RELATIONSHIP OF BROWN ADIPOSE TISSUE PERFUSION AND FUNCTION:
A STUDY THROUGH BETA 2 ADRENORECEPTOR STIMULATION

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ABSTRACT

Brown adipose tissue (BAT) activation increases glucose and lipid consumption; as such, it is been considered as a potential therapy to decrease obesity. BAT is highly vascularized and its activation is associated with a necessary increase in blood flow. However, whether increasing BAT blood flow per se increases BAT activity is unknown. To examine this hypothesis, we investigated whether an isolated increase in BAT blood flow obtained by beta-2 adrenoreceptor (β2-AR) stimulation with salbutamol increased BAT activity. Brown adipose tissue blood flow was estimated in vivo in mice using contrast-enhanced ultrasound. The absence of direct effect of salbutamol on the function of isolated brown adipocytes was assessed by measuring oxygen consumption. The effect of salbutamol on BAT activity was investigated by measuring BAT glucose uptake in vivo. Brown adipose tissue blood flow increased by 2.3 ± 0.6 fold during β2-AR stimulation using salbutamol infusion in mice (P=0.003). β2-AR gene expression was detectable in BAT but was extremely low in isolated brown adipocytes. Oxygen consumption of isolated brown adipocytes did not change with salbutamol exposure, confirming the absence of a direct effect of β2-AR agonist on brown adipocytes. Finally, β2-AR stimulation by salbutamol increased BAT glucose uptake in vivo (991±358 vs. 135±49 ng glucose/mg tissue/45 min in salbutamol vs. saline injected mice respectively, P=0.046). In conclusion, an increase in BAT blood flow without direct stimulation of the brown adipocytes is associated with increased BAT metabolic activity. Increasing BAT blood flow might represent a new therapeutic target in obesity.
New & Noteworthy statement

Brown adipose tissue (BAT) activation is considered as a potential treatment for obesity. Using in vitro and in vivo experiments in isolated brown adipocytes and mice, we demonstrate that beta-2 adrenoreceptor stimulation increased BAT blood flow and glucose uptake despite the absence of a direct effect on adipocytes, suggesting that an increase in BAT perfusion per se leads to an increase in BAT metabolic substrate uptake. Therefore, increasing BAT blood flow might represent an obesity-targeted therapy.
The most abundant adipose tissue, white adipose tissue, is white or yellow in color and stores energy as lipid droplets. In contrast, brown adipose tissue (BAT) is able to consume lipids and glucose to produce heat (thermogenesis) by increasing mitochondrial uncoupling using the uncoupling protein 1 (UCP1) (9). Until recently, it was assumed that in humans, functional BAT was present in infants but disappeared later in life (14). However, functional BAT was recently detected in adult humans (17, 25, 34, 38), increasing the interest in investigating BAT stimulation and expansion as potential therapies to decrease or prevent obesity.

Brown adipose tissue is richly innervated both in mice (9) and humans (54), and is activated by norepinephrine, released by the sympathetic nervous system in conditions such as cold or food intake (7). Norepinephrine binds to BAT beta-adrenoreceptors (β-AR), leading to an activation of UCP1 and to thermogenesis (40).

The search for therapies activating BAT currently relies on direct stimulation of brown adipocytes through β1- and β3-AR stimulation, however, this strategy is limited due to the significant side effects associated with β1-AR agonists and the disappointing results of β3-AR agonists as anti-obesity drugs in humans (1). Beta 2-AR are not present in isolated brown adipocytes (8, 53) and therefore, although β2-AR agonists are extensively used in clinical practice and have few side effects, they have not been studied as potential activators of BAT. BAT is highly vascularized and the increase in BAT blood flow observed during cold-induced and/or β3-AR induced BAT activation is thought to be
necessary both to provide enough oxygen and substrates for thermogenesis and

to avoid thermal injury (15, 36). However, the impact of an increase in BAT

circulation on BAT activation has never been studied.

