In vivo measurement of energy substrate contribution to cold-induced brown adipose tissue thermogenesis

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ABSTRACT The present study was designed to investigate the effects of cold on brown adipose tissue (BAT) energy substrate utilization in vivo using the positron emission tomography tracers [18F]fluorodeoxyglucose (glucose uptake), 14(R,S)-[18F]fluoro-6-thiaheptadecanoic acid [nonesterified fatty acid (NEFA) uptake], and [11C]acetate (oxidative activity). The measurements were performed in rats adapted to 27°C, which were acutely subjected to cold (10°C) for 2 and 6 hours, and in rats chronically adapted to 10°C for 21 days, which were returned to 27°C for 2 and 6 hours. Cold exposure (acutely and chronically) led to increases in BAT oxidative activity, which was accompanied by concomitant increases in glucose and NEFA uptake. The increases were particularly high in cold-adapted rats and largely readily reduced by the return to a warm environment. The cold-induced increase in oxidative activity was meaningfully blunted by nicotinic acid, a lipolysis inhibitor, which emphasizes in vivo the key role of intracellular lipid in BAT thermogenesis. The changes in BAT oxidative activity and glucose and NEFA uptakes were paralleled by inductions of genes involved in not only oxidative metabolism but also in energy substrate replenishment (triglyceride and glycogen synthesis). The capacity of BAT for energy substrate replenishment is remarkable.

Key Words: positron emission tomography • glucose uptake • glycogenesis • lipogenesis • nonesterified fatty acid uptake

The brown adipocyte is a thermogenic cell. It owes its thermogenic potential to the presence of uncoupling protein 1 (UCP1), whose active state leads to heat production while preventing ATP synthesis (1, 2). In small mammals, brown adipocytes are predominantly found in fat depots referred to as classic or constitutive brown adipose tissue (BAT) depots. The thermogenic potential of BAT is remarkable. It confers rodents the ability to live in cold environments without relying on muscle-derived shivering thermogenesis (3). Accordingly, BAT is the primary site of nonshivering thermogenesis in rats (4–6); this tissue can account for up to 75% of the increase in metabolic rate induced by noradrenaline in cold-adapted rats (7).

Recently, the interest for the thermogenic role of brown fat has been rejuvenated with the discovery of active brown adipocyte depots in human adults (8–11). The use of positron emission tomography (PET) coupled to computed tomography has proved to be instrumental in this finding (9–11). The vast majority of the investigators interested in human BAT have used [18F]fluorodeoxyglucose (18FDG) as the PET tracer because brown fat, similar to tumors (12), increases its uptake of glucose when it becomes metabolically active (8, 9, 13–17). We have also used PET tracers such as 14(R,S)-[18F]fluoro-6-thiaheptadecanoic acid [18FTHA; to measure nonesterified fatty acid (NEFA) uptake] and [11C]acetate (to assess oxidative metabolism) (11, 18).

The brown adipocyte is thought to utilize circulating energy substrates as well as its intracellular lipids to produce heat. In the brown adipocyte, triglycerides (TGs) are stored in lipid vacuoles readily accessible for oxidation (19–22). The source of the energy substrate in activated BAT seems to largely depend on the extent to which the tissue is activated and adapted (23). Our current knowledge of the substrates used for BAT thermogenesis is incomplete, and the relative importance of circulating glucose versus circulating lipids versus intracellular TG pool is still not settled. In this study, we investigated in vivo energy substrate utilization in interscapular BAT (iBAT) in rats subjected to acute or chronic cold exposure using PET imaging. iBAT oxidative capacity with [11C]acetate as well as glucose and NEFA uptake with 18FDG and 18FTHA were measured. The measurements were also performed in rats injected with nicotinic acid (NiAc), an inhibitor of

Abbreviations: 18FDG, [18F]fluorodeoxyglucose; 18FTHA, 14(R,S)-[18F]fluoro-6-thiaheptadecanoic acid; Aco2, aconitase 2; BAT, brown adipose tissue; Cd36, cluster of differentiation 36; CoA, coenzyme A; Cox4, cytochrome c oxidase subunit 4; CptIB, carnitine palmitoyltransferase IB; Dgat1, diacylglycerol acyltransferase 1; Dio2, (continued on next page)
in vivo PET procedures and gene or protein expression measurements. We hypothesize that this source of lipid is crucial to ensure BAT oxidative activity. We finally coupled the PET measurements to the expressions of genes coding for proteins involved in metabolic pathways that relate to glucose and lipid metabolism.

**MATERIALS AND METHODS**

**Animal and cold exposure**

Male Wistar rats weighing 200–250 g (Charles River Laboratories, Senneville, QC, Canada) were divided into 2 groups, which were housed either at 27 or 10°C during 3 weeks. Prior to the measurements, rats adapted to 27°C were divided into 3 groups: 27°C: 10°C for 2 hours (acutely exposed to cold for 2 hours), and 10°C for 6 hours (acutely exposed to cold for 6 hours). Cold-adapted rats for 21 days were also divided into 3 groups: 10°C, 27°C for 2 hours (returned to 27°C for 2 h), and 27°C for 6 h (returned to 27°C for 6 h). All animals were singly housed under a 12-hour light-dark cycle (lights on at 7:00 AM) with ad libitum access to pelleted chow (Rodent Laboratory Chow 5001; Purina, St. Louis, MO, USA) and tap water. All experimental protocols were approved by the Animal Ethics Committee of the Université de Sherbrooke in accordance with the guidelines of the Canadian Council on Animal Care. Distinct groups of animals were used for PET procedures and gene or protein expression measurements.

