Cooperation between BAT and WAT of rats in thermogenesis in response to cold, and the mechanism of glycogen accumulation in BAT during reacclimation

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Abstract **Rats were exposed to cold and then reacclimated at neutral temperature. Changes related to fatty acid and glucose metabolism in brown and white adipose tissues (BAT and WAT) and in muscle were then examined. Of the many proteins involved in the metabolic response, two lipogenic enzymes, acetyl-coenzyme A carboxylase (ACC) and ATP-citrate lyase, were found to play a pervasive role and studied in detail. Expression of the total and phosphorylated forms of both lipogenic enzymes in response to cold increased in BAT but decreased in WAT. Importantly, in BAT, only the phosphorylation of the ACC1 isoenzyme was enhanced, whereas that of ACC2 remained unchanged. The activities of these enzymes and the in vivo rate of FFA synthesis together suggested that WAT supplies BAT with FFA and glucose by decreasing its own synthetic activity. Furthermore, cold increased the glucose uptake of BAT by stimulating the expression of components of the insulin signaling cascade, as observed by the enhanced expression and phosphorylation of Akt and GSK-3. In muscle, these changes were observed only during reacclimation, when serum insulin also increased. Such changes may be responsible for the extreme glycogen accumulation in the BAT of rats reacclimated from cold.**—Jakus, P. B., A. Sandor, T. Janaky, and V. Farkas. **Cooperation between BAT and WAT of rats in thermogenesis in response to cold, and the mechanism of glycogen accumulation in BAT during reacclimation.** ***J. Lipid Res.*** **2008.** 49: **332–339.**

Supplementary key words brown adipose tissue • white adipose tissue • lipogenic enzymes • insulin signaling • phosphorylation

Of the different organs involved in thermoregulatory thermogenesis in rodents, brown adipose tissue (BAT) is of particular interest. BAT is responsible for nonshivering thermogenesis in adult, cold-exposed, nonhibernating rodents. During cold exposure (5°C), BAT undergoes hypertrophy (characterized by cell proliferation and differentiation) and then atrophy during reacclimation to a thermoneutral (28°C) environment. These cold-mediated physiological events are triggered by norepinephrine (NE), which induces the enhanced expression of uncoupling protein 1 (UCP1) in differentiated BAT cells. In the expression of UCP1 via NE, leptin is also involved (1, 2). For a comprehensive review, see Ref. 3.

Unlike most studies in this field, the present work focused on the interrelationship that is thought to occur across different tissues during cold acclimation. For example, it is unknown whether a mechanism or organ, besides shivering and BAT, contributes to thermoregulatory thermogenesis. To address this question, we extended our former investigations on BAT to other organs, particularly to white adipose tissue (WAT) and skeletal muscle. In a study by Granneman and coworkers (4) on UCP1$^{-/-}$ mice, it was proposed that WAT may be a major organ contributing to thermogenesis (5). The primary aim of the present study was to find the enzymatic basis for the contribution of WAT. Accordingly, we screened for proteins showing marked changes under the above-described conditions and thereby identified two lipogenic enzymes, acetyl-coenzyme A carboxylase (ACC) and ATP-citrate lyase (CitLy), for further analysis.

Second, based on our earlier findings (6), we developed a more detailed approach to studying the mechanism of glycogen accumulation in BAT and skeletal muscle in the reacclimation period, about which very little is known. One of the few papers published on this subject (which referred to this period as the "deacclimation" period) reported a rapid decrease in UCP1 by day 3 of reacclimation, followed by decreases in the size of BAT and in the number of mitochondria (7). We previously found that within 24 and 48 h of reacclimation, there was a dramatic (10- to 14-fold) accumulation of glycogen in the BAT. In attempting to elucidate the mechanism triggering this event, we had determined that glycogen synthase activity increased,

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whereas glycogen phosphorylase activity decreased (probably as a result of the diminishing influence of NE). GLUT4 is also overexpressed in response to cold (8), and its expression was maintained during reacclimation (9), when glycogen synthesis prevails. Here, we sought to identify the hormonal and enzymatic background characteristic of reacclimation and to study the role of insulin signaling upstream from glycogen synthase and glycogen phosphorylase.

MATERIALS AND METHODS

Materials

2-Deoxy-D-[U-^{14}C]glucose (Amersham), $^{3}H_2O$, and [γ-^{32}P]ATP were purchased from the Institute of Isotopes Co. (Budapest, Hungary). GSK-3β was from Calbiochem (La Jolla, CA). Specific antibodies against Akt (P-Akt), GSK-3β (P-GSK-3β), CitLy (P-CitLy), and ACC (P-ACC) were from Cell Signaling Technology and were purchased from Kvalitex (Budapest, Hungary). The insulin determination kit was from Crystal Chemicals (Downers Grove, IL). Antibody against actin, amyloglycosidase, and other fine chemicals were from Sigma-Aldrich, Ltd. (Budapest, Hungary). SB 415286-specific GSK-3 inhibitor was from Tocris (Avonmouth, UK). GSK-3 substrate peptide was from Upstate.