We hypothesized that increasing BAT blood flow would lead to an

increased BAT substrate availability and uptake and thus result in BAT

activation. Stimulation of glucose uptake by an increase in muscle blood flow has

been described in several studies in the skeletal muscle *in vivo* in humans (6, 12,

51). In these studies, investigators reported that physiological stimuli such as

insulin or exercise induced an increase in muscle blood flow that was

responsible, at least in part, for the muscle glucose uptake increase (6). In

addition, increasing muscle blood flow with a non-physiological stimulus such as

methacholine infusion during either a high- or low-dose insulin clamp also

enhanced glucose uptake (3).

Although they are not expressed in isolated brown adipocytes, β2-AR are

detected in the BAT, leading to the hypothesis that β2-AR might only be present

in BAT blood vessels (8). Despite the absence or low expression of β2-AR gene

expression in isolated brown adipocytes, β2-AR gene expression levels in human

BAT are higher than β1-AR and β3-AR gene expression levels (19).

The purpose of this study was to assess the effect of β2-AR stimulation on

BAT blood flow using a novel *in vivo* contrast ultrasound method (5, 13) and to

determine whether an increase in blood flow *per se* could increase BAT

metabolic activity.
MATERIAL AND METHODS

Mice

All animal studies were performed according to a written protocol approved by the Massachusetts General Hospital Subcommittee on Research Animal Care, and were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (33).

Ten to twelve-week-old male C57BL6 mice (Jackson Laboratory, Bar Harbor, ME) were studied. All animals were fed a standard diet (11.8% kcal from fat; Prolab Isopro RMH 3000 5P75, LabDiet, Richmond, IN) and maintained on a standard 12-hour light/12-hour dark cycle with food and water given ad libitum until the experiments.

Beta 2-AR stimulation was obtained using acute administration of a widely used β2-AR selective agonist, salbutamol (27). Salbutamol was obtained from Sigma Aldrich (S8260) and diluted in sterile 0.9% saline before injection.

BAT tissue perfusion assessment by contrast ultrasound

Brown adipose tissue perfusion was assessed by measuring BAT blood flow using contrast ultrasound as previously described (5, 13). Briefly, mice were anesthetized with an intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg). After orotracheal intubation, animals were mechanically ventilated at a respiratory rate of 110 to 120 breaths/min and a tidal volume of 10μL/g of body weight. In order to avoid any stimulation of BAT by cold, core body temperature was maintained constant at 37°C with a DC Temperature Control System (FHC, Bowdoin, ME). A left carotid catheter (fluid-filled PE-10...
catheter) was surgically placed for continuous invasive measurement of mean blood pressure and heart rate. Similarly, catheters were placed in the left and right jugular veins and used for intravenous infusions of salbutamol and ultrasound contrast agent respectively.

**Ultrasound acquisition**

Acquisitions were performed in the prone position using a commercially available ultrasound system with a 14-MHz linear transducer (Acuson Sequoia C512; Siemens Medical Solutions USA, Inc., Mountain View, CA). Perflutren lipid microbubbles (Definity; Lantheus Medical Imaging, Inc., North Billerica, MA) were diluted in sterile 0.9% saline solution (1:10) and continuously infused intravenously at a rate of 20 μL/min into the right jugular vein. For each acquisition, images centered on the BAT were acquired during 10 seconds after a burst of 10 high-energy frames (mechanical index: 1.8). The first set of acquisitions was performed 10 minutes after the onset of microbubbles perfusion. An intravenous infusion of 0.2 μg/kg.min of salbutamol was then initiated and the second set of acquisitions was performed 10 minutes later.

