In Vivo Noninvasive Characterization of Brown Adipose Tissue Blood Flow by Contrast Ultrasound in Mice

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**Background**—Interventions to increase brown adipose tissue (BAT) volume and activation are being extensively investigated as therapies to decrease the body weight in obese subjects. Noninvasive methods to monitor these therapies in animal models and humans are rare. We investigated whether contrast ultrasound (CU) performed in mice could detect BAT and measure its activation by monitoring BAT blood flow. After validation, CU was used to study the role of uncoupling protein 1 and nitric oxide synthases in the acute regulation of BAT blood flow.

**Methods and Results**—Blood flow of interscapular BAT was assessed in mice (n=64) with CU by measuring the signal intensity of continuously infused contrast microbubbles. Blood flow of BAT estimated by CU was 0.5±0.1 (mean±SEM) dB/s at baseline and increased 15-fold during BAT stimulation by norepinephrine (1 µg·kg⁻¹·min⁻¹). Assessment of BAT blood flow using CU was correlated to that performed with fluorescent microspheres (R²=0.86, P<0.001). To evaluate whether intact BAT activation is required to increase BAT blood flow, CU was performed in uncoupling protein 1–deficient mice with impaired BAT activation. Norepinephrine infusion induced a smaller increase in BAT blood flow in uncoupling protein 1–deficient mice than in wild-type mice. Finally, we investigated whether nitric oxide synthases played a role in acute norepinephrine-induced changes of BAT blood flow. Genetic and pharmacologic inhibition of nitric oxide synthase 3 attenuated the norepinephrine-induced increase in BAT blood flow.

**Conclusions**—These results indicate that CU can detect BAT in mice and estimate BAT blood flow in mice with functional differences in BAT. (Circ Cardiovasc Imaging. 2012;5:652-659.)

**Key Words:** brown adipose tissue ■ imaging ■ contrast ultrasound ■ uncoupling protein ■ nitric oxide synthase

Brown adipose tissue (BAT) can be activated by the sympathetic nervous system or thyroid hormone to produce heat, and is a major contributor to nonshivering thermogenesis. The activation of BAT induces an increase in lipid and glucose oxidation by brown adipocytes, augmenting their mitochondrial respiration and oxygen consumption. In activated brown adipocytes, mitochondrial respiration becomes uncoupled from adenosine triphosphate production, a process mediated by uncoupling protein (UCP) 1. Uncoupling allows protons, produced by the oxidation of lipids and glucose, to re-enter the mitochondrial matrix, while generating heat instead of adenosine triphosphate (for a review, see).

**Clinical Perspective on p 659**

Brown adipose tissue has been identified in human infants and in rodents for several decades. In rodents, it is present in large depots primarily located between the scapulae. Recently, several studies using 18F-fluorodeoxyglucose positron-emission tomography scans coupled with computed tomography have confirmed the presence of metabolically active BAT in adult humans. Stimulation of BAT to increase energy expenditure may potentially be used for antiobesity therapies (for a review, see). Detecting BAT and monitoring its activation state is therefore necessary for the development of BAT-targeted therapies.

BAT is a highly vascularized tissue, and both acute and chronic activation of BAT are associated with increased blood flow to the tissue. Suppressing the increase in blood flow associated with BAT activation impairs thermogenesis. However, it is unknown whether decreased activation of BAT will result in reduced blood flow to the tissue. This question can be approached by investigating the blood flow response to BAT activation in mice deficient in UCP1 (UCP1−/−). These mice have an impaired activation of BAT with a
The increase of BAT blood flow associated with BAT activation can be mediated directly or indirectly by the sympathetic nervous system. Indeed, sympathetic nervous system stimulation can induce vasodilatation, either directly through cyclic adenosine monophosphate or through an interaction of cyclic adenosine monophosphate and nitric oxide (NO).\textsuperscript{16–19} NO plays an important role in BAT-related metabolism and mitochondrial biogenesis of brown adipocytes.\textsuperscript{20} The most prominent pathway for NO production is via NO synthase (NOS) enzymes. In brown adipocytes, NOS3 is the predominantly expressed isoform.\textsuperscript{21} Both NOS1\textsuperscript{12} and NOS3\textsuperscript{16,23} can mediate vasodilatation in mice, and both might have vasodilatory effects in BAT.

