

# Fatty Acid Transport Protein 1 Is Required for Nonshivering Thermogenesis in Brown Adipose Tissue

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**Nonshivering thermogenesis in brown adipose tissue (BAT) generates heat through the uncoupling of mitochondrial  $\beta$ -oxidation from ATP production. The principal energy source for this process is fatty acids that are either synthesized de novo in BAT or are imported from circulation. How uptake of fatty acids is mediated and regulated has remained unclear. Here, we show that fatty acid transport protein (FATP)1 is expressed on the plasma membrane of BAT and is upregulated in response to cold stimuli, concomitant with an increase in the rate of fatty acid uptake. In FATP1-null animals, basal fatty acid uptake is reduced and remains unchanged following cold exposure. As a consequence, FATP1 knockout (KO) animals display smaller lipid droplets in BAT and fail to defend their core body temperature at 4°C, despite elevated serum free fatty acid levels. Similarly, FATP1 is expressed by the BAT-derived cell line HIB-1B upon differentiation, and both fatty acid uptake and FATP1 protein levels are rapidly elevated following isoproterenol stimulation. Stimulation of fatty uptake by isoproterenol required both protein kinase A and mitogen-activated kinase signaling and is completely dependent on FATP1 expression, as small-hairpin RNA-mediated knock down of FATP1 abrogated the effect. *Diabetes* 55:3229–3237, 2006**

**O**besity and the plethora of associated diseases can be viewed as the direct result of an imbalance between energy intake and expenditure. Energy expenditure can drastically increase in response to lower environmental temperatures, and decreased thermogenesis has been linked to enhanced susceptibility to diet-induced obesity (1,2). Brown adipose tissue (BAT) is a major site for nonshivering thermogenesis in small mammals in response to both cold exposure and low protein diets. Most of the thermogenic capacity of

BAT is due to the presence of uncoupling protein (UCP)-1, an inner mitochondrial protein uniquely expressed in brown adipocytes (3). In contrast, the related proteins UCP-2 and UCP-3 are more widely expressed (rev. in 4). UCP-1 uncouples oxidative phosphorylation from the respiratory chain, causing energy dissipation as heat (3,5,6), and mice with a deletion of the *UCP-1* gene are cold intolerant (7). Similarly, mice lacking noradrenalin and adrenalin are unable to respond to a cold challenge (8), demonstrating that thermogenic activity is mainly regulated by the sympathetic nervous system, which heavily innervates BAT. Activation of  $\beta$ -adrenergic receptors ( $\beta$ -ARs) triggers a signaling cascade involving cAMP, protein kinase A (PKA), and mitogen-activated protein kinases (MAPKs), which ultimately results in enhanced UCP-1 expression and heat generation (9,10).

The main fuel for this heat generation in BAT is fatty acids. Maintenance of adequate stores of triglyceride is, therefore, essential for normal BAT function. BAT fatty acid stores are maintained 1) via uptake of free fatty acids (FFAs) from the circulation, 2) through the localized hydrolysis of lipoproteins by lipoprotein lipase, 3) via lipolysis of intracellular lipid droplets driven by hormone-sensitive lipase and adipose triglyceride lipase, and 4) via de novo synthesis by fatty acid synthetase (11). Activation of  $\beta$ -ARs following cold exposure increases serum fatty acid levels by stimulating hormone-sensitive lipase in white adipose tissue (WAT) and upregulation of lipoprotein lipase expression. As a consequence, the fatty acid composition of BAT triglycerides alters rapidly to closely reflect the composition of dietary lipids (12) indicative of rapid cellular uptake of serum FFAs. While mechanisms underlying enhanced glucose uptake by BAT following adrenergic stimulation have been studied intensely in the past (13–15), little is known regarding protein-mediated fatty acid uptake by this tissue.