**BAT tissue perfusion analysis**

Regions of interest were traced manually within the two BAT interscapular lobes (5, 13). The average signal intensity within the region of interest was automatically measured for each frame (syngo ACQ; Siemens Medical Solutions USA, Inc.). The relationship of the signal intensity over time after bubble destruction was obtained in each region of interest and fitted to an exponential function: \( y = A (1-e^{\beta t}) \) where \( y \) is the signal intensity, \( A \) is the plateau intensity, and \( \beta \) is the initial slope of the replenishment curve. Blood flow was estimated by calculating the product of \( A \) and \( \beta \) (52). A minimum of three measurements was
averaged. 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 <0.8 were excluded.

**Cardiac output measurement by echocardiography**

Estimation of cardiac output was based on M-mode echocardiographic measurements using a commercially available ultrasound system with a 10-14 MHz linear transducer (Vivid 7; GE Vingmed Ultrasound AS, Horten, Norway) (47). Mice were anesthetized with the same anesthetic regimen than for the contrast ultrasound experiments. Parasternal 2D-guided M-mode acquisitions were performed in supine position before and 10 minutes after the onset of an intravenous infusion of 0.2 μg/kg.min of salbutamol.

Left ventricular end-diastolic (LVEDD) and end-systolic (LVESD) diameters and heart rate (HR) were measured. Cardiac output (CO) was assessed using the following validated formula: $\text{CO} = (\text{LVEDD}^3 - \text{LVESD}^3) \times \text{HR}$ (47). Five measurements were averaged for each animal.

**Brown adipocytes isolation**

Brown adipocytes were isolated by digestion of BAT interscapular lobes using collagenase as previously described (26). Mice (n=8 for each experiment) were euthanized by an intraperitoneal injection of pentobarbital (200mg/kg) and cervical dislocation. The interscapular BAT lobes were harvested immediately after sacrifice and removal of white adipose tissue and muscles surrounding BAT lobes was performed using a dissecting microscope based on the color and consistence of the different tissues.
Brown adipose tissue lobes were digested in 2 steps using decreasing doses of collagenase (26). To that purpose, the buffer was changed to 3 mL with 1.3 mg/mL collagenase II, and the tissue was incubated in a slowly shaking water bath at 37 °C with vortexing every minute. After 7 min, the buffer was discarded, and the tissue was placed in 6 mL of fresh buffer with 0.67 mg/mL collagenase II, minced with scissors, and incubated for 45 min in the water bath, vortexing every 5 min. The buffer with cells and tissue fragments was filtered through silk cloth, and the filtrate, containing the adipocytes, was centrifuged for 8 min at 30 g. The infranatant was discarded and fresh buffer was added. The remaining tissue was incubated for 45 min in 3 mL of buffer with 0.33 mg/mL collagenase, vortexed every 5 min, and collected. The two cell suspensions were pooled and washed by floating for 1 h. The buffer was removed. The cell suspension (1–4x10^6 cells/mL) was kept at room temperature during the experiment.

Measurement of mRNA expression levels in isolated brown adipocytes and brown adipose tissue

Total RNA was extracted either from isolated brown adipocytes or BAT using the Trizol (Ambion, Life Technologies, Austin, Texas, USA) method according to the manufacturer’s instructions. One µg of RNA was used in the Applied Bioscience Multiscribe Reverse Transcriptase cDNA Synthesis Kit (Applied Bioscience) for RT-PCR to produce cDNA using random hexameric primers. cDNA was subsequently used for relative expression quantitation using the Applied Bioscience Taqman FAST Advanced Master Mix or the FAST SYBR Green I Master mix (Applied Biosciences) in a Lightcycler® 480 (Roche). To this end, subunit specific primer-probe sets were purchased for β2-AR and UCP1.
(Mm02524224_s1 and Mm00494069_m1, respectively, Life Technologies). To normalize the data, the geometric mean of the 2 most stable housekeeping genes, 18s and beta-actin (ACTB) (Mm03928990_g1 and Mm00607939_s1, respectively, Life Technologies) was used as described (48). Each sample was measured in triplicate to determine the threshold cycle (Ct). For each sample, the normalization factor was calculated as the difference between the geometric mean Ct of the housekeeping genes of the sample and the mean Ct of the housekeeping genes of all samples. The level of target mRNA, relative to the mean of the reference housekeeping genes, was calculated by raising 2 to the power of [40 - (Ct of target - housekeeping gene normalization factor)].