We hypothesized that BAT could be detected in vivo by imaging its blood flow. Microspheres have been used in rodents to assess blood flow of BAT,\textsuperscript{9–13} but this method is terminal and cannot be applied to humans. Contrast ultrasound (CU) is a noninvasive technique that estimates microvascular blood flow by visualization and quantification of intravenously infused echogenic microbubbles.\textsuperscript{24} When microbubbles are destroyed by high-energy ultrasound pulses, the time course of their replenishment in a given tissue can be fitted to an exponential curve. The product of the peak signal intensity (A) and the slope of the replenishment (β) of this curve is an estimate of the tissue’s blood flow. Contrast ultrasound has been validated in the noninvasive estimation of myocardial blood flow, both in mice\textsuperscript{25} and in humans.\textsuperscript{26} In a recent study, CU was used to assess microvascular blood volume in muscle and white adipose tissue of humans and rats.\textsuperscript{26}

The present study investigated whether CU could be used to detect BAT and its activation by measuring BAT blood flow in mice, both at baseline and after stimulation with norepinephrine (NE). The estimation of BAT blood flow using CU was validated with that obtained using microspheres. To determine whether BAT activation was required for the increased BAT blood flow detected by CU, we compared wild-type (WT) and UCP1−/− mice. Finally, we investigated whether NOS-dependent NO signaling plays a role in the increase in BAT blood flow induced by NE. The blood flow response to NE was measured in mice deficient in NOS1 (NOS1−/−) or NOS3 (NOS3−/−) and in animals in which NOS was inhibited by N\textsuperscript{3}-nitro-L-arginine methyl ester (L-NNAME).

Methods

Protocol

All animal studies were approved by the Subcommittee on Research Animal Care of the Massachusetts General Hospital, Boston, MA. Two- to 3-month-old male C57BL6 wild-type (WT, n=47), B6.129S4-Nos1\textsuperscript{11}m1Plh (NOS1−/−, n=4), B6.129P2-Nos3\textsuperscript{11}m1Unc (NOS3−/−, n=8) mice (Jackson Laboratory, Bar Harbor, ME), and 2-month-old male B6.129-Ucp1\textsuperscript{11}m1Ks (UCP1−/−, n=5) mice\textsuperscript{14} were studied. UCP1−/− mice and their WT control mice were housed at a room temperature of 26°C for 2 weeks before performing the experiments. Mice were anesthetized with an intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg).\textsuperscript{27,28} After intubation, animals were mechanically ventilated (FiO\textsubscript{2} 1.0, 10 µL/min, 120 breaths/min), and fluid-filled catheters were surgically inserted into the left carotid artery and right jugular vein for continuous measurement of invasive hemodynamic parameters and administration of infusions, respectively. Mice were placed in a prone position for imaging of interscapular BAT. In 1 group of WT mice (n=4), infusions were administered using a less invasive technique via a 3G catheter placed in a tail vein. Because no surgical preparation was performed in the tail-vein experiments, mice were anesthetized with lower concentrations of ketamine (80 mg/kg) and xylazine (8 mg/kg). In all experiments, core body temperature was kept constant at 37°C with a DC Temperature Control System (FHC, Bowdoin, ME).

Acquisition of Contrast Ultrasound

Perflueten lipid microbubbles (Definity; Lantheus Medical Imaging Inc., North Billerica, MA) were diluted 1:10 in a 0.9% saline solution and infused at a rate of 20 µL/min into the right jugular vein or the tail vein. CU was performed with a 14-MHz linear transducer (Sequoia C512, Siemens, Mountain View, CA). Both scapulae were located and used as guiding anatomical landmarks, and interscapular BAT was identified by localization of Sulzer vein via CU.\textsuperscript{13} Ten high-energy ultrasound frames (mechanical index 1.80, frame rate 30 Hz) were used to destroy the contrast microbubbles, and the replenishment time course of the contrast microbubbles in the BAT was recorded for 10 seconds in real-time mode (mechanical index 0.24). Similar acquisitions were obtained in the right kidney and right quadriceps femoris muscle. After the CU acquisitions at baseline, NE (Bedford Laboratories, Bedford, OH) was infused intravenously at 1 µg·kg\textsuperscript{-1}·min\textsuperscript{-1}. The effect of NE was verified by an increase in systemic blood pressure and heart rate after 10 minutes, and CU was repeated.