Cellular fatty acid uptake has both a diffusional and a protein-mediated component (16), with the latter accounting for ~90% of the fatty acid uptake by WAT (17). Fatty acid transport proteins (FATPs) are integral transmembrane proteins (18) that enhance the uptake of long-chain fatty acid into cells (19). Human and mice FATPs comprise a family of six highly homologous proteins, FATP1–6, which are found in all major fatty acid-utilizing tissues (19–22). The scavenger receptor CD36 is also expressed by a number of lipid-metabolizing cell types, including white adipocytes, and has been linked to increased fatty acid uptake (23). However, neither FATP nor CD36 expression by BAT has been studied in detail.

Based on our previous observations that loss of FATP function can drastically reduce fatty acid uptake by WAT

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Received for publication 1 June 2006 and accepted in revised form 12 September 2006.

Additional information for this article can be found in an online appendix at <http://diabetes.diabetesjournals.org>.

$\beta$ -AR,  $\beta$ -adrenergic receptor; BAT, brown adipose tissue; FACS, fluorescence-activated cell sorter; FATP, fatty acid transport protein; FFA, free fatty acid; MAPK, mitogen-activated protein kinase; MEK, MAPK/extracellular signal-regulated kinase; PKA, protein kinase A; shRNA, small-hairpin RNA; UCP, uncoupling protein; WAT, white adipose tissue.

DOI: 10.2337/db06-0749

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(FATP1 knockout [KO] mice) (24) and liver (FATP5 KO mice) (25), we speculated that FATPs could also contribute to cold-induced fatty acid uptake by BAT. Here, we show that upon adrenergic stimulation, BAT increases fatty acid uptake and FATP1 expression, while other FATPs and CD36 are either not expressed or unchanged. Moreover, loss of FATP1 function prevents noradrenalin-induced FFA uptake in BAT, resulting in severely impaired nonshivering thermogenesis.

## RESEARCH DESIGN AND METHODS

Rabbit polyclonal antibodies against FATP1–6 were raised by immunizing rabbits as previously described (20,22,26). The anti-CD36 monoclonal antibody was a gift from Dr. Maria Febbraio. The anti-UCP-1 polyclonal antibody was purchased from Chemicon. Western blot analysis was performed as previously reported (20).

FATP1-null mice, on a mixed 129/Sv and C57BL/6J background, were generated and maintained as previously described (24). All cold exposures were done in the absence of food, unless otherwise noted.

**Body temperature measurements.** Mice were single caged and randomly divided into various groups as indicated. While one group was left at 24°C (ambient temperature), the other group of mice were transferred to a 4°C environment for 24 h. The colonic temperature was measured as an indication of core body temperature at 0, 1.5, 3, 6, 12, and 24 h, respectively. A lubricated thermistor probe (YSI 427; Yellow Springs Instruments) was inserted 2 cm into the rectum and held in place until a stabilized measurement was achieved. An average of three measurements was taken from each mouse at indicated time points. Baseline temperatures, before fasting, were collected from wild-type ( $n = 5$ ) and FATP1-null ( $n = 5$ ) mice. Mice were fasted for 24 h at 4°C. As a control, another group of mice were kept at 4°C for 24 h with free access to food (wild type,  $n = 5$ ; FATP1 null,  $n = 5$ ).

**Indirect calorimetry.** Basal oxygen consumption and carbon dioxide production and activity of individual mice were measured in a comprehensive lab animal monitoring system (Columbus Instruments, Columbus, OH) with an air flow of 0.5 l/min in the absence of food. They were determined at room temperature (25°C) and recorded every 10 min, for a period of 3 h after a single intraperitoneal injection of saline (basal rate) or CL316,243 (1 mg/kg body wt) (stimulated rate). To minimize variability, only values during periods of low locomotive activity were used for calculations. The values were normalized with respect to body weight, corrected according to an effective mass value, and the results were expressed as milliliter per kilogram per hour and then converted into percent increase over basal rate. Heat production was indirectly calculated, based on the observed respiratory exchange ratios.

**Serum analysis.** Serum samples were collected from wild-type and FATP1-null mice fasted overnight at 4°C by orbital bleeding of each mouse at time points 0, 3, 6, 12, and 24 h. Nonesterified fatty acid levels were determined colorimetrically (Wako Chemicals).