Animals and their handling

All experiments were carried out in accordance with the regulations specified by the National Institutes of Health Principles of Laboratory Animal Care, 1985 Revised Version. Male Wistar rats weighing 200–250 g were used. The animals were housed in individual cages, fed standard laboratory chow ad libitum, and exposed to a 12 h/12 h light/dark period (light from 8:00 AM to 8:00 PM) in cold (5°C for 1 week) and neutral (28°C) temperatures. During the experiment, food consumption and weight gain were monitored. At the end of the experiments, animals were decapitated under light ether anesthesia between 8:00 and 9:00 AM (withdrawal from cold took place at the same time to avoid circadian variation).

Tissue sampling and processing

After decapitation, ~3 ml of blood was collected into tubes containing 100 μl of heparin. Interscapular BAT was quickly removed and placed on an ice-cold glass plate, and the BAT was separated within 1 min. At the same time, another member of the staff dissected and freeze-clamped part of the gastrocnemius muscle and epididymal fat pad. The trimmed BAT was also clamp-frozen, and all samples were stored under liquid nitrogen until analysis. BAT and muscle tissues (200 mg) were extracted in 5 volumes and WAT (500 mg) in 2 volumes of extraction buffer in conical glass grinders. The extraction buffer (solution I) contained the following: 50 mmol/l Tris-HCl (pH 7.4), 150 mmol/l NaCl, 1 mmol/l EGTA, 1 mmol/l PMSF, 1 mmol/l Na_3VO_4, 1 mmol/l NaF, 5 mmol/l Na-pyrophosphate, and 100 μl of protease inhibitor cocktail (P-8340; Sigma, Budapest, Hungary). The samples were centrifuged at 10,000 *g* for 10 min. For the skeletal muscle sample, the supernatant was saved, whereas for WAT and BAT, the intermediate phase between the upper lipid phases was removed with a syringe and then this step was repeated. The protein content of the pellet in all samples was determined by bicinchoninic acid (B-9643; Sigma) assay, and each sample was adjusted to 100 μg protein/20 μl. The tissue samples were used either for GSK-3 or for ACC activity measurements. Another aliquot of the samples was analyzed by SDS-PAGE using a Miniprotean II (Bio-Rad Laboratories, Hercules, CA) or, for large (16 × 18 cm) gels, a Sturdier SE 420 (Hoefer Scientific Institute, San Francisco, CA). Electrophoresis was followed by immunoblotting (Western) or Coomassie staining. The relative intensity of the lanes was quantified by UTHCSA Image (IT version 1.27).

Identification of proteins by mass spectrometry after SDS-PAGE analysis

The lanes were loaded with equal volumes of proteins, so differences in the intensities of the bands reflected protein-based changes, including changes in the specific activity of the enzymes of interest (the intensities of several bands did not change, confirming that equal amounts of proteins had been loaded). Proteins in the excised gel bands were reduced, alkalinized, and in-gel digested with trypsin (sequencing-grade, modified trypsin; Promega GmbH, Mannheim, Germany) as described (10). The digests were purified with ZipTipC18 pipette tips (Millipore, Bedford, MA) using a procedure recommended by the manufacturer. One microliter of eluate from the ZipTips was mixed at a 1:1 ratio with saturated 2,5-dihydroxybenzoic acid matrix solution and applied to an Achor chip MALDI plate (Bruker-Daltonics, Bremen, Germany). A Bruker Reflex III MALDI-TOF mass spectrometer (Bruker-Daltonics) was used in positive ion reflector mode with delayed extraction. Autolysis products of trypsin served as internal standards for mass calibration. The monoisotopic masses for all peptide ion signals in the acquired spectra were determined, and the information was entered in a database searching against a nonredundant database (National Center for Biotechnology Information) using the Mascot program (Matrix Science, http://www.matrixscience.com/).

Enzyme activities

GSK-3 activity was determined as follows. Stock buffer (solution II) consisted of 30 ml of solution I containing 250 mmol/l sodium glycerophosphate, 1 mol/l NaCl, 100 mmol/l $MgCl_2$, 5 mmol/l EGTA, 0.5 mmol/l Na_3VO_4, and 5 mmol/l freshly added DTT. Measuring buffer (solution III) for a series of 24 tubes contained, in 600 μl of solution II, 12.5 nmol of ATP (2.5 μl from a 5 mmol/l ATP stock solution) and 8,880 kBq (240 μCi, 5.32×10^8 dpm) in 22 μl of the commercial [γ-^{32}P]ATP preparation. In the resulting solution (calculated with the original radioactivity of the ATP preparation), the specific activity of ATP was 42,500 dpm/pmol. A series of 24 tubes, 2 for each sample, was arranged, each containing 25 μl of measuring buffer. To one tube of each pair, 2.5 nmol of GSK-3 substrate (in 2.5 μl) and 2.5 μl of solution I were added. To the other tube (serving as background), 2.5 μl of solution I (with no substrate) and 1 μg of GSK-3 inhibitor (SB 415286) in 2.5 μl were added. (The inhibitor was used to prevent GSK-3 from phosphorylating other proteins present in the tissue sample.) The reaction was started by adding 20 μl of tissue extract containing 100 μg of protein. In the final incubation medium (50 μl), the ATP concentration was 10 μmol/l, and the actual specific activity was calculated from the radioactive measurement on the day of the experiment. After 15 min of incubation, the reaction was arrested with 10 μl of 1 mol/l HCl and spotted onto a P-81 phosphocellulose filter (Whatman), then the filter was washed with 75 mmol/l H_3PO_4. After drying, the filters were placed into vials containing scintillation fluid, and radioactivity was counted with a Packard Tricarb 2100TR scintillation counter. The enzyme activity was calculated from the dpm difference between the tube with substrate and the tube without substrate and with SB 415286 inhibitor.