One group of WT mice (n=7) received an intravenous bolus injection of 50 mg/kg L-NNAME\textsuperscript{10} after performing CU at baseline conditions, 10 minutes before infusion of NE. After the bolus injection, L-NNAME was continuously infused throughout the experiment at 10 mg·kg\textsuperscript{-1}·min\textsuperscript{-1}.

Histology of BAT

After completion of the CU imaging, mice were euthanized, and the BAT was dissected. In a subgroup of animals, 5-µm thick paraffin-embedded slides of BAT were obtained at 1-mm intervals and stained using hematoxylin and eosin.

Analysis of Contrast Ultrasound

Regions of interest were traced manually within the BAT, kidney cortex, and muscle, excluding the big vessels. Average signal intensity for each region of interest was automatically determined on each frame (Syngo ACQ, Siemens). A curve of signal intensity over time was obtained for each region of interest and fitted to an exponential function: $y = A(1-e^{-βt})$, where $y$ represents the signal intensity, $β$ is the initial slope of the curve, and $A$ the plateau intensity. Blood flow was estimated by the product $AB$ as described previously.\textsuperscript{23,24} Two to 3 curves were averaged for each organ under each condition. A goodness-of-fit coefficient ($R^2$ of the fit) was obtained for each curve, and an arbitrary threshold of 0.8 was set. All curves with a goodness-of-fit of <0.8 were excluded.

Echocardiographic Assessment of Cardiac Output

To estimate cardiac output, mice were anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg) and placed in supine position. Cardiac output was assessed before and after NE infusion using M-Mode echocardiography\textsuperscript{31} in WT mice (n=6), WT mice treated with L-NNAME (n=4), and UCP1−/− mice (n=4).

Assessment of Organ Blood Flow by Fluorescent Microspheres

In separate experiments, blood flow to BAT and kidneys was estimated concurrently using FluoSpheres polystyrene microspheres (15 µm;
Intravital imaging. Intraobserver and interobserver reliabilities were measured in the supernatant at wavelengths of 427 and 468 nm was calculated.

Quantitation of mRNA Levels
BAT was dissected and snap frozen in liquid nitrogen. RNA was extracted from tissues using Trizol (Invitrogen, Carlsbad, CA), and cDNA was synthesized using MMLV-RT (Promega, Madison, WI). Real-time amplification of transcripts was detected using a Mastercycler ep Realplex (Eppendorf, Hamburg, Germany). The relative expression of target transcripts was normalized to levels of 18S RNA. Primer pairs were used to detect transcripts encoding UCP1 (CAAAAACAGAAGGATTGCCGAAA, TCTTGGACTGATGGCTAGAGG), UCP2 (TCTCCTGAAAGCCAACCTCA, CTACGTTCCAGGATCCCAAG), and UCP3 (CGGATTTCAGCCATGATA, GCCATCCATAGTCCCTGATT).

Statistical Analysis
Statistical analysis was done with JMP statistical software (SAS Institute, Cary, NC). Data are expressed as mean±SEM. A 2-way ANOVA for repeated measurements with selective post hoc comparison versus WT mice was used to assess the effect of genotype or treatment on the blood flow response to NE. If the interaction of NE and genotype was significant, unpaired Student t tests were used to compare ultrasound parameters between genotypes or treated mice at the same NE concentration.