**Primary cell culture of brown adipocytes.** The interscapular BAT depots were dissected and trimmed of WAT under sterile conditions. Brown fat pads were then pooled, minced, and placed in 2 ml RIPM/2% BSA with 2 mg/ml type I collagenase (Worthington Biochemicals) for 10–15 min until cells were dissociated. Cells were washed twice in medium/BSA alone and allowed to settle in six-well plates.

**Localization of FATP1 and lipid droplets.** Mice were killed and their interscapular brown fat pads immediately removed, PBS washed, and immersed with cryopreservation medium (30% sucrose, 50% superblock, and 20% optical cutting temperature compound [OCT]). Immunofluorescence procedures of these tissue sections were carried out as previously described (20). Neutral lipid droplets were stained with BODIPY 493/503 (Molecular Probes).

**Cell culture and treatment.** HIB-1B brown preadipocytes were grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum with 1% penicillin/streptomycin. Differentiated cells were prepared as previously described (27). Cells were stimulated with the indicated compounds after a 4-h starvation in serum-free medium.

**Small-hairpin RNA expression constructs.** Oligonucleotides against FATP1 were designed as suggested (28,29) and did not share significant homology with other genes in the mouse genome. The HIB-1.5 cell line was created by transfection with the small-hairpin RNA (shRNA) expression vector pSUPERIOR.retro (OligoEngine) against the FATP1 target sequence (5'ggcgatgatgatcgctg3') and HIB-SCR cell line created by transfections with a scrambled shRNA target sequence (5'agggtgtcgcagtgcgagt3'), with no significant homology to any known gene sequences in the human, mouse, or rat genome. Stable expression was achieved through G418 (Invitrogen) selection at a final concentration of 750  $\mu$ g/ml.

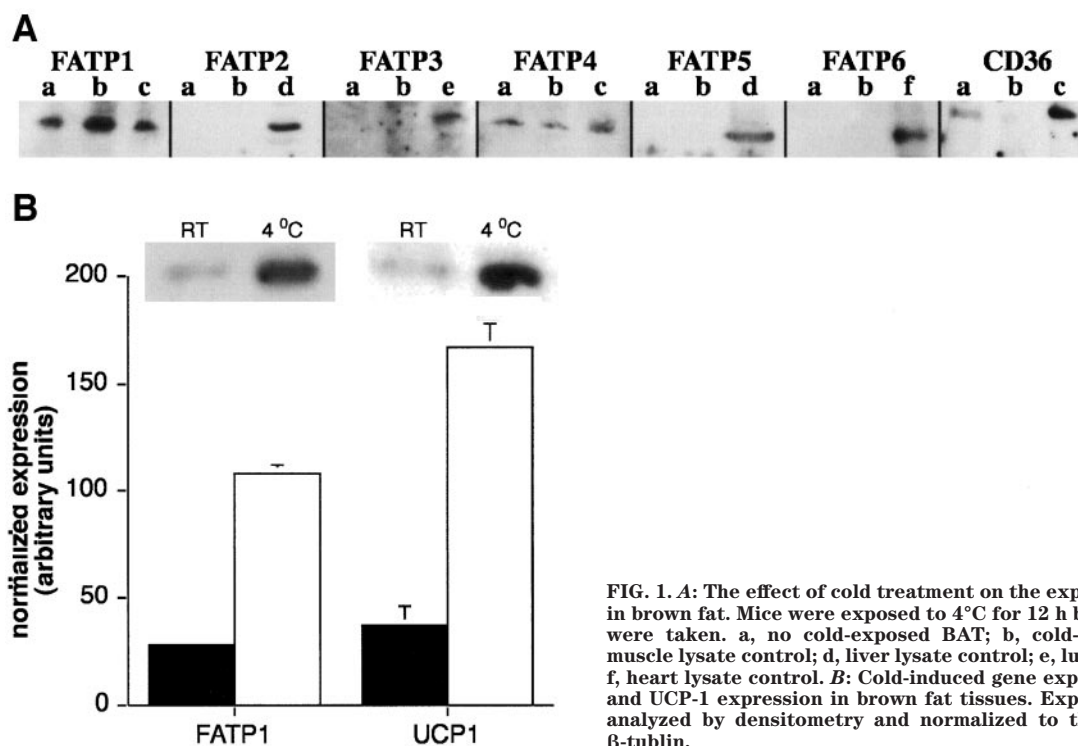
**Fatty acid uptake assays.** After removal of Dulbecco's modified Eagle's medium, cells were washed three times with serum-reduced medium for 10 min, followed by an incubation for 1 min at 37°C in fatty acid uptake solution containing 0.1  $\mu$ mol/l BODIPY-FA and 0.1% fatty acid-free BSA in Hank's balanced solution. Uptake was stopped by washing with ice-cold PBS/0.1% BSA solution, and cells were detached with trypsin/EDTA, resuspended in fluorescence-activated cell sorter (FACS) buffer including propidium iodide, and analyzed on a FACS calibur (Becton Dickinson), as previously described for 3T3 L1 adipocytes (20). Real-time fatty acid uptake assays were performed using the QBT kit (Molecular Device), as previously reported (30).