**Small animal PET protocol**

All the *in vivo* PET experiments were initiated immediately after the insertion of a cannula in the tail vein for the injection of the PET tracers. All imaging experiments were performed on the Avalanche avalanche photodiode-based small animal PET scanner (LabPET/Triumph; Gamma Medica, Northridge, CA, USA) of the Sherbrooke Molecular Imaging Centre, having a 7.5 cm axial field of view. The animals were anesthetized with isoflurane (1.5%) delivered through a nose cone and were placed in the supine position on the scanner bed with the rat heart centered within the field of view of the scanner to include the interscapular region. The 27°C-exposed rats not subjected to cold exposure were placed on a heated bed during the scanning procedure in order to avoid any cold effect in these control animals (24). On the other hand, 10°C-exposed rats were not put on the heated bed because we wanted to maintain the effects of cold. It is important to note that the time lapse between the end of the 10°C stimulation and the first radioactive tracer injection was less than 5 minutes. Boluses of each radiopharmaceutical compound (15–20 MBq, in 0.5 ml 0.9% NaCl) were injected via the caudal vein over 30 seconds after starting PET data acquisition. In one set of experiments, a 20 min dynamic data acquisition with [11C]acetate was done to determine tissue blood flow and oxidative metabolism, followed, 10 minutes later, by a 40 minute dynamic data acquisition with either [18F]FDG or [18F]THA to also determine glucose or NEFA utilization, respectively, as previously described (25). List-mode dynamic data acquisitions allowing flexible time framing of the data for kinetic modeling were performed for all tracers. When used, NiAc (150 mg/kg per gavage) or saline (1 ml/kg) was given 30 minutes before the end of the acute cold exposure. Blood samples were taken at the end of experiments by heart punctures and were rapidly collected and frozen at −80°C for further analysis.

**Imaging data analysis**

For [11C]acetate images, dynamic series of 28 frames (1 × 30, 12 × 10, 8 × 30, 6 × 90, and 1 × 300 sec) each were sorted out, whereas 30 frames (1 × 30, 12 × 10, 8 × 30, 6 × 90, and 3 × 300 sec) were used for [18F]FDG and [18F]THA imaging and 3D images were reconstructed using 15 iterations of a maximum-likelihood expectation-maximization (MLEM) algorithm incorporating physical description of the detector response function. Regions of interest (ROIs) were drawn on transaxial images. Time-activity curves were quantified by matching ROIs of [11C]acetate with either [18F]FDG or [18F]THA. Input curves were extracted by means of an ROI drawn on the left ventricular cavity blood pool in summed last-frame images to seek better contrast. The sizes of these almost-circular ROIs were compared with images of 8 cylinders of different diameters from which a recovery factor was extracted and applied to the ROIs for partial volume correction (25).

For [11C]acetate, we used a 3-compartment kinetic model that estimates blood flow from the constant 1 (26) and also estimates the generation of CO2 [a measure of the Krebs cycle activity and surrogate for the volume of oxygen consumed (VO2)] from the citric acid cycle in BAT using the constant 2 (27). The net BAT glucose and NEFA uptake was determined by a Patlak graphic analysis (28).

**Plasma assays**

Plasma glucose, NEFA, and TG levels were measured as previously described (25).

**Quantitative real-time RT-PCR**

Total mRNA was isolated from BAT using QiAzo1 and the RNAeasy Lipid Tissue Kit (QIAGEN, Mississauga, ON, Canada). The RNA concentrations were estimated from absorbance at 260 nm. cDNA synthesis was performed using the expand reverse transcriptase (Invitrogen, Burlington, ON, Canada). mRNA extraction and cDNA synthesis were performed according to the manufacturer’s instructions, and cDNA was diluted in DNase-free water (1:25) before quantification by real-time PCR. mRNA transcript levels were measured in duplicate samples using a CFX96 touch real-time PCR (Bio-Rad Laboratories, Mississauga, ON, Canada). Chemical detection of the PCR products was achieved with SYBR Green I (Sigma-Aldrich, Oakville, ON, Canada). The primers used for the PCRs are shown in Table I. At the end of each run, melt curve analyses were performed, and representative samples of each experimental group were run on agarose gel to ensure the specificity of the amplification. Fold differences in target mRNA expression were measured using the Δ-cycle threshold method by comparison with the housekeeping gene β2 microglobulin and expressed as fold change versus warm-adapted group.
Western blotting