**Measurement of oxygen consumption rates from isolated brown adipocytes**

Oxygen consumption in freshly isolated brown adipocytes was measured at 37°C using a Clark-type oxygen electrode (Hansatech Oxygraph, Amesbury, MA) as previously described (26). Brown adipocytes (100 000 to 200 000 cells/experiment) were suspended in a Krebs-Ringer bicarbonate buffer (145 mmol/L Na+, 6.0 mmol/L K+, 2.5 mmol/L Ca²⁺, 1.2mmol/L Mg²⁺, 128 mmol/L Cl⁻, 1.2 mmol/L SO₄²⁻, 25.3 mmol/L HCO₃⁻, 1.2 mM H₂PO₄⁻) with 10 mmol/L glucose, 10 mmol/L fructose, and 4% fatty-acid-free bovine serum albumin. A volume of 900 μL of the solution containing cells and buffer was placed in an oxygen electrode closed chamber, stirred continuously and maintained at 37°C. The oxygen consumption rate was continuously recorded during the whole experiment. The basal oxygen consumption rate was determined after 4 minutes of incubation. Salbutamol was then added with a Hamilton syringe (100μL) through the cover
of the closed chamber in increasing concentrations (10 ng/mL, 100 ng/mL, 12 μg/mL, 10 μg/mL and 100 μg/mL). At the end of the experiment, 100 μL of a 0.1 g/L norepinephrine solution were added as a positive control. Maximal oxygen consumption during the 120 seconds following the addition of salbutamol or norepinephrine in the closed chamber was recorded.

**Brown adipose tissue glucose uptake in vivo**

Glucose uptake in vivo was measured as previously reported (43). Mice were fasted overnight (10 pm to 9 am). Body weight was measured just before the beginning of the experiment. Thirty minutes after anesthesia with sodium pentobarbital (60 mg/kg mouse body weight, intraperitoneal injection), blood was taken from the tail to assess basal glucose concentrations and background radioactivity levels. Mice were injected intraperitoneally with either saline or salbutamol at a dose of 10 μg/g mouse body weight. Ten minutes later, 1 mg glucose in combination with 0.33 μCi [³H]2-deoxyglucose/g mouse body weight was administered via the retro-orbital sinus. After 45 min, animals were sacrificed by cervical dislocation, and BAT, subcutaneous white adipose tissue, and visceral white adipose tissue were harvested and immediately frozen in liquid nitrogen. Accumulation of [³H]2-deoxyglucose was assessed in BAT, subcutaneous and visceral white adipose tissues using a perchloric acid/Ba(OH)²/ ZnSO₄ precipitation procedure as previously described (43).

**Statistical analysis**

Statistical analysis was performed using SPSS statistics software (version 17.0.0, SPSS Inc, Chicago; IL, USA). Values are expressed as mean ± SEM.
Brown adipose tissue blood flow, blood pressure, heart rate and cardiac output before and during salbutamol infusion were compared using paired t-tests. Comparison of β2-AR gene expression in isolated brown adipocytes and BAT was performed using an unpaired t-test. Maximal oxygen consumption of isolated brown adipocytes at baseline, with salbutamol and with norepinephrine was compared using ANOVA for repeated measurements. If the effect of treatments (ANOVA) was significant, the treatments were compared to baseline conditions and to each other using paired t-tests. The difference in BAT and white adipose tissues glucose uptake between mice with salbutamol and saline injection was tested by unpaired t-test.

All tests were two-tailed and a $P$ value < 0.05 was considered significant.

