood can be obtained from the Sulzer's vein, and arterial blood from the left ventricle. (C) Incorporating recent metabolomics, stable isotope tracing, and arteriovenous metabolomics data with existing studies, a model emerges indicating that glucose, when available, is the preferred BAT fuel. In fed mice eating a standard chow diet (in which carbohydrates are plentiful), acute cold stimulates a moderate uptake of circulating nutrients into BAT because it mainly relies on its intracellular lipid stores for fuel. During chronic cold adaptation,

glucose and lactate are more important sources of carbon, while amino acid uptake also greatly increases relative to the other conditions, providing an additional carbon source and a major source of increased nitrogen. Lipid uptake is relatively similar across these conditions. (D) However, BAT must be flexible in its fuel utilization. When mice are fasting or consuming a lipid rich diet i.e., when glucose is less available, a greater proportion of BAT carbon flux comes from lipids. It is important that BAT maintain fuel flexibility to defend body temperature regardless of nutrient availability.

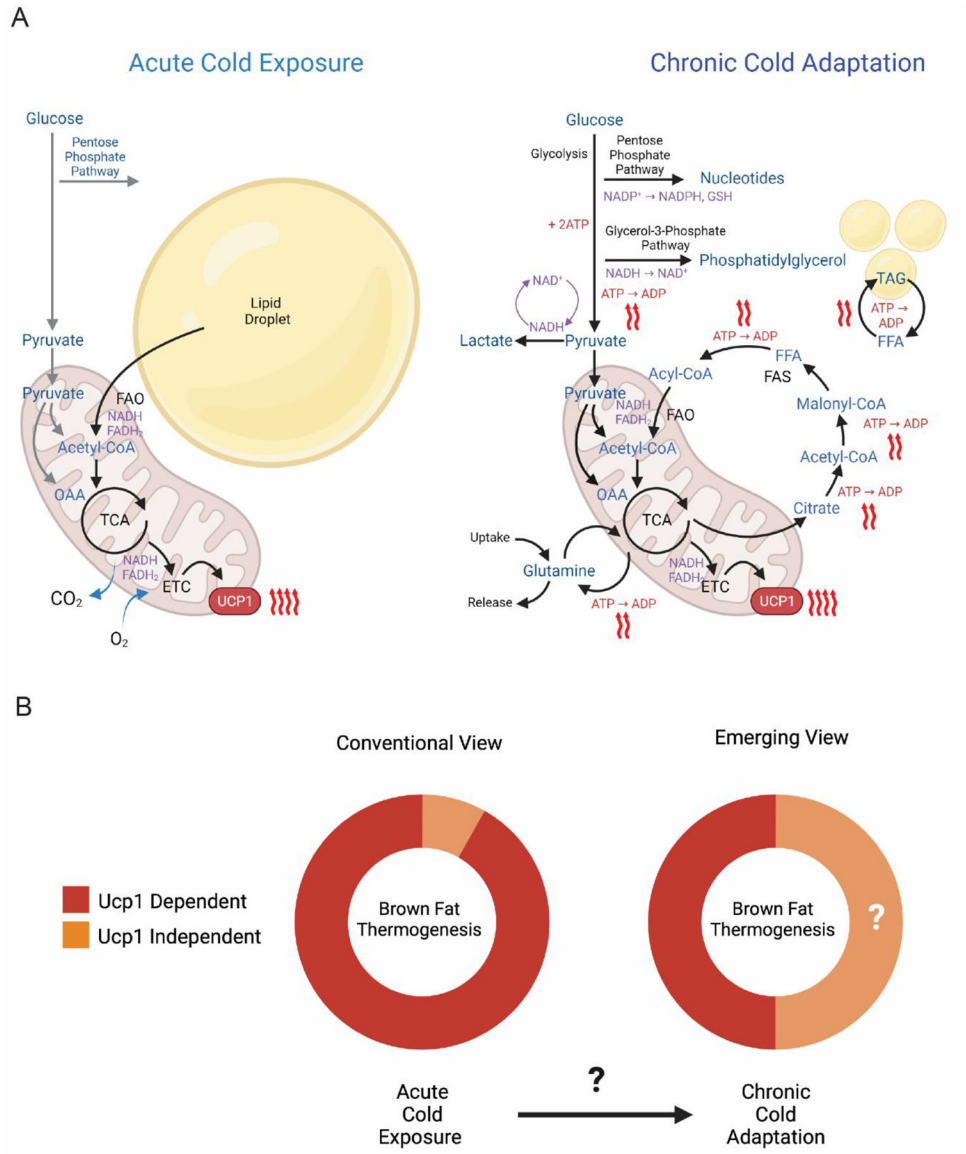

Figure 3. Potential advantages of using a glucose-based fuel economy during prolonged adaptive thermogenesis.

(A) *Left* - During acute cold exposure, intracellular lipid droplets are a major source of acetyl-CoA for the TCA cycle and uncoupled respiration while glucose uptake mildly increases. Glucose derived pyruvate could contribute to the acetyl-CoA pool, boosting the TCA cycle and ROS production. Alternatively, or in addition, with such a large influx of mitochondrial acetyl-CoA coming from fatty acid oxidation, there is evidence to suggest that pyruvate could also be used anaplerotically by pyruvate carboxylase (PC) to make oxaloacetate (OAA) that could help support the citrate synthase reaction (which catalyzes the condensation