Antibodies against protein kinase B (PKB/Akt) (#4685) and hormone-sensitive lipase (HSL) (#4107) were purchased from Cell Signaling Technology (Davers, MA, USA). Antibody against UCP1 (#ab10983) was purchased from Abcam Incorporated (Cambridge, MA, USA). Secondary antibody was purchased from Cell Signaling Technology. BAT (50 mg) was homogenized in a lysis buffer composed of 50 mM Tris (pH 7.4), 40 mM NaCl, 2 mM EDTA, 10 mM sodium pyrophosphate (#86422; Sigma-Aldrich), 10 mM sodium glycerophosphate (#C9422; Sigma-Aldrich), 50 mM sodium fluoride (#86770; Sigma-Aldrich), and 2 mM sodium orthovanadate (#86508; Sigma-Aldrich), supplemented with 0.1% sodium dodecyl sulfate (SDS), 1% sodium deoxycholate (#D6759; Sigma-Aldrich), and 1% Nonidet-P40 (#74895; Sigma-Aldrich). One tablet of protease inhibitor cocktail (#11836153001; Roche, Basel, Switzerland) and 1 tablet of phosphatase inhibitor cocktail (#04906837001; Roche) were added per 10 ml lysis buffer. Tissues were rotated at 4°C for 10 minutes and then the soluble fractions were isolated by centrifugation at 12,000 g for 10 minutes in a refrigerated microcentrifuge. Protein levels were then quantified using a protein assay dye reagent concentrate (#500-0006; Bio-Rad Laboratories) and analyzed by Western blotting. After the transfer, the PVDF membranes were washed in PBS-Tween (0.1% Tween and 1% nonfat milk in PBS) and incubated in blocking buffer (5% nonfat milk in PBS-Tween) for 1 hour. The membranes were washed and incubated for 1 hour at room temperature. The membranes were then washed and incubated overnight at 4°C with Akt/PKB, HSL, or UCP1 antibodies (1:1000 in 5% nonfat milk in PBS-Tween) for 1 hour at room temperature. The membranes were washed and incubated in blocking buffer (5% nonfat milk in PBS-Tween) for 1 hour.
at room temperature with the secondary antibody (1:5000 in 5% nonfat milk in PBS-Tween). After washing 4 times, the membranes were incubated with the chemiluminescent substrate ECL (#RPN-2109; Amersham Biosciences, Quebec, QC, Canada) for 1 minute, and signal was detected by exposing the membranes to ECL hyperfilm. The pixel density of each band was analyzed using ImageJ 1.45s (National Institutes of Health, Bethesda, MD, USA).

Statistical analyses

Results are expressed as the mean ± SEM. Comparisons were done on normally distributed data using ANOVA followed by Bonferroni post hoc tests to assess the differences between various exposure conditions with GraphPad Prism Software version 6.0 (San Diego, CA, USA) for Mac (Apple Incorporated, Cupertino, CA, USA). Differences were considered statistically significant when P values were <0.05.

RESULTS

After 21 days, cold-adapted rats exhibited a weight gain lower than that of rats of the same age kept at 27°C (112 ± 5 g vs. 134 ± 8 g; P < 0.001). This lower weight gain was accompanied by an increase in total food intake (759 ± 11 g in cold-adapted rats vs. 549 ± 20 g in rats adapted to 27°C; P < 0.001) and a decrease in energetic efficiency, e.g., grams of body weight gain per grams of food consumed (0.15 ± 0.01 g/g in cold-adapted rats vs. 0.24 ± 0.01 g/g in rats adapted to 27°C; P < 0.001).

Effect of cold exposure on oxidative metabolism

The PET tracer [11C]acetate was used to assess the oxidative metabolic activity of iBAT in rats exposed to 10°C (Fig. 1). Acute exposure to cold led to a rapid increase in iBAT oxidative activity, which became highly significant after 6 hours of cold exposure (Fig. 1A). Chronic (21 days) exposure to cold (cold adaptation) also led to an increase in iBAT weight (1.11 g in cold-adapted rats vs. 0.29 g in rats adapted to 27°C; P < 0.001; Fig. 1B), which in turn contributed to the enhanced iBAT total activity (iBAT oxidative activity × iBAT weight; Fig. 1C). The return to a warm environment of cold-adapted rats readily reduced iBAT oxidative activity, regardless of whether results were expressed per grams of tissue (Fig. 1A) or whole tissue (Fig. 1C). iBAT metabolic activity of cold-exposed rats (both for 6 h and 21 d) reached the level of highly metabolically active tissues such as heart and liver (Fig. 1D). It is noteworthy that iBAT was the only tissue showing an increased metabolic activity in response to cold. Cold adaptation also increased iBAT blood flow (Fig. 1E). This increase was accompanied by a 300% elevation in the expression of Vegfa (Fig. 1F), which was used as an angiogenesis stimulation marker. It is noteworthy that the increase in Vegfa gene expression readily occurred in rats acutely (for 2 and 6 hours) exposed to cold. The rise in blood flow in the 2 and 6 hours cold-exposed rats did not however reach statistical significance. The return to warm temperature blunted the elevations in blood flow and Vegfa gene expression in rats chronically exposed to cold. As expected, the change in total oxidative activity was accompanied by variations in the interscapular skin temperature during cold exposure (Fig. 1G, H). The changes in iBAT total oxidative activity also went along with an increase in iBAT expression of Ucp1 mRNA, which was significant 2 hours following cold exposure (Fig. 1J). Ucp1 mRNA expression decreased quickly after the return to thermoneutral temperature.