**RESULTS**

**Effect of β-2 adrenoreceptor stimulation on BAT perfusion**

In thermoneutral conditions with core body temperature maintained at 37°C, salbutamol intravenous infusion (0.2µg/kg.min) increased BAT blood flow by 2.3 ± 0.6 fold compared to baseline (Aβ product 4.2 ± 0.6 dB/s vs. 1.4 ± 0.2 dB/s, $P=0.003$, Figure 1). During salbutamol infusion, the mean blood pressure decreased from 79 ± 3 mmHg to 65 ± 4 mmHg ($P=0.008$) whereas the heart rate remained stable (386 ± 13 bpm and 387 ± 12 bpm at baseline and during salbutamol infusion respectively).

In order to evaluate whether an increase in cardiac output was responsible for the BAT perfusion response, the effect of salbutamol infusion on
cardiac output was tested in a separate set of mice (n=6). A slight increase in cardiac output was observed (9.6 ± 0.8 vs. 11.5 ± 0.7 mL/min at baseline compared with salbutamol infusion; \( P=0.04 \)). The mean percentage change in cardiac output between baseline and salbutamol infusion was of 25 ± 10%.

Beta 2 adrenoreceptor expression in isolated brown adipocytes and brown adipose tissue

Expression of β2-AR and UCP1 was assessed in isolated brown adipocytes and total BAT by RT-PCR. As expected, β2-AR mRNA expression level was extremely low in isolated brown adipocytes whereas a significant expression was found in total brown adipose tissue (Figure 2A). UPC1 gene expression was higher in isolated cells as compared to brown adipose tissue (Figure 2B).

Effect of β2-AR stimulation on oxygen consumption in isolated brown adipocytes

In order to test the hypothesis that β2-AR stimulation has no direct metabolic effect on brown adipocyte cells, oxygen consumption was measured in freshly isolated brown adipocytes at baseline, in the presence of increasing doses of salbutamol (ranging from \( 10^{-5} \) to 0.01 g/L) and in the presence of norepinephrine. No change in oxygen consumption was observed in presence of β2-AR agonist, confirming the absence of a direct effect of β2-AR stimulation on brown adipocytes (Figures 3A and 3B). In contrast, a marked increase in oxygen consumption was observed in presence of norepinephrine (2.1-fold increase of maximal oxygen consumption compared to baseline, \( P=0.001 \)).
Effect of β2-AR stimulation on BAT glucose uptake

To establish whether β2-AR stimulation increased glucose uptake in vivo, mice were injected with labeled [3H]2-deoxyglucose and with either saline or salbutamol. Beta-2 adrenoreceptor stimulation by salbutamol resulted in a significant increase in BAT glucose uptake whereas no change was observed in subcutaneous and visceral white adipose tissue (Figure 4).

DISCUSSION

The present study demonstrates that β2-AR stimulation increases BAT glucose uptake despite the absence of a direct activation effect on brown adipocytes. Beta 2 adrenoreceptor stimulation, however, increases BAT blood flow, suggesting that an increase in BAT perfusion per se leads to an increase in BAT metabolic substrate uptake.

Beta 2 adrenoreceptor stimulation in vivo by salbutamol, a selective β2-AR agonist, increased BAT blood flow. Beta 2 adrenoreceptor gene expression by isolated brown adipocytes was extremely low. The absence of effect of β2-AR stimulation in vitro by salbutamol on the oxygen consumption of isolated brown adipocytes confirmed the absence of a direct effect of salbutamol on the metabolic activity of these cells. Interestingly, however, β2-AR stimulation in vivo increased BAT glucose uptake, suggesting an effect on the metabolic activity of BAT.

The critical role of BAT vascularization in the functionality of BAT has been recently demonstrated (41). In a high fat diet obesity-induced murine
model, obesity caused capillary rarefaction and hypoxia in BAT, leading to BAT whitening with a diminished response to beta adrenergic signaling, accumulation of lipid droplets and mitochondrial dysfunction and loss (41). Importantly, BAT revascularization led to an improvement in BAT function. Similarly, we reported a decreased BAT capillary density in a genetic murine model of obesity (db/db mice) (13). These data suggest that BAT vascularization and blood flow play a major role in the function of BAT and in the association between BAT dysfunction and obesity.

