Increased Reliance on Muscle-based Thermogenesis upon Acute Minimization of Brown Adipose Tissue Function*§

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Skeletal muscle has been suggested as a site of non-shivering thermogenesis (NST) besides brown adipose tissue (BAT). Studies in birds, which do not contain BAT, have demonstrated the importance of skeletal muscle-based NST. However, muscle-based NST in mammals remains poorly characterized. We recently reported that sarco/endoplasmic reticulum Ca$^{2+}$/H$^{+}$ cycling and that its regulation by SLN can be the basis for muscle NST. Because of the dominant role of BAT-mediated thermogenesis in rodents, the role of muscle-based NST is less obvious. In this study, we investigated whether muscle will become an important site of NST when BAT function is conditionally minimized in mice. We surgically removed interscapular BAT (iBAT, which constitutes ~70% of total BAT) and exposed the mice to prolonged cold (4 °C) for 9 days. The iBAT-ablated mice were able to maintain optimal body temperature (~35–37 °C) during the entire period of cold exposure. After 4 days in the cold, both sham controls and iBAT-ablated mice stopped shivering and resumed routine physical activity, indicating that they are cold-adapted. The iBAT-ablated mice showed higher oxygen consumption and decreased body weight and fat mass, suggesting an increased energy cost of cold adaptation. The skeletal muscles in these mice underwent extensive remodeling of both the sarcoplasmic reticulum and mitochondria, including alteration in the expression of key components of Ca$^{2+}$ handling and mitochondrial metabolism. These changes, along with increased sarcolinip expression, provide evidence for the recruitment of NST in skeletal muscle. These studies collectively suggest that skeletal muscle becomes the major site of NST when BAT activity is minimized.

Thermogenesis is one of the most important homeostatic mechanisms that play a key role in the evolutionary expansion of mammals and birds. Despite its importance for the survival of the organism, the mechanistic details of various thermogenic mechanisms in birds and mammals remain far from completely understood. Heat production through muscle shivering is well known as the first line of defense to acute cold exposure. However, it remains unclear whether muscle can produce heat through non-shivering thermogenesis (NST). It is well known that mammals rely on brown adipose tissue (BAT) as a site for NST. BAT plays a dominant role in thermogenesis especially in neonatal mammals and in rodent species. BAT cells express uncoupling protein 1 (UCP 1) in the inner membrane of mitochondria which, upon activation, depletes the proton gradient (or uncouples oxidative phosphorylation) and generates heat rather than ATP (1–3). However, the predominant use of rodents as the experimental animal model defined BAT as the only site of NST but ignored the existence of other NST sites. Interestingly, BAT is either absent, as in birds and pigs, or is only a minor component, especially in adult large mammals, including humans, bringing into question of other sites for NST. Previous studies have proposed that, in addition to shivering, skeletal muscle also plays a role in NST (4–7); however, the molecular details were not fully understood. Studies using birds, which lack BAT, suggested that NST in skeletal muscle is vital during cold adaptation (8–12). These studies have shown that sarco/endoplasmic reticulum (SER) Ca$^{2+}$ cycling plays an important role in muscle-based thermogenesis (10–12). Recent studies have suggested that skeletal muscle could also serve as a site of NST in mammals, including humans; thus, the question is open to re-examination (Refs. 13–19 and reviewed in Ref. 20, 21). Recently, we reported that modulation of SER Ca$^{2+}$ cycling by sarcolipin (SLN) in skeletal muscle plays an important role in NST (13, 14, 22–24). Our data indicated that binding of SLN to sarcoendoplasmic reticulum Ca$^{2+}$ ATPase (SERCA) uncouples ATP hydrolysis from its Ca$^{2+}$ uptake function, leading to futile

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2 The abbreviations used are: NST, nonshivering thermogenesis; BAT, brown adipose tissue; SER, sarco/endoplasmic reticulum; SLN, sarcolipin; SERCA, sarcoendoplasmic reticulum Ca$^{2+}$ ATPase; iBAT, interscapular brown adipose tissue; Tc, core body temperature; VO$$_2$$, oxygen consumption; CASQ, calsequestrin; PLB, phospholamban; CaM-KII, Ca$^{2+}$/calmodulin-dependent protein kinase II; RyR, ryanodine receptor; TA, tibialis anterior; SDH, succinate dehydrogenase; SR, sarcolipin reticulum; VDAC, voltage-dependent anion channel; Tricine, N-(2-hydroxy-1,1-bis(hydroxymethyl) ethyl)glycine.
Ca\textsuperscript{2+} cycling and heat production (22, 25). We have shown that mice lacking SLN were cold-sensitive, indicating a role of SLN in muscle-based NST (13). In addition, we showed that cold acclimatization of UCP 1 knockout mice (UCP 1-KO) up-regulated SLN expression in skeletal muscles, suggesting that muscle thermogenesis is recruited to a greater extent when BAT function is lost (14). Furthermore, cold adaptation studies using neonatal mice firmly suggest that muscle serves as a site of thermogenesis beyond shivering (23).