Effect of cold exposure on glucose uptake

The PET tracer [18F]FDG was used to assess iBAT glucose uptake in rats exposed to cold (Fig. 2). Acute and chronic exposure to 10°C led to an increase in iBAT glucose uptake, which became highly significant after 6 hours of cold exposure (Fig. 2A, B). Return to 27°C of cold-adapted rats readily reduced iBAT glucose uptake, regardless of whether uptakes were expressed per grams of tissue (Fig. 2A) or per whole tissue (Fig. 2B). Blood glucose also decreased during acute cold exposure (Fig. 2C). During the first 6 hours of cold, we estimated iBAT glucose uptake to be ~33 nmol/min. After cold adaptation, we estimated that the uptake rose to 153 nmol/min. Glucose uptake, however, decreased quickly to 60 nmol/min in cold-adapted rats 6 hours after returning to 27°C (Fig. 2B).

Blood glucose levels were higher in cold-adapted rats than in rats housed at 27°C (8.53 vs. 7.64 mmol·L⁻¹; P < 0.05). Return to 27°C after cold adaptation decreased blood glucose in a time-dependent manner (Fig. 2C). Representative PET images from each group are shown in Fig. 2D–I. Along with changes in glucose uptake, we also observed alterations in the expression of genes involved in glucose metabolism. Interestingly, the transporter encoded by glucose transporter 1 (Glut1) seemed to be the main glucose transporter (Fig. 2F) in iBAT of rats acutely exposed to cold. Indeed, Glut1 expression was increased after 6 hours of acute cold, whereas that of Glut4 remained unchanged. Conversely, Glut4 expression was stimulated (8.4-fold increase) following cold adaptation, whereas that of Glut1 was unaffected. Expression of genes coding for enzymes involved in glycolysis, such as hexokinase and phosphofructokinase 1 (Pfk1), was also gradually increased with cold exposure and adaptation (Fig. 2K) and tended to gradually decrease with return to 27°C (not significantly). However, the modulation of pyruvate dehydrogenase a1 (Pdhha1) expression was only observed after cold adaptation (8.7-fold increase) and was not affected with return to 27°C. Data expressed in Fig. 2L demonstrate the stimulating effects of chronic cold on uridine diphosphate-glucose pyrophosphorylase (Udpgh) and glycogen phosphorylase (Pygh), which are involved in glycogen synthesis and...
Figure 1. Effect of cold on iBAT activity and capacity. Open bars represent rats adapted to thermoneutrality (27°C), acutely exposed to cold for 2 hours (10°C – 2 hours) or 6 hours (10°C – 6 hours). Gray bars represent rats adapted to cold (10°C), acutely exposed to 27°C for 2 hours (27°C - 2h) or 6 hours (27°C – 6 hours) (n = 8 rats for each experimental group). A) iBAT oxidative activity index determined using [11C]acetate. B) iBAT weight expressed in grams. C) iBAT total oxidative activity index, as a relation between iBAT oxidative activity and total iBAT weight. D) Comparison of iBAT oxidative activity index to heart and liver after acute or chronic cold exposure. E) Determination of iBAT blood flow using [11C]acetate. F) Vegfa mRNA expression in iBAT. G) Average interscapular skin temperature during PET procedure expressed in degrees Celsius (°C). H) Relation between iBAT total oxidative activity index and interscapular skin temperature. I) mRNA expression of mitochondrial (Aco2 and Cox4) and thermogenic capacity (Dio2 and Ucp1) genes in iBAT. J) UCP1 protein content in iBAT with corresponding blot. Data are expressed as the mean ± SEM. °P < 0.05, **P < 0.01, and ***P < 0.001 vs. 27°C, and °°P < 0.05, **°P < 0.01, and ***°P < 0.001 vs. 10°C, assessed by post hoc Bonferroni test following 2-way ANOVA. °P < 0.05 vs. iBAT, ANOVA with Bonferroni post hoc test.
**Figure 2.** Effect of cold on iBAT circulating glucose metabolism. Open bars represent rats adapted to thermoneutrality (27°C), acutely exposed to cold for 2 hours (10°C – 2 hours) or 6 hours (10°C – 6 hours). Gray bars represent rats adapted to cold (10°C), acutely exposed to 27°C for 2 hours (27°C – 2 hours) or 6 hours (27°C – 6 hours) (n = 8 rats for each experimental group). A) iBAT dynamic glucose uptake per gram of tissue determined using the Patlak graphic approach following 18FDG. B) Total iBAT dynamic glucose uptake determined using 18FDG. C) Blood glucose levels expressed in millimoles l⁻¹. D–I) Representative PET images following 15 MBq 18FDG injected intravenously. White circles and arrows show positive 18FDG uptake in iBAT. Yellow arrows show periaorta BAT, red arrows show heart, and green arrows show liver. D) Rat adapted at 27°C. E) Rat acutely exposed (2 hours) to 10°C. F) Rat acutely exposed (6 h) to 10°C. G) Rat adapted at 10°C. H) Rat acutely exposed (2 hours) to 27°C. I) Rat acutely exposed (6 h) to 27°C. J–M) iBAT gene expression profile expressed as fold increase relative to 27°C-adapted rats. J) Genes involved in glucose uptake. K) Genes involved in glycolysis. L) Genes involved in glycogen cycle. M) Genes involved in de novo fatty acid and TG synthesis. Data are expressed as the mean ± SEM. *P < 0.05, **P < 0.01, and ***P < 0.001 vs. 27°C, and #P < 0.05, ##P < 0.01, and ###P < 0.001 vs. 10°C, assessed by post hoc Bonferroni test following 2-way ANOVA.
hydrolysis, respectively. Finally, Fig. 2M illustrates the expression of genes coding for enzymes involved in de novo fatty acid and TG synthesis. Expressions of glycerol-3-phosphate dehydrogenase (Gpd1) (4.8-fold), glycerol kinase (Gyk) (38-fold), and phosphoenolpyruvate carboxykinase (Pepck) (3.5-fold) were increased following acute cold exposure, whereas expression of acyl-coenzyme A carboxylase B (Acacb) (2.1-fold), fatty acid synthase (Fasn) (89-fold), Gpd1 (9.3-fold), Gyk (16-fold), and Pepck (3-fold) was increased following chronic adaptation to cold.