Vasculature in BAT might be different than that of other tissues. Our team (5) and others (21) previously reported that norepinephrine induces a significant increase in BAT blood flow. Vascular response to norepinephrine is dependent to the predominance of β- or α-AR (α1/β2 ratio) in the vessels. A norepinephrine induced-vasodilation is observed in specific tissues with β-AR predominance such as the iliac arteries (50) whereas an important norepinephrine induced-vasoconstriction is observed in the vast majority of tissues and is related to the α-AR predominance (49). Therefore, we can hypothesize that β2-AR might be predominant as compared to α1-AR in the BAT vasculature.

The present study demonstrates the importance of BAT perfusion in rodents: the vascularity of BAT in humans may be less homogeneous as BAT depots are not as well delineated as in rodents (30, 31). Nonetheless, BAT perfusion in humans is significant, being twice that of muscle in warm conditions and increasing to 4 times that of muscle when cold-stimulated (36). Similarly, our data was obtained in rodents, in which the importance of BAT for glucose regulation is crucial (43, 45). In humans, several studies suggest that activated
BAT has the ability to remove glucose from circulation but whether this response is clinically relevant is still debated (42). Recent data suggest that this response may in certain cases at least be clinically relevant, as stimulation of BAT in human volunteers with detectable BAT (using 18FDG PET/CT scanning) increases insulin sensitivity (10).

We used salbutamol, a selective β2-AR agonist (27) routinely used in clinical practice (in pathologies such as asthma or preterm labor) to increase BAT perfusion. Using an in vivo technique recently validated by our team (5, 13), we demonstrated that a dose of 0.2 µg/kg.min of salbutamol (equivalent to the dose of 5 to 20 µg/min used in adults in clinical practice (46)) increases BAT blood flow. The effect of β2-AR stimulation was significant but smaller than the effect of norepinephrine, with a two-fold increase in BAT blood flow with salbutamol as compared to a 15-fold increase previously observed with norepinephrine (5). Of note, BAT blood flow was assessed in thermoneutral conditions with core body temperature maintained at 37°C in order to avoid BAT stimulation by cold.

Salbutamol infusion induced a small but significant decrease in mean blood pressure (46). A slight increase in cardiac output was observed, mainly due to an increase in stroke volume, possibly related to the decrease in left ventricular afterload. However, the increase in cardiac output was limited and lower than the increase in BAT blood flow. Therefore, it is unlikely that changes in cardiac output can fully account for the salbutamol-induced increase in BAT blood flow.

The absence of a direct effect of β2-AR stimulation on brown adipocytes was demonstrated by the extremely low β2-AR gene expression and the absence
of effect of salbutamol on oxygen consumption in isolated brown adipocytes. The extremely low β2-AR gene expression in brown adipocytes confirms previous results (8, 9, 53). In addition, no effect of salbutamol on the oxygen consumption of isolated brown adipocytes was detected, in contrast to norepinephrine which markedly increased oxygen consumption. Of note, the doses of salbutamol were chosen to reflect the plasma concentrations reported with clinical doses of salbutamol (28, 44).

Despite the absence of a direct effect of β2-AR stimulation on brown adipocytes, studies have suggested that β2-AR play an important role in BAT function and morphology. The morphological abnormalities of BAT in mice deficient in the three β-AR (with features of both brown and white adipose tissue) do not exist in mice deficient in both β1 and β3-AR, in which β2-AR are present (2). In addition, β2-AR have clinical relevance as they are the most abundant β-AR in human BAT, representing 63% of the total β-AR mRNA compared to 28 and 9% for β1-AR and β3-AR mRNA, respectively (19).