The major objective of this study is to demonstrate that skeletal muscle-based thermogenesis can become prominent when BAT function is minimized. We hypothesized that the contribution of skeletal muscle to NST is masked in mice because of the dominant role of BAT-dependent thermogenesis in cold adaptation. In mice, a major depot of BAT is located between the two scapulae, called interscapular BAT (iBAT), and constitutes more than 70% of total BAT depots present. Therefore, in this study, we surgically removed iBAT and exposed the mice to cold (4 \degree C) for a period of 9 days. We demonstrate here that the iBAT-ablated mice were able to maintain body temperature but induced substantial remodeling of both SER and mitochondria in their skeletal muscles. We found significant changes in mitochondrial architecture and SER protein expression as evidence for the recruitment of NST in skeletal muscle. Our studies show that skeletal muscle can substitute for the loss of BAT function and can serve as an important site of NST.

**Results**

**Ablation of iBAT Does Not Impair Body Temperature Maintenance**—iBAT constitutes \textgeq\textasciitilde70\% of the total BAT depots in rodents. We surgically removed iBAT to minimize the contribution from BAT to reveal the importance of skeletal muscle NST (supplemental Movie 1). This method precludes compensatory changes that can be induced in the UCP 1-KO model. Following iBAT ablation, mice were allowed to recover for 2 days at thermoneutrality (29 \pm 1 \degree C). iBAT ablation in mice did not affect food intake, body weight, core body temperature (Tc), routine behavior, or serum metabolite levels (data not shown) when housed individually at 29 \pm 1 \degree C or 22 \pm 1 \degree C. Next we challenged these mice to acute cold (4 \degree C) for a period of 9 days. The iBAT-ablated mice showed a greater drop in Tc (0.8 \pm 0.3 \degree C lower) than sham-operated (+iBAT) mice during the first 4 h of the cold challenge (Fig. 1A). However, they were able to maintain Tc (~35–37 \degree C) similar to sham controls and did not develop hypothermia during the 9-day period of cold challenge. The mice were quite active at temperatures between 29 \pm 1 \degree C and 22 \pm 1 \degree C (Fig. 1B). When switched to 4 \degree C, long-range activity (high bursts of movement) was significantly decreased in both groups. Interestingly, on the first day of the cold challenge, the iBAT-ablated group (487.0 \pm 13.0) showed significantly higher (p = 0.0002) physical activity than the sham group (333.0 \pm 14.0), which is mainly due to increased shivering and grooming behavior. The physical activity data show that high-intensity shivering was prominent during the first 48 h in both groups, but from day 4 onward, shivering was not evident. By day 4, both groups showed similar long-range physical activity, and by day 8 of cold adaptation, the difference in physical activity between the two groups became nonsignificant (547.4 \pm 25.5 \textit{versus} 508.4 \pm 32.7, p = 0.383). These data indicate that, during prolonged cold challenge, NST mechanisms must be activated to maintain Tc in iBAT-ablated mice.

**Cold Adaptation in the Absence of iBAT Is Energetically Costly**—Next we investigated the effect of iBAT ablation on whole body metabolism. Both the iBAT-ablated and sham groups showed similar oxygen consumption (VO\textsubscript{2}) rates at 29 \pm 1 \degree C (p = 0.258) and 22 \pm 1 \degree C (p = 0.078). When challenged to 4 \degree C, both groups up-regulated their VO\textsubscript{2} significantly (Fig. 1C). Interestingly, the iBAT-ablated mice showed higher VO\textsubscript{2} during the whole period of cold exposure. Their mean VO\textsubscript{2} was higher by 9.2\% (5225 \pm 30.5 versus 5705 \pm 34.1, p < 0.0001) on the first day and 3.2\% (5435 \pm 34.5 versus 5620 \pm 34.0, p = 0.011) on day 8 than that of the sham group. Both sham and iBAT-ablated mice consumed approximately three times as much food during the first day of cold exposure relative to housing at 22 \pm 1 \degree C. The iBAT-ablated group consumed 24.3\% more food (15.3 \pm 0.73 g \textit{versus} 12.3 \pm 0.55 g, p < 0.001) on the first day in the cold (Fig. 2A), but on day 8, food intake (9.9 \pm 0.54 g \textit{versus} 10.5 \pm 0.78 g) was similar to controls. There was no significant difference in body weight when housed at 29 \pm 1 \degree C or 22 \pm 1 \degree C, but cold challenge resulted in a significant loss of body weight in iBAT-ablated mice (Fig. 2B). The iBAT-ablated mice showed a greater decrease, from 27.0 \pm 0.25 g to 23.7 \pm 0.38 g compared with sham controls (27.1 \pm 0.54 g to 25.4 \pm 0.68 g). Next we measured the weight of the intraperitoneal fat pad, which is the major energy reserve during increased physiological demand such as prolonged cold adaptation. The sham group lost only 10\% of intraperitoneal fat mass (3.2 \pm 0.22 g \textit{versus} 2.84 \pm 0.2 g), but the iBAT-ablated group lost 33.4\% (3.4 \pm 0.2 g \textit{versus} 2.7 \pm 0.15 g, Fig. 2C) after 9 days in the cold. Serum metabolite measurement showed that glucose was higher whereas triglyceride was lower in cold-adapted iBAT-ablated mice (Fig. 2D).