Pyruvate dehydrogenase (PDH) and phosphofructokinase (PFK) activities were measured as described (11, 12). ACC and CitLy activities were measured spectrophotometrically as reported previously (13, 14). Glycogen content was measured as described previously (6).

Glucose uptake

Glucose uptake was measured using 2-deoxy-[^{14}C]glucose, basically as described (15). Briefly, 1 h after an intraperitoneal injection of 7.4 kBq of radioactive 2-deoxy-glucose, the glucose concentration was measured in plasma and radioactivity was measured in plasma and BAT. The plasma specific radioactivity was then used to calculate glucose uptake (15).

In vivo fatty acid synthesis

FA synthesis was evaluated in vivo by measuring the incorporation of $^{3}H_2O$ into FA, basically as described (16). This method measured the rate of de novo FFA synthesis independent of the precursor carbon source. An intraperitoneal injection of 370 kBq of tritiated water in 1 ml of saline was administered to the animals, and 1 h later they were euthanized. At this early stage of incorporation, the specific radioactivity of FA was low and increasing, so the FFA transported from the total pool hardly changed the radioactivity in other tissues. Thus, the radioactivity found in FA in a given tissue can be regarded as in situ synthesized. (Cold-adapted animals were injected with $^{3}H_2O$ in the cold room.) The tissues were processed, and the radioactivity in FFA in BAT and WAT was counted in a liquid scintillation counter. Results are given as micrograms of H atom incorporated into 1 g of tissue in 1 h (16).

Statistical analyses

Data were evaluated by ANOVA and are presented as means ± SEM. The relative intensity of lanes was quantified by UTACSA Image (IT version 1.27).

RESULTS

Cooperation of WAT with BAT in thermoregulatory thermogenesis

BAT proteins were screened for changes in their expression during a control/cold-exposure/reacclimation cycle. As **Fig. 1** shows, the expression of several enzymes varied markedly in response to the changes in temperature. Of these, two lipogenic enzymes, ACC and CitLy, were chosen for further analysis. In addition, malic enzyme was of interest because it catalyzes the synthesis of NADPH + H^+, which is an obligatory coenzyme for the de novo FFA synthesis. In the next step, the lipogenic enzymes were subjected to detailed investigation by immunoblotting using specific antibodies, and the study was extended to WAT.

Because ACC and CitLy are regulated by phosphorylation, immunoblotting with specific antibodies was carried out to distinguish between the total amounts of these enzymes and their phosphorylated forms. Comparison of the changes in the total amounts of the two enzymes (**Fig. 2A, B**) showed that, in the cold, their expression increased markedly in BAT but decreased in WAT. The

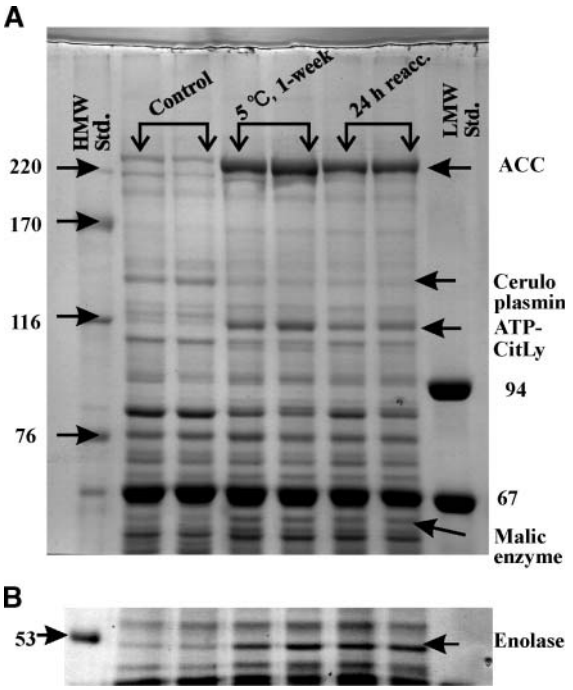


Fig. 1. Resolution of proteins of brown adipose tissue (BAT) from animals under the indicated conditions. The changes from the control animals (neutral temperature of 28°C) through the cold exposure (1 week at +5°C) and in the subsequent 24 h reacclimation were detected. In each lane, 90 μg of protein was loaded. The proteins were run with a Sturdier instrument on a large gel in the presence of 5% PAGE. ACC, acetyl-coenzyme A carboxylase; CitLy, ATP-citrate lyase; HMW Std., high molecular weight standard; LMW Std., low molecular weight standard. The bands showing marked changes are labeled with arrows. The proteins were identified by MALDI-TOF MS. A: Upper part of the gel. B: Lower part of the gel from another run.