**Effect of cold exposure on lipid uptake**

The PET tracer $^{18}$FTHA was used to assess NEFA uptake in iBAT of rats exposed to cold (Fig. 3). Acute exposure to 10°C led to an increase in iBAT NEFA uptake, which became highly significant after 6 hours of cold exposure (Fig. 3A, B). The acute return to 27°C of cold-adapted rats did not readily reduce iBAT NEFA uptake, regardless of whether uptakes were expressed per grams of tissue (Fig. 3A) or per whole tissue (Fig. 3B). Our results demonstrated that iBAT took up $\sim 12.5$ mmol NEFA per minute following a 6 hour period of cold exposure. Chronic cold raised this uptake to 25 mmol NEFA per minute, which remained relatively stable at 21 mmol NEFA per minute 6 hours following the return to 27°C. This total dynamic uptake was also 2-fold larger than that led to by acute cold exposure (Fig. 3B). Blood NEFA slightly increased according to the duration of cold exposure and significantly so in cold-adapted rats (Fig. 3C). Representative PET images from each group are shown in Fig. 3D–I. It is noteworthy that $^{18}$FTHA also allowed for the detection of periaortic and cervical BAT depots in contrast to the $^{18}$FDG scan. Exposure to cold significantly reduced plasma TG levels (Fig. 3J). TG levels rose again following the return to 27°C of cold-adapted rats. After chronic exposure to 10°C, the increase in iBAT NEFA uptake and circulating TG clearance were accompanied by an increase in the expression of genes involved in lipid transport, such as cluster of differentiation 36 (Cd36), fatty acid transport protein 1 (Fatp1), and fatty acid binding protein 4 (Fabp4) (Fig. 3K). To further investigate TG clearance, we evaluated lipoprotein lipase (Lpl) expression in iBAT. As illustrated in Fig. 3L, Lpl expression rapidly increased after 6 hours of 10°C exposure (9-fold; $P < 0.001$) or in response to chronic cold exposure (5-fold; $P < 0.001$). We also found a direct correlation between Lpl expression in iBAT and circulating TG (Fig. 3M, Pearson’s $r = -0.83$ and $P < 0.05$). This prompted us to measure the expression of genes involved in lipid synthesis and oxidation (Fig. 3N). After acute cold exposure, there was an increase in the lipogenic genes diacylglycerol acyltransferase 1 (Dgat1) and glycerol-3-phosphate acyltransferase (Gpat3) expression. After cold adaptation, Dgat1 and Dgat2 were significantly increased (2- and 4-fold, respectively), whereas Gpat3 remained similar to rats at thermoneutrality. We finally investigated the transport of acyl-coenzyme A (CoA) to the mitochondria by looking at carnitine palmitoyltransferase IB (Cpt1B) expression, which was only modulated after cold adaptation (Fig. 3O).