These data suggest that cold adaptation in the absence of iBAT is energetically costly.
Upon exposure to 4.0 °C (first inset), there was no significant difference in activity between the iBAT and sham groups (Fig. 1, B). Physical activity was measured at various housing temperatures. At 29.0 ± 1.0 °C (first inset) and 22.0 ± 1.0 °C (second inset), there was no significant difference in activity between the iBAT and sham groups. At 4 °C, the iBAT group exhibited higher local activity with increased grooming behavior (third inset) for 3 days. Gradually, at 4 °C, they resumed long-range activity, and by day 8, physical activity of both the iBAT and sham groups did not show any statistical difference (fourth inset). Unpaired Student’s t-test was applied. *** p < 0.00001; ns, p > 0.05 (nonsignificant).

Mitochondrial Architecture and Metabolism Are Altered in the Skeletal Muscle of iBAT-ablated Mice—We observed that iBAT-ablated mice were able to maintain Tc but at a significantly higher energy cost (Fig. 1C). We hypothesized that this is due to increased reliance on muscle-based NST and therefore wanted to investigate whether iBAT ablation caused a substantial remodeling of mitochondrial architecture and metabolism in the skeletal muscles. Electron microscopic analyses of tibialis anterior (TA) muscle revealed that there was a higher abundance of intramyofibrilar mitochondria and lipid droplets surrounded by mitochondria in the iBAT-ablated group (Fig. 5A). At a higher magnification, the intramyofibrilar mitochondria showed more elaborate cristae in the iBAT-ablated mice (Fig. 5B), suggesting increased mitochondrial activity in the muscle.

alteration in Ca²⁺ cycling, we examined Ca²⁺ signaling pathways. Interestingly, we found that the phosphorylated form of Ca²⁺/calmodulin-dependent protein kinase II (CaMKII) α at threonine 286 was increased in cold-adapted iBAT-ablated mice, although total CaMKIIα expression was unaffected (Fig. 4, A and B). CaMKII is a known mediator of ryanodine receptor (RyR) 2 phosphorylation at residue serine 2814 (26), which corresponds to threonine 2848 in RyR 1 (Fig. 4C). Therefore, we analyzed RyR 1 expression and its phosphorylation status at Thr²⁸⁴⁸ (Thr(P)²⁸⁴⁸-RyR 1). We found both an increase in RyR 1 expression (Fig. 4, D and E) and RyR 1 phosphorylation (Thr(P)²⁸⁴⁸-RyR 1) upon cold adaptation. These data suggest that significant changes in the SER Ca²⁺ handling machinery are important for increased recruitment of muscle-based NST.

FIGURE 1. Body temperature maintenance, physical activity, and oxygen consumption in iBAT-ablated mice. A, iBAT-ablated mice (−BAT, n = 8) were able to maintain body temperature similar to sham controls (+BAT, n = 8) when housed at 29.0 ± 1.0 °C (green line) and at 22.0 ± 1.0 °C (blue line). Upon exposure to 4.0 °C (brown line) the −iBAT group maintained a slightly lower body temperature 0.8 ± 0.3 °C (inset) compared with the sham group during the first 4 h (black box). B, physical activity was measured at various housing temperatures. At 29.0 ± 1.0 °C (first inset) and 22.0 ± 1.0 °C (second inset), there was no significant difference in activity between the −iBAT and +iBAT groups. At 4 °C, the −iBAT group exhibited higher local activity with increased grooming behavior (third inset) for 3 days. Gradually, at 4 °C, they resumed long-range activity, and by day 8, physical activity of both the groups did not show any statistical difference (fourth inset). Unpaired Student’s t-test was applied. ***, p < 0.0001; ns, p > 0.05 (nonsignificant). C, VO₂ of the −iBAT (iBAT ablated) group was similar to that of the +iBAT (sham) group at housing temperatures of 29.0 ± 1.0 °C (first inset) and 22.0 ± 1.0 °C (second inset). Upon acute cold (4 °C) challenge, the −iBAT group up-regulated VO₂ significantly compared with the +iBAT group on the first day (third inset). By day 8 of cold adaptation, VO₂ of the −iBAT group was still higher than that of controls (fourth inset). Unpaired Student’s t-test was applied. *, p < 0.05; ****, p < 0.00001; ns, p < 0.05 (nonsignificant).
In addition, we found an increase in succinate dehydrogenase (SDH) activity staining in fast-twitch muscle, indicating an increase in oxidative metabolism (Fig. 5C) in iBAT-ablated mice. This is further supported by the data showing higher mitochondrial respiration (O2 consumption) and ATP synthesis activity in response to different substrates (glutamate + malate, ADP, and succinate) in muscles from iBAT-ablated mice (Fig. 5D). Next we studied the expression levels of mitochondrial electron transport chain proteins (Fig. 5E). The electron transport chain complex proteins (II, III, and IV) along with ATP synthase (complex V) were significantly up-regulated after cold adaptation, and iBAT-ablation led to up-regulation of complexes I, III, and V (Fig. 5F). These data suggest that the mitochondria are more active and that fatty acid metabolism is recruited to a greater extent in skeletal muscle in iBAT-ablated mice. We found up-regulation in peroxisome proliferator-activated receptor β, an indicator of higher fatty acid utilization, in skeletal muscles from cold-adapted iBAT-ablated mice (Fig. 5, E and F). This was also supported by data showing that fatty acid transport proteins, lipoprotein lipase and CD36, were up-regulated in skeletal muscles in sham mice and were further up-regulated in iBAT-ablated mice (Fig. 5F). Interestingly, cold-adapted iBAT-ablated mice had lower serum triglyceride (Fig. 3D), suggesting greater fatty acid uptake and utilization in muscle.