of acetyl-CoA with OAA) or other OAA fates, such as aspartate production. Notably, there could be different populations of mitochondria, some that use pyruvate in the pyruvate dehydrogenase reaction, some that use the PC reaction (see Outstanding Questions). Regardless, acute BAT thermogenesis may be more dependent overall on lipid oxidation and UCP1-dependent heat production. *Right* - During chronic cold

adaptation, brown fat metabolism may reprogram to a more glucose-based fuel economy if glucose is plentiful. There are several advantages of doing this: (1) Glycolysis is a significant source of ATP; (2) glycolytic intermediates support the pentose phosphate pathway, a source of NADPH, GSH and nucleotide precursors; (3) glycolytic intermediates support glycerol-3-phosphate production, which provides electron donors and precursors for phospholipids and TAGs; (4) mitochondrial exported metabolites can be used in anabolic reactions--one example being citrate, which supports *de novo* synthesis of lipids for oxidation, signaling metabolites, and lipid building blocks; (5) interestingly, many of the pathways that use glucose also contain an ATP hydrolysis step, which collectively may provide a significant source of UCP1-independent heat production. This could lower the overall demand for oxygen and relieve pressure on the mitochondria, preventing excessive ROS and organelle damage. (B) The conventional view of BAT thermogenesis is that heat is mainly produced via UCP1. Several studies suggest that UCP1-indepenent pathways may also contribute to heat production in certain cases. One interesting possibility is that during chronic cool or cold adaptation, BAT increases the use of UCP1-independent pathways because it provides better fuel economy and supports homeostatic processes important to the overall health of the tissue.

Table 1.

Overview of studies using *in vivo* stable isotope tracers in BAT.