Linear regression analysis was used to determine the correlation coefficient (R²) between BAT blood flow estimated by CU and fluorescent microspheres. In all experiments, P<0.05 was considered significant. The interobserver reliability of CU measurements was performed by 2 independent observers (M.C. and M.S.-C.) in 10 mice. To compute intraobserver reliability, a single observer (M.C.) repeated the measurements on the same loops several weeks after the first measurement set. Intraobserver and interobserver reliabilities were assessed using intraclass correlation coefficients.

Results
BAT Blood Flow Assessed by CU: Feasibility, Detection Range, and Variability
Precise localization of the BAT was confirmed by histology in the first 5 mice studied (Figure 1A and 1B). To examine the feasibility of using CU to measure BAT blood flow, a total of 424 contrast microbubbles replenishment curves in 55 animals were analyzed. Sixty-five curves (15%) had a goodness-of-fit <0.8 and were not used; however, no animals had to be excluded from the analysis. No saturation of the CU signal intensity was noted in the tissues investigated either before or after NE infusion. After destruction of contrast microbubbles with high-energy frames, the signal intensity in the tissues returned to the predestruction plateau (Figure 2A and 2B).

In WT mice, when contrast microbubbles were administered via the jugular vein, baseline blood flow of BAT estimated by Aβ was 0.5±0.1 dB/s (Table). Infusion of 1 µg·kg⁻¹·min⁻¹ NE induced a 15-fold increase in BAT blood flow (P=0.026, Table and Figure 2C and 2D). Similar results were observed in WT mice infused via the tail vein (Table). The intraclass correlation coefficients for the intraobserver and interobserver reliability of Aβ were 0.99 and 0.96, respectively.

Correlation of CU-Estimated Blood Flow to Microspheres-Derived Blood Flow
Organ blood flow estimated by CU was compared with that obtained using microspheres. All tissues contained >500 microspheres/mg. The microsphere concentrations in BAT were higher in mice that received an infusion of 0.5 or 1 µg·kg⁻¹·min⁻¹ NE than in mice that received an infusion of saline (Figure 3A). Conversely, both doses of NE resulted in reduced microsphere concentrations in the kidney. The concentration of microspheres was similar in both kidneys, indicating that the microspheres were evenly distributed in the circulation after injection into the left carotid artery. The ratio of BAT to right kidney blood flow, as measured by CU, closely correlated with that obtained using microspheres (R²=0.86, P<0.001; Figure 3B).

NE-Induced BAT Blood Flow Is Impaired in UCP1−/− Mice
To investigate whether intact activation is required to increase BAT blood flow, CU studies were performed in UCP1−/− mice. Blood flow of BAT at baseline was similar between WT and UCP1−/− mice housed at thermoneutral conditions (0.8±0.1 versus 1.1±0.1 dB/s, respectively). NE infusion increased BAT blood flow both in WT and UCP1−/− mice (P=0.003 and
The increase in BAT blood flow, however, was markedly blunted in the UCP1−/− mice when compared with WT mice (5-fold versus 14-fold, respectively, \( P = 0.026 \); Figure 4A). Blood flow in kidney (Figure 4B) and muscle (Figure 4C) did not significantly differ between WT and UCP1−/− mice (\( P = 0.261 \) and 0.557, respectively).

**Real-Time Polymerase Chain Reaction of BAT in WT and UCP1−/− Mice**

To account for possible compensatory changes in the expression of other uncoupling proteins in UCP1−/− mice, relative mRNA expression of UCP1, UCP2, and UCP3 was measured in the BAT of both WT and UCP1−/− mice. As expected, mRNA expression of UCP1 was not detectable in UCP1−/− mice (Figure 5). mRNA expression levels of UCP2 and UCP3 did not differ in BAT from WT and UCP1−/− mice (\( P = 0.077 \) and 0.185, respectively).