Evidence for Increased Mitochondrial Fusion in Cold-adapted Skeletal Muscle of iBAT-ablated Mice—We next studied key proteins involved in mitochondrial dynamics, including fusion and fission in skeletal muscle. The expression of mitochondrial fusion proteins (mitofusin (Mfn) 1, Mfn 2, and optic atrophy (Opa) 1) were increased slightly in cold-exposed sham controls, but their expression was significantly up-regulated in iBAT-ablated mice (Fig. 6, A and B), indicating increased mitochondrial fusion in skeletal muscle. Interestingly, studies have shown that Mfn 2 also plays a role in SR-mitochondrial membrane tethering. Therefore, the up-regulation of Mfn 2 in the iBAT-ablated mice (Fig. 6B) indicates increased SER-mitochondrial Ca2+ cross-talk. On the other hand, expression of mitochondrial fission proteins (fission (Fis) 1, dynamin-related protein (Drp) 1, and mitochondrial fission factor) was unaltered by cold exposure (Fig. 6, C and D). Interestingly, we found a significant up-regulation of voltage-dependent anion channel (VDAC) 1 in the skeletal muscle upon cold adaptation (Fig. 6, E and F). VDAC 1 is known to facilitate mitochondrial Ca2+
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Despite the suggestion that skeletal muscle also plays a role in NST during cold adaptation, especially after iBAT ablation (Fig. 6, E and F). These data strongly suggest that there is increased mitochondrial fusion over fission during skeletal muscle adaptation to cold, which is important to meet the energy demand of elevated muscle-based NST.

Discussion

Despite the suggestion that skeletal muscle also plays a role in NST during cold adaptation (4, 5, 7, 28, 29), it remains unclear to what extent muscle-based NST is activated during prolonged cold adaptation. Also, the presence of functionally active BAT may influence the contribution from muscle. We hypothesized that the presence of BAT can minimize the importance of muscle-based NST, especially in rodents such as mice and rats (30–32). Therefore, our objective was to unravel NST mechanisms in the skeletal muscle when BAT function is acutely minimized by surgical ablation of iBAT. Conditional ablation of iBAT is superior over using a global UCP 1-KO mouse model because these animals were found to be highly heterogeneous in their cold sensitivity (33). Furthermore, there have been limited studies addressing how loss of BAT activity impacts skeletal muscle, especially Ca\(^{2+}\) cycling and mitochondrial metabolism in rodents during cold adaptation. Here we demonstrate that, when BAT function is acutely minimized, skeletal muscle becomes the major site of NST during prolonged cold adaptation. We further show that increased reliance on skeletal muscle-based NST requires substantial remodeling of the SER Ca\(^{2+}\) handling machinery and synergy with mitochondrial metabolism.

**SER Ca\(^{2+}\) Handling Plays a Central Role in Muscle NST—** Studies carried out in birds showed that cold adaptation increases SER Ca\(^{2+}\) cycling and RyR1 content in skeletal muscles (11, 12). Our data showing similar adaptation in mouse skeletal muscles indicate that the molecular mechanisms of muscle-based NST are analogous in both birds and mammals. Recent studies from our laboratory have shown that uncoupling of SERCA activity by SLN plays an important role in muscle NST (13, 34). Uncoupled SERCA activity increases ATP hydrolysis and heat production, thereby contributing to muscle-based adaptive thermogenesis (13, 25, 35, 36). We also found that SLN expression was increased significantly upon iBAT ablation and a preferential expression of the SERCA 2a isoform, which suggests that SLN/SERCA interaction plays an important role in muscle NST. We also found that there is an increase in both RyR 1 expression and phosphorylation status in iBAT mice. It has been shown that phosphorylation of RyR leads to enhanced Ca\(^{2+}\) leaking (26, 37, 38). Here we show, for the first time, that Thr\(^{2844}\) in RyR 1 (a site analogous to the well characterized Ser\(^{2814}\) on RyR 2) (26) is phosphorylated during cold exposure. RyR1 phosphorylation by PKA at RyR1-Ser(P)\(^{2844}\) was reported previously (39). We propose that SLN-mediated uncoupling of SERCA leads to an increase in cytosolic Ca\(^{2+}\)\(^{2+}\) and activation of CaMKII by phosphorylation at Thr\(^{286}\). Phosphorylation of RyR1 increases Ca\(^{2+}\) leaking from the SR, increasing SERCA activity, setting up a leak reuptake cycle analogous to the functioning of the heater organ in endothermic fishes (21). Based on our data and results published previously, it seems possible that activation of CaMKII is a cold-induced, Ca\(^{2+}\)-sensitive molecular response conserved in diverse life forms (40).