phosphorylation of ACC and CitLy corresponded to the pattern of total enzyme expression in the two tissues. Of the two isoenzymes of ACC in BAT, the phosphorylation only of ACC1 (ACCα) was enhanced upon cold treatment. The relative optical densities in this band in the lanes were as follows: lanes 2 and 3, 100%; lanes 4 and 5, 280%; lanes 6 and 7, 220%; lanes 8 and 9, 170%.

The increased expression and phosphorylation of these lipogenic enzymes indicated opposite effects: enzyme activity was expected to increase in response to increased expression and to decrease as a result of increased phosphorylation. Accordingly, the activities that resulted from the two opposite influences had to be measured. As shown in Fig. 2, the activities of ACC (Fig. 2C) and CitLy (Fig. 2D) in BAT increased significantly in the presence of cold ambient temperature, implying that total enzyme expression overcame the effect of phosphorylation. Then, the activities began to return to normal during reacclimation. By contrast, in WAT, the activities of the lipogenic enzymes trended to decrease in the cold. (It may seem contradictory that in the control bands, ACC activity was similar in BAT and WAT, whereas the intensity of the ACC band was stronger in WAT. However, phosphorylation of ACC in WAT was also much stronger, which entailed decreased

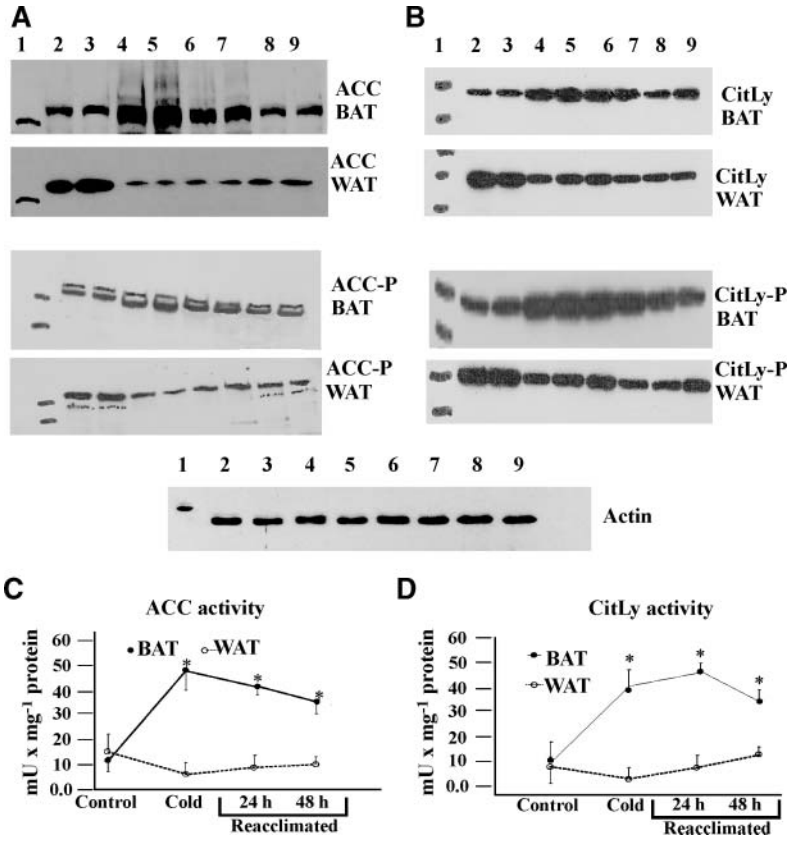


Fig. 2. Comparison of the expression of ACC and CitLy in BAT and white adipose tissue (WAT), as detected by immunoblotting, and actual activities of these enzymes. The animal conditions were as described for Fig. 1. Lane 1, high molecular weight standard; lanes 2 and 3, control; lanes 4 and 5, cold-exposed for 1 week; lanes 6 and 7, 24 h reacclimation; lanes 8 and 9, 48 h reacclimation. A: Expression of total ACC and P-ACC. Note that both isoenzymes of P-ACC were detected: the thin, upper band is ACC2 (or ACCβ) and the lower band is ACC1 (or ACCα). B: Expression of total and phosphorylated CitLy. The lane for muscle actin shows no change, demonstrating that equal amounts of protein were loaded. C: ACC enzyme activity. Values shown are means ± SEM (n = 5). * $P < 0.01$, compared with controls. D: CitLy activity. Values shown are means ± SEM (n = 5). * $P < 0.01$, compared with controls.

activity.) The consequences of changes in lipogenic enzymes were tested by in vivo measurement of FA synthesis rates in both adipose tissues (**Fig. 3**).