Selective β2-AR stimulation (using salbutamol) increased BAT glucose uptake whereas no effect on WAT glucose uptake was observed. A dose of 10 μg/g body weight was chosen based on previous reports of active doses of salbutamol in other indications in murine model (11, 24). Salbutamol induced a 7-fold increase in BAT glucose uptake whereas a previous study reported a 2-fold increase after 0.2 mg/kg norepinephrine intraperitoneal injection (22).

Assessment of BAT activity is challenging. BAT glucose uptake is imperfect but has been recognized as a marker of BAT activity in previous publications (17, 25, 34). In addition, direct assessment of thermogenesis is
challenging and would not have reflected only BAT activity but also vasodilation induced by β2-AR stimulation in our study.

In humans, β2-AR agonists such as salbutamol increase both glucagon and insulin secretion and have effects on liver metabolism, which cause an overall increase in blood glucose level (37). In rat isolated islets of Langerhans, the β2-AR agonist clenbuterol induced a rise in glucagon secretion whereas no direct effect on insulin secretion was observed (23). The potential impact of salbutamol on glucose homeostasis might be involved in the BAT increased glucose uptake that we observed. This hypothesis is counterbalanced by the fact that no increase in WAT glucose uptake was observed in the same conditions. Furthermore, it has been recently shown that acute β2-AR stimulation by salbutamol has no effect on skeletal muscle glucose uptake (29).

An association between activation of BAT, either acute or chronic, and increase in BAT blood flow has been previously reported (5, 15, 20, 21, 32, 36, 39). Stimuli that increase BAT metabolic activity such as cold exposure (21, 36), β3-AR agonist treatment (15), or norepinephrine treatment (5, 20, 32, 39) also increase BAT blood flow. In addition, a positive association between the whole body energy expenditure and BAT blood flow was reported in humans (36).

Conversely, when the response of BAT blood flow to norepinephrine is inhibited, the thermogenic ability of BAT is suppressed (32). As in many other tissues, it is likely that blood flow increases in response to an increased metabolic demand. The present study demonstrates the distinct and reciprocal finding that an increase in BAT blood flow induces an increase in BAT metabolic activity.

The mechanism by which an increase in tissue blood flow stimulates glucose uptake is unknown; in muscles, it may involve an increase in glucose
gradient between plasma and interstitium. This gradient in turn facilitates glucose delivery to the interstitium resulting in an increased membrane transport into cells (4). Both glucose transporters GLUT 1 and GLUT 4 are present in brown adipocytes (18). Whereas insulin-stimulated glucose uptake is well characterized with the involvement of the rapid translocation of GLUT 4 from intracellular vesicles to cell membrane, insulin-independent BAT glucose uptake is less well understood. Recently, the mechanisms involved in the β3-AR stimulation induced glucose uptake have been studied and involve a rapid de novo synthesis of GLUT 1 and the translocation of the newly synthesized GLUT 1 (35). Whereas β3-AR has a direct effect on brown adipocytes, we showed that β2-AR stimulation does not. Increased blood flow associated with β2-AR stimulation might result in an increase in glucose gradient between plasma and interstitium and a stimulation of GLUT 1 transporter.

There are several limitations to the present study. Although the salbutamol doses given *in vivo* were selected to produce relevant plasmatic doses, they may not reflect the doses used in clinical practice. In addition, salbutamol is a selective β2-AR agonist but a marginal effect on β1-AR cannot be excluded.

During in vivo experiments, salbutamol infusion was associated with a slight decrease in mean blood pressure (from 79 ± 3 mmHg to 65 ± 4 mmHg, *P*=0.008). Therefore, the potential influence of the baroreflex, including a systemic increase in sympathetic activity that may stimulate BAT, cannot be completely excluded even though the decrease in blood pressure was small and no increase in heart rate was noted.
Despite the routine use of BAT glucose uptake as a marker of BAT activity in clinical studies using $^{18}$F-FDG-PET/CT, glucose is not the only substrate used for thermogenesis and may underestimate the thermogenic activity (16). In addition, glucose uptake by BAT endothelial cells (as opposed to uptake by brown adipocytes) cannot be excluded using this technique.