**Increased Mitochondrial Dynamics in iBAT-ablated Mice Suggests Enhanced Thermogenic Capacity in Muscle**—We observed that, in the absence of iBAT, the recruitment of muscle-based NST is energetically costly, requiring substantial remodeling of the mitochondrial machinery, including mitochondrial abundance and architecture. An important finding is that proteins involved in mitochondrial fusion were significantly increased in muscle during cold adaptation. Mitochondria are highly dynamic organelles that continually undergo fusion and fission in response to increased intracellular energy demand (41). High-energy demand states in muscle activate mitochondrial fusion, which increases proton motive force, thereby enhancing ATP production, whereas mitochondrial fission decreases proton motive force (42, 43). In addition, mitochondrial ATP production can be boosted by Ca\(^{2+}\) influx from the cytosol, activating Ca\(^{2+}\)-sensitive matrix dehydrogenases and ATP synthase (36). Mitochondrial Ca\(^{2+}\) uptake is mediated by VDAC 1 located on the outer mitochondrial membrane and also affected by the spatial distance from the SER (44). Recent studies suggest that mitofusins, especially Mfn 2,
function as a tether, bridging the SER (45, 46) and mitochondria, thereby facilitating interorganelle Ca^{2+} cross-talk (47). Further data showing superinduction of Mfn 2 upon iBAT-ablation along with cold-mediated up-regulation of VDAC 1 may suggest increased Ca^{2+} cross-talk between the SER and mitochondria plays a crucial role in muscle-based NST. The mechanisms that contribute to muscle-based NST is schematically depicted in Fig. 7.

**Increased Recruitment of Muscle-based NST in iBAT-ablated Mice**—Previous studies have speculated that increased shivering is the basis for cold adaptation in UCP 1-KO mice (39). Shivering is an acute response and can last only few seconds at a time. Continued shivering during prolonged cold adaptation is detrimental for muscle function, as it can cause fatigue and injury and weaken muscle function and metabolic capacity. Prolonged shivering can be energetically costly and can severely compromise survival of the organism in its wild habitat, as both shivering and coordinated fight-or-flight response cannot be recruited simultaneously. Therefore, NST needs to be activated during the transition from shivering to a cold-acclimated state. Furthermore, activation of NST in skeletal muscle will preserve its function and, at the same time, produce heat. We showed previously that iBAT-ablated mice can survive an acute cold (4 °C) challenge even when shivering is minimized by administration of curare (an agent that blocks acetylcholine receptors at neuromuscular junctions), suggesting the existence of BAT-independent NST even in mice (13). The drastic changes in skeletal muscle reported here, especially increased reliance on oxidative metabolism, during prolonged cold acclimatization in iBAT-ablated mice is evidence for the existence of muscle-
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**Figure 5.** IBAT ablation causes a significant up-regulation of oxidative metabolism in skeletal muscle. A, low magnification electron micrographs of cold-adapted TA, a fast-twitch skeletal muscle. Removal of IBAT leads to an increase in the abundance of intramyofibrillary mitochondria and lipid droplets (yellow arrowheads). B, high-magnification electron micrographs of cold-adapted skeletal muscles. The intramyofibrillary mitochondria in the skeletal muscles from IBAT-ablated mice are ultrastructurally much better organized with densely packed cristae. C, representative images showing SDH activity in cold-adapted TA-skeletal muscle samples. The –IBAT group shows denser staining, indicating higher SDH activity, suggesting higher oxidative metabolism. D, muscles from IBAT-ablated mice show higher oxygen consumption. Glut, glutamate; Mal, malate; wt, weight. E, representative Western blot showing the expression of the electron transport complex proteins peroxisome proliferator-activated receptor β (PPARβ), CD36, lipoprotein lipase, and citrate synthase (CS) in gastrocnemius muscle. 20 μg of muscle homogenate was analyzed. F, densitometric quantification of the proteins shown in E. The levels of complexes II, III, IV, and ATP synthase (V) were higher in muscles from sham mice compared with controls from 22 °C. The levels of complexes I, III, and V were higher in muscles from IBAT-ablated mice compared with sham controls. Values are normalized to the GAPDH level. All Western blotting experiments were repeated at least twice, and data were included for statistical analysis. Unpaired Student’s t test was applied. *, p < 0.05; **, p < 0.001; ***, p < 0.0001; ns, p > 0.05 (nonsignificant).