**Effect of NiAc on iBAT metabolic activity and energy substrate uptake**

NiAc was used to investigate the impact of inhibiting lipolysis in adipocytes on iBAT metabolic activity and energy substrate uptake. As illustrated in Fig. 4A, NiAc suppressed the increase in oxidative metabolism in iBAT after acute (84%) and chronic (73%) cold exposure. Similarly, interscapular skin temperature was decreased by 90% after acute cold and by 71% after cold adaptation (Fig. 4B) in NiAc-treated rats. These effects of NiAc were accompanied by changes in glucose and NEFA uptakes. In response to acute cold (6 hours), iBAT glucose uptake (based on $^{18}$FDG) went from 33 to 13 nmol glucose per minute after NiAc (Fig. 4C). After chronic cold exposure, the inhibition of intracellular lipid utilization produced by NiAc was accompanied by a decrease in glucose uptake (Fig. 4D–G). In parallel, NiAc completely blunted NEFA uptake during acute cold exposure (Fig. 4H). After chronic cold exposure, NiAc also reduced NEFA uptake by 77%. Corresponding PET images are shown in Fig. 4I–L. Figure 4 also illustrates the effect of cold on Hsl gene expression (Fig. 4M) and HSL protein levels (Fig. 4N) in iBAT. Acute cold gradually, but not significantly, increased HSL protein ($P = 0.15$), whereas chronic cold increased both Hsl expression and HSL levels, which were not altered by the return to 27°C.

**DISCUSSION**

The present study was aimed at investigating the effects of cold exposure and cold adaptation on in vivo energy substrate utilization in iBAT using 3 PET tracers, namely $^{18}$FDG, $^{18}$FTHA, and $[^{11}$C]acacetate, which respectively assess glucose uptake, NEFA uptake, and oxidative metabolism. The study was carried out in rats kept at 27°C (thermoneutrality) and acutely exposed to 10°C (cold) for 2 and 6 hours and in rats chronically exposed to cold for 21 days (cold adapted) and returned to thermoneutrality after cold adaptation for 2 and 6 hours prior to being sacrificed. Our study also reports the variations in the expression of genes involved in thermogenesis as well as in glucose and lipid metabolism. The results demonstrate the rapid in vivo changes in iBAT metabolic activity and blood flow occurring in response to cold. The changes in metabolic activity were accompanied by variations in glucose and NEFA ($^{18}$FDG) and NEFA ($^{18}$FTHA) uptakes as well as induction of genes involved in oxidative metabolism and energy substrate (TG and glycogen) replenishment. Finally, experiments using the lipolysis inhibitor NiAc emphasize, in vivo, the essential contribution of BAT intracellular lipids in thermogenesis.

The present results add to previous ones (29–31) by highlighting the remarkable thermogenic properties attributable to brown adipocytes with $[^{14}$C]acetate utilization in vivo. Using $[^{14}$C]acetate in humans, our group has also recently demonstrated the ability of cold exposure and cold adaptation to increase the oxidative metabolic activity/capacity of BAT (11, 18). It is noteworthy that the use of the PET tracer $[^{14}$C]acetate in rats allowed for comparing the metabolic activity of iBAT to that of other
tissues such as the heart and liver. In this study, neither acute cold exposure nor cold adaptation noticeably affected the metabolic activity of the heart and liver, demonstrating that enhanced whole-body metabolic activity with cold exposure is mainly attributable to iBAT metabolism in rats. The increase in iBAT metabolic activity was predictably accompanied by an increased expression of Ucp1 and Dio2 mRNA and by a rise in interscapular skin temperature. The use of [11C]acetate also allows for measuring blood flow. In that respect, our results showed that acute cold exposure (from 0 to 6 hours) did not readily increase blood flow even in the presence of an increased iBAT oxidative activity. However, acute cold exposure led to a rapid increase in Vegfa mRNA expression, suggesting

Figure 3. Effect of cold on iBAT circulating lipid metabolism. Open bars represent rats adapted to thermoneutrality (27°C), acutely exposed to cold for 2 hours (10°C - 2 hours) or 6 hours (10°C - 6 hours). Gray bars represent rats adapted to cold (10°C), acutely exposed to 27°C for 2 hours (27°C - 2hours) or 6 hours (27°C - 6hours) (n = 8 rats for each experimental group). A) iBAT dynamic NEFA uptake per gram of tissue determined using the Patlak graphic approach following 18FTHA. B) Total iBAT dynamic NEFA uptake determined using 18FTHA. C) Blood NEFA level expressed in millimoles l^-1. D–I) Representative PET images following 15 MBq 18FTHA injected intravenously. White circles and arrows show positive 18FTHA uptake in iBAT. Yellow arrows show periaorta BAT, orange arrows show cervical BAT, red arrows show heart, and green arrows show liver. D) Rat adapted at 27°C. E) Rat acutely exposed (2 hours) to 10°C. F) Rat acutely exposed (6 hours) to 10°C. G) Rat adapted at 10°C. H) Rat acutely exposed (2 hours) to 27°C. I) Rat acutely exposed (6 hours) to 27°C. J) Blood TG level expressed in millimoles l^-1.