Based on the activities of ACC and CitLy in BAT and WAT as outlined above (Fig. 2), the in vivo synthesis rate of FFA was measured during a control/cold-exposed/reacclimation cycle using the currently most accepted ^3H_2O method (Fig. 3). FFA synthesis rate (per gram of tissue) increased by 3-fold in BAT. This level was maintained during the first 24 h of reacclimation and decreased by 48 h. In WAT, the opposite was found (i.e., synthesis declined 3-fold and then started slowly to recover during reacclimation) (Fig. 3A).

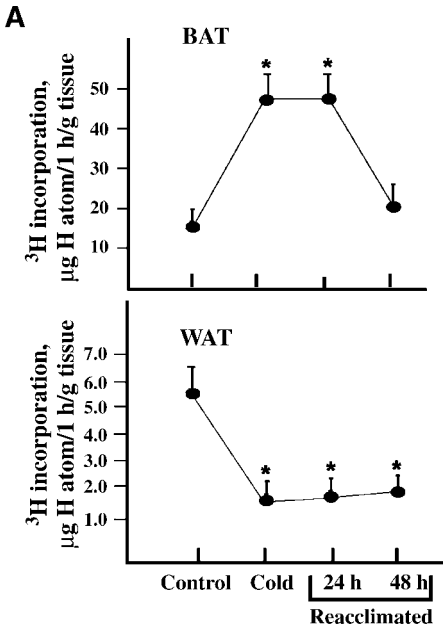
Despite the accelerated rate of FFA synthesis (Fig. 3B), the FA content decreased markedly in BAT in the ambient cold temperature (17). Thus, the rate of FFA breakdown (i.e., β-oxidation) also increased (16) and was greater than the enhanced synthesis rate, resulting in a reduction of relative FA content.

Changes in signal transduction and other conditions lead to glycogen accumulation in BAT during reacclimation

The results outlined above (Figs. 1–3) mostly referred to FA metabolism. As for glucose metabolism, we reported previously a marked accumulation of glycogen in BAT of rats during reacclimation (6). Here, further investigations are presented on the mechanism of this effect. **Figure 4** shows that, although serum insulin level decreased in the cold (Fig. 4A), glucose uptake by BAT increased (Fig. 4B). In previous reports (18, 19), this behavior was attributed to an "increased insulin sensitivity." In the present work, it was important that serum insulin increased even above the control level during reacclimation (Fig. 4A) and, not surprisingly, so did glucose uptake into BAT (Fig. 4B). The change in activity of PFK was negligible (Fig. 4C). In the case of PDH, however, the change in activity was opposite that of the other measured parameters: after a decrease in the activity of this enzyme in the cold, during reacclimation, instead of recovering, it continued to decline, remaining significantly below control levels (Fig. 4D). This result implied that there was less conversion of three-carbon pyruvate to two-carbon active acetate, allowing an increased gluconeogenesis, which can entail enhanced glycogen synthesis (Fig. 4E).

Figure 5 shows the expression and phosphorylation pattern of two members of the insulin signal cascade in BAT, Akt (PKB) and GSK-3. In the cold, P-Akt (active form) was reduced, as was P-GSK-3 (inactive form), because GSK-3 is the substrate of Akt (Fig. 5A). Accordingly, this resulted in a marked increase of GSK-3 activity (Fig. 5B) and explains why glycogen synthesis is a negligible process in BAT under conditions of cold ambient temperature (6). However, there was a striking increase in Akt and P-Akt during reacclimation, as shown by the increased ratio of phosphorylated GSK-3. As a consequence, the synthesis of glycogen was facilitated under these conditions. (6).

The investigation of insulin signaling was extended also to skeletal muscle (**Fig. 6**), but, in contrast to the above-described results in BAT and consistent with a previous



B

Composition of BAT in %

			Reacclimated	
	Control	Cold	24 h	48 h
FA	87.7	56.0	68.5	83.4
NFDM	13.3	38.3	27.7	14.4

Fig. 3. Opposing changes of in vivo fatty acid synthesis in BAT and WAT. The animal conditions were as described for Fig. 1. A: Velocity of ^{3}H incorporation into the indicated tissues at 1 h after ^3H_2O injection. Values shown are means ± SEM (n = 5). * $P < 0.001$, compared with controls. B: Composition of BAT. NFDM, non-fat dry material.

report (19), no changes were observed in cold ambient temperature. Therefore, in cold, insulin signaling appears to be tissue-specific, where BAT is the target tissue. However, during reacclimation, the patterns of Akt and GSK-3 expression in skeletal muscle were similar to that of BAT (Fig. 5), which indicates that during reacclimation, an enhanced glycogen synthesis is taking place in muscle as well.