Based on the results, IBAT ablation causes a significant up-regulation of oxidative metabolism in skeletal muscle. This is supported by an increased intramyofibrillary lipid droplet and increased expression of fatty acid-transporting proteins in skeletal muscles, decreased fat mass (indicating lipolysis from the fat store). It is known that, during shivering, muscles predominantly utilize glucose, so increased fatty acid utilization in IBAT-ablated mice indicates increased recruitment of muscle-based NST. The reliance of muscle-based NST on lipids as fuel may provide metabolic flexibility to the animal during cold adaptation by sparing glucose to be used by other organs, including the brain.

**The Evolutionary Significance of Skeletal Muscle-based NST**—The importance of muscle as a site of NST precedes the origin of BAT among eutherian mammals just before their diversification from metatherians (21). The origin of BAT has been a significant step in mammalian endothermy. BAT-based NST is evolutionarily advantageous, as it can generate a significant amount of heat during acute demand in a short period of time. Heat production from BAT is more efficient and is advantageous especially where heat loss is huge, such as newborn and small mammals having a high surface-to-body ratio. Hibernating mammals, during arousal from hibernation, need rapid heating of the whole body (in some cases from 0 °C to 37 °C) within a few minutes (20–120 min), where BAT is better suited to meet the thermogenic demand (48, 49). This study provides important insights into this topic. Unlike BAT-based NST, muscle-based NST is energetically costly, requiring increased food intake. It is evident from this study that loss of IBAT (~200 mg less than 1% body weight) causes substantial remodeling of the skeletal muscle. Muscle-based NST is better suited in an ecological niche where food (energy) availability is not a limiting factor and heat production through Ca^{2+} cycling is affordable (21). Therefore, mammalian taxa inhabiting nutrient-rich ecological niches could rely on muscle NST, remain active throughout the year, and evade hibernation. Considering that skeletal muscle constitutes more than 40% of body mass, using multiple NST mechanisms (protein synthesis, degradation,
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**A**

![Control at 22°C](image1)

**B**

![Control at 22°C](image2)

**C**

![Control at 22°C](image3)

**D**

![Control at 22°C](image4)

**E**

![Control at 22°C](image5)

**F**

![Control at 22°C](image6)

**FIGURE 6. Increased expression of proteins associated with mitochondrial fusion in iBAT-ablated mice.** A, Mfn 1, Mfn 2, and Opa 1 associated with mitochondrial fusion were up-regulated during cold adaptation and further increased upon removal of iBAT. B, densitometric quantification of mitochondrial fusion proteins. C, the expression of mitochondrial fission proteins was not altered in skeletal muscle. D, cold adaptation and iBAT removal did not alter level of mitochondrial fission proteins. E, the mitochondrial proteins VDAC 1 (Ca⁺⁺ channel), ANT 1 (ATP transporter), and TFAM (regulator of mitochondrial DNA synthesis) were increased. F, quantification of VDAC 1, TFAM, and ANT 1. All Western blotting experiments were repeated at least three times, and data were included for statistical analysis. Values were normalized to the GAPDH level. Unpaired Student’s t test was applied. *, p < 0.05; **, p < 0.01; ***, p < 0.001; ns, p > 0.05 (nonsignificant).

metabolic activities, ion transport in the plasma membrane, SER, and mitochondrial heat production), it is capable of producing heat comparable to BAT in many organisms.

**Experimental Procedures**

**Animals**—12-week-old C57BL/6J male mice were obtained from Harlan Laboratories for this study. The study protocol was approved by the Ohio State University Institutional Animal Care and Use Committee. All animal procedures were carried out in our Association for Assessment and Accreditation of Laboratory Animal Care-accredited animal facility and conducted in accordance with the Guide for the Care and Use of Laboratory Animals. Mice were housed in groups of five in polycarbonate cages before surgery. The animals were housed in a controlled environment at a temperature of 22 ± 1 °C with a 12:12 h light: dark cycle and relative humidity of about 50%. Mice were fed a regular rodent diet (2014, Harlan Teklad, Madison, WI) and provided with water ad libitum.

**Surgical Removal of iBAT**—iBAT ablation surgery was performed with slight modifications as described previously (50, 51). The surgical procedure is shown in supplemental Movie 1. The mice were allowed to recover after surgery in a prewarmed empty cage with a heat lamp. After the mice became completely conscious, they were singly caged and observed for signs of pain or discomfort for 2 days.

**Metabolic Measurements and Acute Cold Challenge**—To determine whether iBAT removal affected the metabolic state of the mice, we measured VO₂ and physical activity using the Comprehensive Lab Animal Monitoring System (oxymax/CLAMS) equipped with a temperature-controlled environmental chamber from Columbus Instruments (Columbus, OH). First, we measured the VO₂ in both sham-operated and iBAT-removed mice at 29 ± 1 °C to determine the basal metabolic rate. After 1 day, the ambient temperature was dropped to 22 ± 1 °C and maintained for 2 days and then dropped to 4 ± 1 °C and maintained for 9 days. The Tc was monitored twice daily using thermal transponders (IPTT300, Bio Medic Data System, Seaford, DE) implanted in the back of the mouse below the skin during the surgical or sham procedure. Food intake was measured once daily.