K–O) iBAT gene expression profile expressed as fold increases relative to 27°C-adapted rats. K) Genes involved in lipid uptake. L) Lpl mRNA expression. M) Relation between blood TG level and Lpl expression in iBAT. N) Genes involved in lipid synthesis. O) Cpt1b mRNA expression. Data are expressed as the mean ± SEM. *P < 0.05, **P < 0.01, and ***P < 0.001 vs. 27°C, and #P < 0.05, ##P < 0.01 vs. 10°C, assessed by post hoc Bonferroni test following 2-way ANOVA.
Figure 4. Effect of cold on iBAT intracellular TG metabolism. Open bars represent rats adapted to thermoneutrality (27°C), acutely exposed to cold for 6 hours (10°C – 6 hours). Gray bars represent rats adapted to cold (10°C) \((n = 6\) rats for each experimental group). Effect of NiAc, 150 mg/kg (black bars) \(\alpha\) saline treatment (open bars), on (A) iBAT oxidative activity index determined using \[^{11}C\]acetate, (B) average interscapular skin temperature expressed in degrees Celsius \(^\circ\), and (C) total iBAT dynamic glucose uptake determined using \[^{18}F\]DG. D–G) Representative PET images following 15 MBq \[^{18}F\]DG injected i.v. White circles and arrows show positive \[^{18}F\]DG uptake in iBAT. Red arrows show heart, and green arrows show liver. D) Rat adapted at 27°C and acutely exposed (6 hours) to 10°C exposure and treated with saline. E) Rat adapted at 27°C and acutely exposed (6 hours) to 10°C exposure and treated with NiAc. F) Rat adapted at 27°C and treated with saline. G) Rat adapted to 10°C and treated with saline. H) Total iBAT dynamic NEFA uptake determined using \[^{18}F\]THA. I–L) Representative PET images following 15 MBq \[^{18}F\]THA injected intravenously White circles and arrows show positive \[^{18}F\]THA uptake in iBAT. Yellow arrows show periaorta BAT, orange arrows show cervical BAT, red arrows show heart, and green arrows show liver. I) Rat adapted at 27°C and acutely exposed (6 hours) to 10°C exposure and treated with saline. J) Rat adapted at 27°C and acutely exposed (6 hours) to 10°C exposure and treated with NiAc. K) Rat adapted at 10°C and treated with saline. L) Rat adapted at 10°C and treated with NiAc. (continued on next page)
that cold promptly stimulates the angiogenesis process to allow for later recruitment of iBAT blood vessels in cold-adapted animals. Vegf expression represents an acknowledged marker of angiogenesis (32) and has recently been reported to increase in iBAT of cold-adapted animals (33, 34). Increased blood flow certainly represents a metabolic requirement for brown adipocyte development and heat dissipation from BAT.

The present study also validates the usefulness of the PET tracers $^{18}$FDG and $^{18}$FTHA to investigate iBAT glucose and NEFA uptakes in vivo, in rats throughout the process of cold adaptation. Exposure to cold readily increased both glucose and NEFA uptakes in iBAT together with stimulating the expression of the genes coding for the glucose transporter Glut4 and the fatty acid uptake transporters Cd36, Fatp1, and Fabp4, which is in agreement with previous studies (35–38). It is noteworthy that acute cold exposure preferentially stimulated Glut1 rather than Glut4. We additionally determined that iBAT cleared $-0.048$ mmol glucose per day ($-0.79\%$ circulating glucose) in rats acutely (for 6 hours) exposed to cold and $0.228$ mmol/d ($-1.7\%$ circulating glucose) in cold-adapted rats, which is in accordance with estimations done in previous experiments in rats (39) and with our previous estimations in humans (11, 18). We also determined that iBAT took up $0.018$ mmol NEFA per day in rats acutely exposed to cold (for 6 hours) and $0.036$ mmol/d in cold-adapted rats. Importantly, our PET dynamic acquisition procedure and data analyses allow for the real-time in vivo measurement of glucose and NEFA uptake rates, quantified in nanomole per gram of BAT per minute. This allows a quantitative assessment of substrate uptake by BAT compared to standardized uptake value (SUV) or percentage of injected dose values, which are semiquantitative measures. We also recently described the dynamic uptake of glucose and NEFA by iBAT in cold-exposed humans with (18) or without (11) adaptation to cold. It is noteworthy that in most human and animal studies carried out so far by others, $^{18}$FDG with static PET scanning has been used to detect semiquantitative glucose uptake in active brown fat expressed as the injected dose per gram of tissue (SUVs) (8, 9, 40–42).