DISCUSSION

The pivotal organ of nonshivering thermogenesis in rodents is the BAT. The first discovered uncoupling protein (UCP1) is located exclusively in this tissue and, either alone (20, 21) or together with UCP2/3 (22, 23), is thought to be responsible for nonshivering thermogenesis. In UCP1$^{-/-}$ mice exposed to cold over a long period of time, shivering is the only survival mechanism, but it is not thought to be thermogenically adaptive (21). Even if it is, organs other than BAT most likely cooperate in the thermogenic response (4).

Here, the contribution of BAT was explored in greater detail with regard to the enzymes involved and the study was extended to WAT. Of the screened proteins showing marked changes (Fig. 1), ACC and CitLy were studied further (Fig. 2). The increased expression of these enzymes and their activities in BAT, together with their decrease in WAT, indicated that different changes of lipid synthesis play important roles in the cooperation between organs in favor of thermogenesis.

It was noteworthy that in cold exposure, only the phosphorylation of ACC1 (ACCα) and not of ACC2 (ACCβ) increased in BAT (Fig. 2A). ACC1 isoenzyme plays a role in FFA synthesis in lipogenic tissues, including WAT and BAT, whereas ACC2 is thought to regulate carnitine palmitoyltransferase I via malonyl-CoA (24, 25). The increase of mRNAs of these enzymes in BAT of cold-exposed mice has been reported (16), and our findings support this in rats at the level of protein expression.

The changes in lipogenic enzymes observed in response to cold (Fig. 2) raised the question of whether these enzyme activities are in accordance with in vivo FA synthesis. Early experiments using carbon sources as precursors (acetate or glucose) suffered from the problem of unknown dilution of the precursor. By contrast, tritium (^3H_2O) incorporation into FFA allows the measurement of de novo FFA synthesis independent of the carbon source. All studies, independent of the method and species (mice or rats) used, have found a marked increase of FFA synthesis in BAT in response to cold ambient temperatures, whereas in the case of WAT in rats, the results are controversial (26–29). A study using the ^3H_2O method in cold-acclimated rats reported a 13-fold increase of FFA synthesis in BAT versus a 1.5-fold increase in WAT (30). The same approach used in mice resulted in a >10-fold increase in FFA synthesis in BAT, WAT (parametrical), and liver. The different findings with respect to WAT were attributed to the species difference (31). An ~3-fold increase in FFA synthesis in BAT of cold-challenged mice was also reported (16), consistent with our findings in rats; however, we also found a similar fold decrease in WAT.

Besides the increased activities of lipogenic enzymes in BAT, the increased FFA synthesis was also supported by the overexpression of NADPH + H^+-producing enzymes (i.e., malic enzyme) (Fig. 1) and glucose-6-phosphate dehydrogenase (32). Although Trayhurn (30) found a 1.5-fold increase of FFA synthesis in WAT (per gram of tissue) of cold-exposed rats, in that study the total WAT mass decreased in the whole animals. The shrinkage of WAT mass in response to cold has also been confirmed by others (33). Thus, changes in synthesis rate are magnified on a whole organ basis because of the proliferation of BAT and the shrinkage of WAT. Early studies raised the possibility that FFAs present in the BAT are derived partly from other tissues, such as from WAT (17), proposing a mechanism for the collaborating action. Cats and young rats exposed to intermittent cold stress developed morphological characteristics in epididymal WAT resembling those of BAT cells (33). As mentioned above, other studies have also proposed WAT as an organ contributing to thermogene-