**Blood Sampling and Clinical Chemistry**—Blood samples were collected by incising the right submandibular vein of anesthetized mice with a sterile 4-mm lancet (MediPoint, Mineola, NY). The serum metabolite profile in freshly collected blood was measured by a CardioChek PA analyzer using separate ketone (REF 1718) and lipid panel (REF 1710) strips. Plasma glucose was analyzed using Autodisc blood glucose test strips on a Breeze2 glucometer from Bayer HealthCare (Mishawaka, IN).

**Electron Microscopy and SDH Activity Staining of Muscle Tissues**—Longitudinal Sections of TA muscle were taken after 9 days of cold adaptation and fixed with 1% glutaraldehyde solution. Samples were processed by the core facility, and images were obtained using a Tecnai G2 Spirit transmission electron microscope (FEI, Hillsboro, OR) at various magnifications. SDH activity staining was performed on sections of TA muscles as described earlier (52).

**Measurement of Mitochondrial Respirations**—Measurement of mitochondrial function (oxygen consumption) in permeabilized muscle fibers of red gastrocnemius was performed at 37 °C using Oxygraph 2K (Oroboros Inc., Innsbruck, Austria). State 4
FIGURE 7. Schematic showing the structural remodeling of the SR and mitochondria during adaptation to cold in iBAT-ablated mice. iBAT ablation leads to significant recruitment of skeletal muscle-based NST during cold adaptation. NST in muscle relies on increased heat production through uncoupling of SERCA activity by SLN. Increased SLN/SERCA interaction promotes uncoupling of SERCA activity, leading to heat production but creating increased energy demand. To support the increased energy demand, there is remodeling of mitochondria and the SR structure, including increased mitochondrial size (elaborate cristae), up-regulation of oxidative phosphorylation proteins, and increased SR-mitochondrial contacts that will allow increased Ca\(^{2+}\) influx into mitochondria, leading to elevated ATP production. In addition, SLN uncoupling of SERCA results in increased local cytosolic Ca\(^{2+}\) concentration, thereby activating Ca\(^{2+}\)-dependent signaling pathways, including CAMKII, known to hyperphosphorylate RyR. Phosphorylation of RyR1 leads to spontaneous Ca\(^{2+}\) release from the SER, leading to increased SERCA activity, ATP hydrolysis, and heat production. This model proposes how increased Ca\(^{2+}\) leakage from the SER and reuptake of Ca\(^{2+}\) by SERCA can provide the basis for NST in muscle.

respiration (non-phosphorylating inner membrane proton leak) was measured following the addition of saturating concentrations of malate (2 mM) and glutamate (500 mM). Maximal respiration supported by electron flux through complex I was measured with the addition of ADP (500 mM). State 3 respiration (maximal coupled respiration) with convergent electron flux through complex I and complex II was achieved by adding saturating concentrations of succinate (500 mM). Maximal respiration (maximal coupled respiration) with convergent electron flow was achieved by adding saturating concentrations of oxalate and 0.5 mM EGTA. The SERCA ATP hydrolysis reaction was incubated for 20 min and stopped using 100 mM EGTA. The SERCA ATP hydrolysis reaction was incubated for 20 min and stopped using 100 mM EGTA. A 20-µl aliquot was used for color development using a Biozyme green phosphate assay. The amount of P, released was calculated as nanomoles P, per minute per milligram of protein.