The increases in $^{18}$FDG and $^{18}$FTHA uptake in cold-adapted rats were also accompanied by changes in the expression of genes involved in glycogenesis, de novo fatty acid synthesis, and TG synthesis. All those genes code for proteins that are involved in pathways supporting the enhanced BAT metabolic activity seen after cold adaptation (Fig. 5). Activation of these pathways ultimately contributes to replenish or sustain the intracellular reserves of TG. It is of interest that cold adaptation led to an increase in the expression of Udg2 and Pdgk, which are genes involved in glycogenesis that might provide a readily available glucose pool usable to ensure the enhanced glycolysis and rapid turnover of acetyl-CoA in active iBAT. Through the measured high rate of [$^{13}$C]acetate oxidation, we also confirmed that acetyl-CoA turnover is elevated in cold-exposed and cold-adapted rats. The increased expression of glycolytic enzymes such as Pfk1 and Pdhα observed in cold-adapted rats supports the expected cold-induced activation of glycogenesis (43–45). Cold adaptation also led to a marked increase in the expression of Acacb and Fas, which tends to further support the genuine ability of BAT of cold-exposed and cold-adapted rats for de novo fatty acid synthesis (46–49). In iBAT, fatty acids can either be oxidized or serve for TG synthesis. Cold is a strong inducer of TG formation in BAT. Lipogenic genes (Acacb, Dgat1, Dgat2, Fasn, Gpat3, Gpdl1, Gyk, and Pepck) are in fact highly induced in cold-exposed animals. In cold-adapted rats, the levels of TG remain constant despite enhanced fatty acid oxidation, implying that the intracellular TG pool is efficiently replenished. In acutely exposed rats, the TG levels drop (50, 51), which seemingly further stimulates the induction of lipogenic genes. Circulating TGs also constitute an important source of NEFA for BAT. It has been previously shown that cold exposure markedly increases BAT lipid clearance in rodents (37, 52, 53). In the present study, we further emphasize the importance of iBAT metabolic activity to lower circulating TG and increase expression of Lpl in cold-exposed, cold-adapted rats. These 2 variables were also highly correlated. Our results are in line with those of another group of investigators (37) that demonstrated the importance of BAT in the clearance of circulating TGs.

The experiments carried out with NiAc further demonstrate the importance of intracellular TG as the main fuel to sustain BAT energy metabolism during cold exposure in mammals (19–21). NiAc primarily acts on the nicotinic receptor 1, a GPCR that inhibits the activation of PKA, thereby inhibiting intracellular TG lipolysis (54–56). We estimated that the intracellular TG pools contributed to up to 84% of thermogenesis during an acute cold challenge. This estimation was based on [$^{13}$C]acetate data and was corroborated by the associated decrease (90%) in interscapular skin temperature. During chronic cold exposure, BAT TG pools were estimated to contribute to $\sim 74\%$ of BAT thermogenesis (from [$^{13}$C]acetate data). Accordingly, the interscapular skin temperature dropped by 71%. Recently, isolated adipocyte experiments demonstrated that intracellular TG contributes to 80–97% of total brown adipocyte respiration (57). These data are certainly in line with our in vivo findings. We also found that inhibition of BAT intracellular TG lipolysis results in a major decrease in glucose and NEFA uptake, demonstrating that TG lipolysis precedes de novo fatty acid synthesis, TG synthesis, and glyceroneogenesis (57, 58). The oxidation and de novo fatty acid synthesis cycle may contribute to create a sink for the excess glucose and lipid and hence be seen as being of importance in the role of BAT in the control of glycemia and hyperlipidemia (59). The NiAc experiments also suggest that glucose can contribute to BAT metabolic activity. Assuming that NiAc blocks 100% of intracellular fat breakdown, the data suggest that glucose can also minimally contribute directly to thermogenesis. We cannot exclude the possibility that the reduction in circulating NEFA from inhibition of white adipose tissue (WAT)
lipolysis contributed to the reduced BAT activation in response to NiAc. However, we do not believe that such reduction contributed markedly. In our cold-adapted rats, the contribution was estimated to be 24 nmol per total BAT per minute, which cannot account for the high metabolic rate of the tissue. Additionally, based on our human data (11), we estimated that BAT NEFA uptake upon cold exposure ranged from 0.054 to 0.792 mmol over 3 hours. Such amounts were negligible compared to the estimated 356 mmol fatty acid (e.g., the 28 g intracellular BAT TGs) likely mobilized over the same period. Therefore, we believe that the inhibition of WAT lipolysis played a negligible role in the inhibition of BAT metabolic activation with NiAc. BAT intracellular lipids likely represent the major source of free fatty acids used, and this process would per se not acutely block BAT thermogenesis because the BAT intracellular pools are the ones most readily used, based on previous studies (11, 18).

In conclusion, the present study demonstrates a predominant role of BAT (over the heart and liver) in cold-induced thermogenesis based on [11C]acetate data. Additionally, our study also demonstrated, in vivo, that the observed changes in metabolic activity were followed by changes in glucose ([18F]FDG) and NEFA ([18F]THA) uptake. All these alterations were accompanied by changes in the expression of genes involved in favoring substrate oxidation and TG replenishment in iBAT. Because inhibition of intracellular lipolysis using NiAc profoundly suppressed iBAT thermogenesis and metabolism, we also conclude, from our in vivo experiments, that intracellular TGs are the primary energy source for iBAT thermogenesis. However, what contributes the most to the intracellular TG pool between being derived from circulatory lipids or derived from de novo fatty acid synthesis from glucose (and other substrates) still remains to be elucidated.

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Figure 5. Schematic and hypothetical representation of the main pathways involved in substrate utilization and replenishment in cold-adapted brown adipocytes. A small fraction of glucose and NEFA seems to be oxidized, whereas the majority is sent to de novo fatty acid and triacylglycerol (TAG) synthesis for storage as TAG or stored as glycogen (the case of glucose) to allow a rapid turnover of acetyl-CoA. Consequently, all the substrate taken up by BAT is stored as TAG, and lipolysis of these generated TAG pools appears essential for thermogenesis.


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