Authors	Tissues	Tracer	Route of Administration	Tracing Conditions	Notable Findings
Jung et al.[24]	BAT, Serum	U-^{13}C6 Glucose	Oral gavage	Thermoneutral, room temperature, acute cold, 4-week cold adaptation	• Acute cold and cold adaptation increase glucose oxidation and flux into the TCA cycle. • Cold adaptation utilizes glucose for a number of other pathways including PPP, DNL, acyl-carnitines, n-acetyl amino acids. • Acute cold has minimal glucose shunting compared to cold adaptation.
Wang et al.[26]	BAT	U-^{13}C6 Glucose	Intraperitoneal injection	10-day thermoneutral or cold exposure	• Chronic cold exposure increases glucose oxidation and utilization in the TCA cycle. • MPC inhibition in cold exposed mice blocks glucose oxidation and impairs body temperature regulation.
Hui et al.[27]	Brain, BAT, Muscle, Heart, Kidney, Spleen, Pancreas, Small Intestine, Lung, Liver, WAT	U-^{13}C6 Glucose, U-^{13}C3 Lactate, 1-^{13}C Lactate, U-^{13}C5 Glutamine, U-^{13}C3 Alanine, U-^{13}C4 3-Hydroxybutyrate, U-^{13}C16 Palmitate, U-^{13}C18 Oleate, U-^{13}C18 Linoleate, U-^{13}C3 Serine, U-^{13}C2 Glycine, U-^{13}C6 Citrate, U-^{13}C2 Acetate, U-^{13}C6 Leucine, U-^{13}C6 Isoleucine, U-^{13}C5 Valine, U-^{13}C6,^{15}N3 Histidine, U-^{13}C6,15N2 Lysine, U-^{13}C5, ^{15}N Methionine, U-^{13}C9,^{15}N Phenylalanine, U-^{13}C4,^{15}N Threonine, U-^{13}C11,^{15}N2 Tryptophan	Jugular vein infusion	Room temperature: Fed or Fasted, chow diet or keto diet	• BAT in the fed state utilizes large amounts of glucose and lactate to fuel the TCA cycle. • BAT in the fasted state switches to use less glucose and more lipids to fuel the TCA cycle. • When mice are fed a ketogenic diet, BAT predominately uses lipids to fuel the TCA cycle regardless of fed or fasted state.
Park, Haley, Le, Jung et al.[37]	BAT, Liver, Serum	U-^{15}N2,^{13}C5 Glutamine, ^{15}N Ammonium Chloride, U-^{15}N2 Glutamine	Retro-orbital injection	Thermoneutral, room temperature, 4-week cold adaptation	• BAT predominately uses glutamine carbons for the TCA cycle, while it's nitrogen predominately label amino acids and n-acetyl amino acids. • A single nitrogen from the glutamine tracer rapidly and abundantly labels a *de novo* synthesized glutamine in a temperature dependent manner in BAT.

Authors	Tissues	Tracer	Route of Administration	Tracing Conditions	Notable Findings
					• Free ammonia also rapidly and abundantly labels glutamine in BAT in a temperature dependent manner.
Neinast et al.[61]	BAT, Pancreas, Heart, Liver, gWAT, Muscle, Lung, Plasma	U-^{13}C6 Leucine, U-^{13}C6 Isoleucine, U-^{13}C5 Valine, U-^{13}C5 α-Ketoisovalerate	Oral gavage, intravenous injection, jugular vein infusion	Room temperature: WT, MCKa, BCKDK KO, db/db	• BCAAs are rapidly and abundantly oxidized in BAT. • Insulin-resistant mice have blunted BCAA oxidation in BAT.
TeSlass et al.[45]	BAT, iWAT, gWAT, Brain, Muscle, Heart, Lung, Liver, Kidney, Pancreas, Spleen, Small Intestine.	U-^{13}C6 Glucose, U-^{13}C3 Lactate, U-^{13}C3 Alanine, U-^{13}C3 Glycerol, U-^{13}C5 Glutamine.	Jugular vein infusion	Room temperature: Fasted and Fed	• BAT in the fed state predominately uses glucose and glycogen for glycolysis. • In the fasted state, BAT switches to predominately using glycogen, along with lactate, glycerol and glutamine.
Mills et al.[65]	BAT, Kidney, eWAT, sWAT, Liver, Heart, Plasma	U-^{13}C4 Succinate, U-^{13}C6 Glucose, U-^{13}C16 Palmitate	Tail vein Injection	Room temperature, thermoneutral, cold exposure	• Succinate is substantially accumulated in cold activated BAT. • BAT succinate accumulation drives thermogenesis independent of adrenergic signaling. • Succinate induced thermogenesis in BAT is driven by succinate dehydrogenase in a ROS dependent mechanism.
Mills et al.[66]	BAT, sWAT	U-^{13}C4 succinate,	Intravenous tail vein injection	Room temperature, acute and chronic CL-316,243 injection	• Mice without UCP1 are unable to clear circulating succinate. • Mice without UCP1 have higher succinate accumulation in the liver causing inflammation. • Increases BAT and beige content in mice antagonizes succinate accumulation in the liver and subsequent liver inflammation.