**NOS-Dependent Regulation of BAT Blood Flow Response**

Infusion of 1 \( \mu g \cdot kg^{-1} \cdot min^{-1} \) NE increased CU-derived BAT blood flow in both NOS1−/− and NOS3−/− mice, as well as in WT mice treated with the NOS inhibitor L-NAME (ANOVA, \( P < 0.001 \), Figure 6A). The NE-induced increase in BAT blood flow in NOS3−/− mice and L-NAME–treated mice was lower than that in WT mice (\( P = 0.034 \) and 0.016, respectively), whereas the NE-induced increase in BAT blood flow in NOS1−/− mice did not differ from WT mice (\( P = 0.456 \)). In contrast to NOS1−/− and NOS3−/− mice, kidney blood flow was lower in WT mice treated with L-NAME than in untreated WT mice (\( P = 0.018 \)). Blood flow to the kidney did not change in any of the groups during infusion of 1 \( \mu g \cdot kg^{-1} \cdot min^{-1} \) NE (Figure 6B, \( P = 0.964 \)). Muscle blood flow was not significantly influenced by genotype or treatment with NE (Figure 6C, \( P = 0.134 \)).

**Effect of NE on Cardiac Output of Mice**

To exclude the possibility that variations in cardiac output were responsible for the differences in the BAT blood flow responses observed between mouse strains, cardiac output was estimated by echocardiography. Continuous infusion of 1 \( \mu g \cdot kg^{-1} \cdot min^{-1} \) NE increased cardiac output in WT and UCP1−/− mice, and in WT mice treated with L-NAME (\( P < 0.001 \)). Cardiac output in WT mice (6.4±0.5 mL/min at baseline versus 12.5±1.0 mL/min) and UCP1−/− mice (7.8±0.9 mL/min at baseline versus 12.1±2.2 mL/min) increased to a similar degree, whereas cardiac output increased to a lesser degree in WT mice treated with L-NAME (7.0±1.0 mL/min at baseline versus 8.8±0.9 mL/min; \( P = 0.03 \) compared with untreated WT animals).
The present study describes and validates CU as a novel noninvasive method that is able to identify and localize BAT and characterize the regulation of its blood flow in mice. CU detected increases in BAT blood flow associated with NE-induced BAT stimulation. Although stimulation of BAT by NE was accompanied by an increase in BAT blood flow in UCP1−/− mice, this increase was markedly blunted compared with the increase observed in WT animals. The response of BAT blood flow after stimulation by NE was also measured in mice with reduced NOS-dependent NO synthesis. The increase in blood flow of BAT was reduced in NOS3 −/− mice and mice treated with L-NAME when compared with WT animals.

Detection of BAT blood flow by CU was feasible in all mice studied. Interobserver and intraobserver variabilities in CU-estimated BAT blood flow were similar to those reported for CU-estimated myocardial blood flow in mice. Continuous infusion of contrast microbubbles yielded a constant concentration in the organs imaged (plateau), and the length of the loop acquisition allowed the replenishment of contrast microbubbles to the plateau after their destruction by high-energy ultrasound pulses. Both conditions are necessary to fit an exponential curve to the contrast microbubble signal intensity over time. Despite the high magnitude of changes in BAT blood flow, which was far greater than that of myocardial blood flow before and after vasodilation, curves were analyzable both at low- and high-BAT blood flow. CU was feasible using a jugular venous line for infusion, as well as the less invasive approach of infusion via the tail vein.
Early studies of BAT in rodents measured its blood flow with nonsurvival approaches. Using radioactive microspheres, Foster et al demonstrated a 13-fold increase of BAT blood flow in rats infused with NE, similar to what we found in mice both with microspheres and CU. Of note, the magnitude of the NE-induced increase in BAT blood flow appears comparable with that detected with the more physiologic stimulus of cold exposure. In addition, Virtanen et al reported a 15-fold increase in uptake of 18F-fluorodeoxyglucose into supraclavicular BAT after cold exposure in humans, and Carter et al observed a 15- to 16-fold activation of BAT in mice exposed to cold. These data indicate that, irrespective of species and mode of BAT activation, the degree of metabolic activation appears to be of similar magnitude as the blood flow increase of BAT.