Western Blotting—Protein levels were quantified using a Western blotting technique following a protocol published previously (14, 52). 20 µg of protein prepared from gastrocnemius muscle was analyzed on 10% Tris/glycine SDS-PAGE. For detecting RyR 1 and Thr(P)\(^{2845}\)-RyR1, 50 µg of total protein was analyzed on 5% SDS-PAGE. SLN and PLB were analyzed in total protein prepared from the red portion of gastrocnemius muscle using 16% Tris/Tricine SDS-PAGE. After electrophoresis, proteins were transferred to 0.45 µm nitrocellulose membranes. Membranes were blocked for nonspecific binding with Odyssey® blocking buffer (TBS, LI-COR Biosciences) for 1 h at room temperature. The primary antibodies used included oxidative phosphorylation antibody mixture (catalog no. MS604, used at 1:2000 dilution) from MitoSciences USA; anti-mitochondrial fission factor (catalog no. 17090-1-AP, used at 1:500 dilution) from Proteintech Group Inc.; anti-VDAC 1 (catalog no. AVC-001, used at 1:1000 dilution) from Alomone Labs; anti-Opa 1 (catalog no. 612606, used at 1:500 dilution) from BD Transduction Laboratories; anti-FIS 1 (catalog no. MABN391, used at 1:500 dilution) from EMD Millipore; anti-lipoprotein lipase (catalog no. ab21356, used at 1:1000 dilution) and anti-Mfn 2 (catalog no. ab56889, used at 1:2000 dilution) from Abcam; anti-DRP 1 (catalog no. 8570, used at 1:1000 dilution) from Cell Signaling Technology; and anti-Mfn 1 (catalog no. sc-50330, used at 1:1000 dilution), anti-ANT 1 (catalog no. sc-9299, used at 1:2000 dilution), anti-peroxisome proliferator-activated receptor β (catalog no. sc-7197, used at 1:1000 dilution), anti-cluster of differentiation (CD) 36 (catalog no. sc-9154, used at 1:1000 dilution), anti-CaMKII (catalog no. sc-9035, used at 1:500 dilution), anti-p-CaMKIα (catalog no. sc-12886-R, used at 1:500 dilution), and anti-GAPDH (catalog no. sc-25778, used at 1:2000 dilution) from Santa Cruz Biotechnology, Inc. Anti-vinculin antibody (catalog no. MA6896, used at 1:1000 dilution) was from R&D Systems. Anti-CASQ 1 (catalog no. MA5–913, used at 1:2000 dilution) was from Thermo Fisher Scientific. SERCA1a (1:2000 dilution), SERCA 2a (1:1000 dilution), CASQ 2 (1:1000 dilution), and SLN (1:1000 dilution) antibodies were custom-generated in the laboratory of Dr. Periasamy. RyR1 and Thr(P)\(^{2845}\)-RyR 1 antibodies (used at 1:1000 and 1:500 dilutions, respectively) were generated in the laboratory of Dr. Wehrens using the regular and phospho-peptide QSAqTYPD-PREGYC. After the primary treatment, the blots were washed with 0.05% Tris-buffered saline, 0.1% tween 20 and treated with IRDye secondary antibody (LI-COR Biosciences), and the blots were imaged using the IR imaging system ODYSSEY® CLx (LI-COR Biosciences).

SERCA-mediated ATP Hydrolysis Assay—The functional activity of SERCA was performed using SR vesicles prepared from gastrocnemius muscle as described in Ref. 25. ATP hydrolysis activity was measured using the Biolum green phosphate assay. The reaction was initiated by addition of 20 µg of crude supernatant in assay buffer containing 20 mM MOPS (pH 7.0), 5 mM MgCl, 100 mM KCl, 5 mM Na3, 5 mM ATP, 5 mM K+ oxalate, and 0.5 mM EGTA. The SERCA ATP hydrolysis reaction was incubated for 20 min and stopped using 100 mM EGTA. A 20-µl aliquot was used for color development using a Biolum green phosphate assay. The amount of P, released was calculated as nanomoles P, per minute per milligram of protein.
alignments using Clustal Omega. The accession numbers of the sequences used are as follows. The RyR1 sequences used were rabbit (NP_010195188), American pika (XP_004592526), chinchilla (XP_013377862), dog (XP_003638882), cattle (NP_001193706), sheep (XP_011986639), chimpanzee (XP_009433809), pig (NP_001015534), green monkey (XP_007994884), human (NP_0005351), jerboa (XP_004659847), mole (XP_008852255), walrus (XP_004396552), small-earred galago (XP_003796202), golden mole (XP_006871604), mouse (NP_0031315), rat (F1LMY4.1), camel (XP_014411102), panda (XP_011231434), lizard (XP_008118153), frog (XP_012824470), horse (XP_014587158), whale (XP_004284315), alligator (XP_014379036), cat (XP_012882424), hamster (XP_007625324), vole (XP_005371360), hedgehog (XP_016048603), bat (XP_006761508), deer mouse (XP_006982164), Tasmanian devil (XP_012401108). The RyR2 sequences used were human (NP_001026), rat (NP_0114467), mouse (NP_076357), rabbit (NP_010706226), bison (XP_0010585419), panda (XP_002923703), horse (XP_014702004), pigeon (XP_013224527), monkey (XP_010382984), lizard (XP_008123140), eagle (XP_010562147), hedgehog (XP_005729763), alligator (XP_014452198), mole (XP_006872072), jerboa (XP_004662223), Tasmanian devil (XP_003767843), American pika (XP_004578917), bat (XP_008148100), bear (XP_008687891), deer mouse (XP_015854219), camel (XP_010961169), vole (XP_013208064), hamster (XP_00350533), chinchilla (XP_013360160), sheep (XP_011980516), marmoset (XP_008984013), marmoset (XP_009440004), pig (XP_013838665), and dog (XP_013968196). The illustrations showing the percentage of conservation of residues around the Thr(P)CH3 or RYR1 antibody epitope were generated using Weblogo 3.0 (53).

Author Contributions—N. C. B. conceived the study, N. C. B., S. K. M., and S. S. collected data. N. C. B., S. K. M., S. S., X. H. T. W., and M. P. analyzed data. All authors contributed to the writing of the manuscript.

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