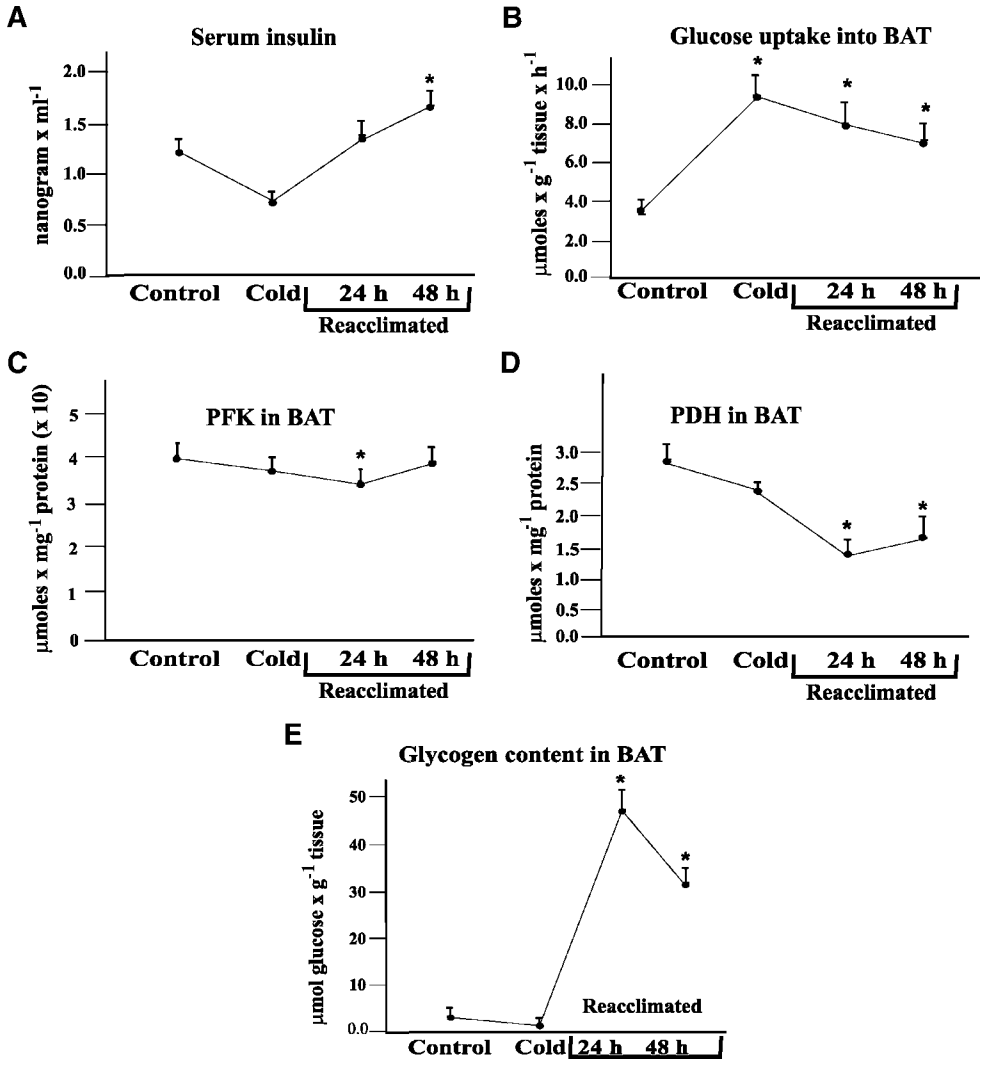


Fig. 4. Changes in related physiological events when glycogen accumulates in BAT. The animal conditions were as described for Fig. 1. A: Serum insulin level. Values shown are means ± SEM (n = 5 in each group). * $P < 0.01$, compared with controls. B: BAT uptake of 2-deoxy-D-[U-^{14}C]glucose for 1 h. Values shown are means ± SEM (n = 5 in each group). * $P < 0.01$, compared with controls. C: Phosphofructokinase (PFK) activity. Values shown are means ± SEM (n = 5 in each group). * $P < 0.05$, compared with controls. D: Pyruvate dehydrogenase complex (PDH) activity. Values shown are means ± SEM (n = 5 in each group). * $P < 0.01$, compared with controls. E: Glycogen content in BAT. Values shown are means ± SEM (n = 5 in each group). * $P < 0.001$, compared with controls.

sis: β_3-agonist (CL-316243) treatment was found to cause a BAT-like histological appearance of WAT (4). In leptin-treated UCP1 knock-out mice, another interesting inter-organ relationship was revealed. Leptin reduced the WAT size in wild-type mice but not in UCP1$^{-/-}$ mice (34). A theoretical explanation could be that because the BAT is not turned on with leptin in UCP1$^{-/-}$ mice (blood flow measurement, UCP1 expression), the FFA released from WAT is not used by the BAT and instead might go back to the WAT.

In conclusion, in cold-exposed rodents, the pattern of lipid metabolism in general is opposite in BAT versus WAT. This observation supports the hypothesis that WAT acts primarily as a net FFA provider, whereas BAT acts as a net FFA user. Our results indicate the pivotal role of two lipogenic enzymes in this process. Nevertheless, it should be kept in mind that the role of WAT, even if it assists in thermogenesis, is secondary to the shivering and BAT-mediated nonshivering thermogenesis (21), because white adipose cells have not been shown to express UCP1 protein. Kozak and coworkers (35) reported that in WAT of wild-type mice, UCP1 mRNA was expressed in response to cold and β_3-agonist treatment. However, this should be attributed to the appearance of brown adipocytes in the WAT of treated animals, as demonstrated previously (36, 37). Also, in the study of Kozak and coworkers (35), the expression of UCP1 mRNA and protein was accompanied by a proportional appearance of multilocular brown adipocytes. Furthermore, the appearance of brown adipose cells in WAT showed an age dependence (38) and