The kinetics of the increase in blood flow and glucose uptake were not assessed during the same experiment. Therefore, we cannot determine whether the glucose uptake increase lasted only as long as the blood flow increase.

Finally, BAT blood flow and glucose uptake measurement were not performed under the exact same conditions. This methodological difference was due to a technical issue. Brown adipose tissue perfusion assessment by contrast ultrasound required a complete anesthesia of the mice because of the need for microbubbles intravenous continuous injection. As we aimed to perform experiments in the most physiological conditions and BAT glucose uptake experiment did not require intravenous injections, anesthesia was less profound and no intravenous catheter was implanted.

In conclusion, an increase in BAT blood flow without direct stimulation of brown adipocytes is associated with an increased BAT metabolic activity. Therefore, increasing BAT blood flow might represent an alternative or additional target in the development of therapies aimed at stimulating BAT.
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**FIGURE CAPTIONS**

**Figure 1.** Brown adipose tissue blood flow estimated by contrast-ultrasound before and during β2-adrenoreceptor stimulation.

Individual data of Aβ product at baseline and 10 minutes after the onset of salbutamol infusion at 0.2 µg/kg.min are presented (n=7). Brown adipose tissue blood flow was significantly higher during β2-adrenoreceptor stimulation using salbutamol as compared to baseline. **: *P*<0.001 vs. baseline.

**Figure 2.** Gene expression of beta-2 adrenoreceptor and uncoupling protein 1 in isolated brown adipocytes and in the brown adipose tissue.

A. Beta-2 adrenoreceptor gene expression level was extremely low in isolated brown adipocytes whereas mRNA level beta-2 adrenoreceptor was significantly higher in the tissue. B. Uncoupling protein 1 (UCP1) mRNA gene expression level was higher in isolated brown adipocytes than in brown adipose tissue. Data are presented as mean ± SEM, *: *P*<0.05 vs. isolated brown adipocytes.

**Figure 3.** In vitro measurements of oxygen consumption of isolated brown adipocytes at baseline, in presence of selective beta 2 adrenoreceptor agonist and in presence of norepinephrine.

A. Oxygen consumption curve of isolated brown adipocytes measured using a Clark-type oxygen electrode. No change in oxygen consumption was observed in presence of increasing doses of salbutamol whereas a marked increase immediately followed addition of norepinephrine in the closed chamber. Black arrows represent addition of 100µL salbutamol solution at the following
concentrations: $10^{-5}$ g/L, $10^{-4}$ g/L, $10^{-3}$ g/L, 0.01 g/L, and 0.1 g/L and the dotted arrow represent addition of 100 μL of a 0.01 g/L norepinephrine solution.

B. Maximal oxygen consumption in isolated brown adipocytes measured at baseline, in presence of salbutamol and norepinephrine. **: $P<0.01$ vs. baseline.

Measurement was repeated in 4 sets of cells during each experiment and 4 independent experiments were performed.

Figure 4. Brown adipose tissue (BAT, panel A) and visceral (panel B) and subcutaneous white adipose tissue (WAT, panel C) glucose uptake after beta2 adrenoreceptor stimulation.

Mice were treated with 10 μg/g mouse body weight of salbutamol intraperitoneally (n=10) and their glucose uptake was compared to mice injected with saline (n=8). An increase of glucose uptake after treatment with salbutamol was noted in BAT (991±358 vs. 135±49 ng glucose/mg tissue/45 min in salbutamol vs. saline injected mice respectively, $P=0.046$) but not in subcutaneous or visceral white adipose tissue. * $P<0.05$ vs. saline.
Visceral WAT

ng glucose/mg tissue/45 min

Saline

Salbutamol