Activation of BAT by the sympathetic nervous system results in increased blood flow to the tissue, which is necessary to provide a sufficient supply of oxygen, lipids, and glucose to BAT. Additionally, the increased blood flow helps dissipate the heat produced in BAT throughout the rest of the body. One objective of this study was to investigate whether the changes in BAT blood flow are coupled to the changes in BAT activation. CU revealed that BAT blood flow during infusion of NE was lower in UCP1−/− mice than in WT mice. UCP1−/− mice demonstrate intact lipolysis and oxidative capacity at baseline, but treatment with NE fails to increase oxygen consumption and thermogenesis in these animals. Thus, our findings demonstrate that when BAT activation is decreased, as in UCP1−/− mice, BAT blood flow is reduced.

In UCP1−/− mice, 1 µg·kg·min−1 NE still induced a 5-fold increase in blood flow, which corresponds to approximately 30% of the increase in BAT blood flow observed in WT mice. One possible explanation for the NE-induced increase in BAT blood flow in UCP1−/− mice is that it is due to an increase in cardiac output. As the increase of cardiac output in UCP1−/− mice was markedly lower than the increase in blood flow of these mice (55% versus 500%, respectively), it is unlikely that changes in cardiac output can fully account for the augmented BAT blood flow. One limitation of our study, however, is that the estimation of cardiac output using M-Mode echocardiography, although easy to obtain and to interpret, relies on several geometrical assumptions.

Another possible explanation for the increase in BAT blood flow noted in NE-treated UCP1−/− mice is that there is a concomitant increase in BAT oxygen consumption mediated by other UCP proteins. Several investigators have...
reported that UCP2 mRNA levels are increased in the BAT of UCP1−/− mice, suggesting a compensatory increase in UCP synthesis.14,15 In contrast, the current study did not reveal increased UCP2 gene expression in UCP1−/− mice, possibly due to their housing at thermoneutral conditions. Finally, it is conceivable that a limited vasodilatory response to NE in BAT occurs even though there is no increase in oxygen consumption and thermogenesis. Such dissociation of the vascular and thermogenic response has been reported with chronic BAT activation. Both increased expression of vascular endothelial growth factor and angiogenesis are preserved in BAT of UCP1−/− mice chronically exposed to cold.38,39

NO plays an important role in mitochondrial biogenesis and function of BAT.20 The most prominent pathway for NO production is via NOS enzymes. Both NOS122 and NOS323,24 can mediate vasodilation induced by β-adrenergic stimulation. Our data suggest that NOS3-dependent NO production is involved in the acute regulation of BAT blood flow. NE infusion increased BAT blood flow in both NOS3−/− mice and mice treated with the NOS inhibitor L-NAME, but the increase in BAT blood flow was less than that observed in WT mice (32% and 40%, respectively). Nagashima et al previously reported that L-NAME completely abolished the NE-induced increase in BAT blood flow in rats.13 The doses of both NE and L-NAME reported by Nagashima et al, however, were markedly higher (12-fold and 10-fold, respectively) than those used in the present experiments, and may have impaired BAT blood flow by decreasing the cardiac output.40

In summary, the current findings validate CU as a noninvasive method to estimate BAT blood flow in vivo in mice. Furthermore, the data suggest that CU can detect changes in blood flow induced by stimuli that activate BAT. Our data imply that BAT blood flow and activation are at least partially coupled, and that NOS3-dependent NO production plays a role in induction of BAT blood flow by NE. CU could be used to monitor the effect of antiobesity therapies that modulate BAT function. Furthermore, if translated into humans, CU could be performed serially, and thus be applied in a longitudinal follow-up of BAT-modulating therapies.

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Disclosures
None.

References
Several recent studies have confirmed the presence of metabolically active brown adipose tissue (BAT) in adult humans. Interventions to increase BAT volume and activation are being extensively investigated as therapies to decrease the body weight in obese subjects. The objective of this study was to validate contrast ultrasound (CU) as a method to noninvasively estimate BAT blood flow in mice. Our findings suggest that CU can not only detect BAT but also estimate increases in blood flow associated with BAT activation. CU could potentially be used in preclinical trials to measure the effect of antiobesity therapies that modulate BAT function. If translated to humans, CU could be performed serially in longitudinal studies of BAT-modulating therapies.
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The version of this article published ahead of print on July 11 includes a correction to Figure 2.