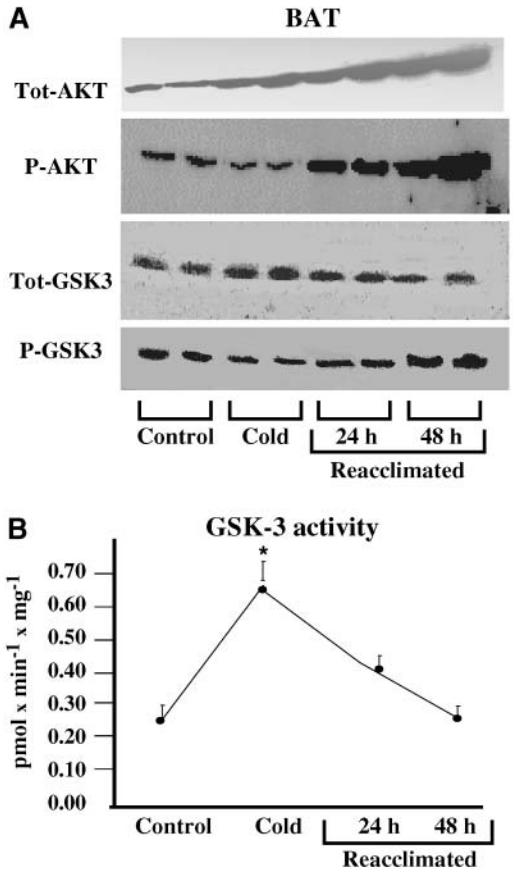


Fig. 5. Expression of components of the insulin signal cascade affecting GSK-3 activity in BAT. A: Western immunoblotting of Akt and GSK-3 total enzymes and their phosphorylated forms. B: GSK-3 activity under the indicated conditions. Values shown are means ± SEM (n = 6 in each group). * $P < 0.001$, compared with controls.

a strain dependence, confirming an earlier report (37). Most recently, this group reported a UCP1-independent thermogenic effect of leptin in mice, probably made through the thyroid axis (39).

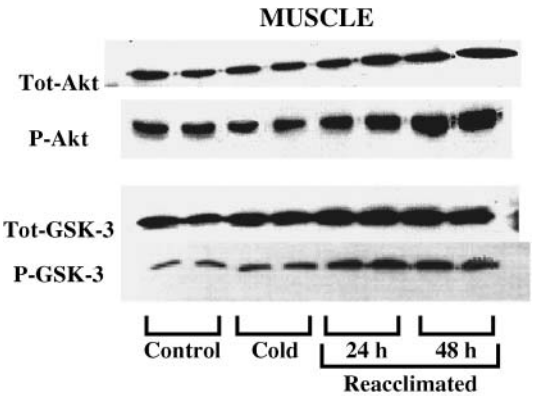


Fig. 6. Expression of components in the signal transduction cascade affecting Akt and GSK-3 in skeletal muscle. The conditions for the animals were as described for Fig. 1. Western immunoblotting of Akt and GSK-3 total enzymes and their phosphorylated forms is shown.

The second part of this work was devoted to an examination of glucose metabolism, namely, the mechanism of glycogen accumulation in BAT and muscle during reacclimation (6). The endocrine and enzymatic changes under this condition, as well as glucose metabolism, especially the insulin signaling cascade, were investigated. The changes in insulin level and glucose uptake occurred in the reacclimation period (Fig. 4), strongly suggesting an enhanced glycogen synthesis. The decrease of PDH activity was particularly noteworthy. PDH would degrade pyruvate to a two-carbon compound (acetyl-CoA), which cannot be used for glucose synthesis. Together, these results are consistent with and well explain glycogen accumulation during reacclimation.

It is well documented that cold exposure of rats markedly increases the turnover of glucose and its uptake into BAT, WAT, and skeletal and heart muscles (40). Because the serum insulin level was concurrently reduced, an increased insulin sensitivity of these tissues was proposed. Recently, Velloso and coworkers (19) gave a molecular basis for this insulin sensitivity regarding the BAT, via increased activity of IRS-2 and Akt. However, in muscle and WAT, the insulin-triggered molecular signaling events were impaired; thus, tissue-specific insulin signaling was postulated (19). The present work confirms these data obtained in BAT regarding Akt (Fig. 5) and completes it with the involvement of GSK-3. Our investigations into events occurring in the reacclimation period showed that in BAT there is marked activation of Akt (P-Akt) and inactivation of GSK-3 (P-GSK-3) (Fig. 5), both of which facilitate glycogen synthesis. By extending our study to skeletal muscle (Fig. 6), we were able to confirm the previous report (19) that in cold there were no changes in skeletal muscle. However, in the reacclimation period, the phosphorylation of Akt and GSK-3 increased markedly, indicating that enhanced insulin signaling during reacclimation is not restricted to BAT. This finding supports our earlier proposal concerning a "post-cold glycogen replenishment" in muscle (6).

A remaining question is how the glucose turnover could increase in cold-exposed rats while enhanced expression of insulin signaling members was observed only in BAT. The answer has to involve an insulin-independent mechanism, such as NE (41, 42). In fact, BAT has the benefit of both insulin-dependent and insulin-independent glucose uptake in cold, which together results in a 110-fold increase of uptake (40). These factors likely explain glycogen accumulation in BAT when the cold exposure is suddenly stopped. Glucose (glycogen) in BAT is a good substrate for thermogenesis and also for lipogenesis (15, 30). Indeed, the huge amount of glycogen accumulated in the BAT of reacclimated rats disappears when the animals are re-exposed to cold for 30 min (6).

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