## RESULTS

**FATP1 is the major cold-inducible fatty acid transporter in BAT.** To determine which fatty acid transporters (FATPs and CD36) are present in BAT, we performed Western blot analyses of BAT lysates from mice kept either at ambient temperature or at 4°C for 12 h, using antibodies specific for FATP1–6 and CD36 (Fig. 1). As a positive control, we included tissue lysates from either WAT, liver, lung, skeletal muscle, or heart to validate negative results. FATP1 was robustly expressed by BAT and, importantly, strongly induced on cold stimulation (Fig. 1A). In contrast, FATP4 was expressed at lower levels and unaltered by cold exposure. All other FATPs were negative. CD36 was weakly expressed by BAT from mice kept at 24°C but, unexpectedly, absent from cold-induced BAT. The induction of FATP1 expression was rapid, as a 3.8-fold increase was observed after a 12-h cold challenge. In comparison, UCP-1 protein was induced 4.4-fold (Fig. 1B).

To study the subcellular localization of FATP1, BAT cryosections were coincubated with anti-FATP1 and anti-integrin  $\alpha 6$  antibodies. We used integrin  $\alpha 6$  as a marker for plasma membrane localization since it had been shown to be highly expressed on the cell surface of white adipocytes (31). Three-dimensional reconstructions of confocal microscopy optical sections showed that FATP1 is expressed throughout BAT and is clearly upregulated following a 12-h cold exposure (Fig. 2A). Using the same sensitivity settings, FATP1 was undetectable in FATP1-null BAT. Surprisingly, the integrin  $\alpha 6$  signal was also weaker both in cold exposed and normal (data not shown) FATP1-null BAT. At higher detector sensitivity settings outside the linear range of the BAT wild-type tissues, integrin  $\alpha 6$  signal was detectable on the plasma membranes of FATP1 KO BAT, while FATP1 was still negative (data not shown) and could not be detected by Western blot (Fig. 3). Comparison with the integrin  $\alpha 6$ -staining pattern, particularly at higher magnifications of cold-induced tissues (Fig. 2B), showed that FATP1 is mostly present on the plasma membrane. In addition, some FATP1 was observed intracellularly in smaller round organelles (Fig. 2B). The observed plasma membrane localization is consistent with the proposed role of FATP1 in FFA uptake from circulation.

**FATP1 is required for cold-induced FFA uptake by BAT.** Based on our observation that FATP1 is a major fatty acid transporter in BAT, we took advantage of the previously characterized FATP1-null mice (24,32) to determine FATP1-dependent alterations in BAT morphology and FFA uptake. We analyzed paraffin-embedded hematoxylin and eosin-stained BAT sections from both FATP1-null and wild-type mice at ambient temperature and after fasting/cold treatment for 12 h (Fig. 3A). At ambient temperature, BATs of wild-type and FATP1-null mice were filled with multilocular lipid droplets that, overall, appeared larger in wild-type mice (Fig. 3A). Preliminary

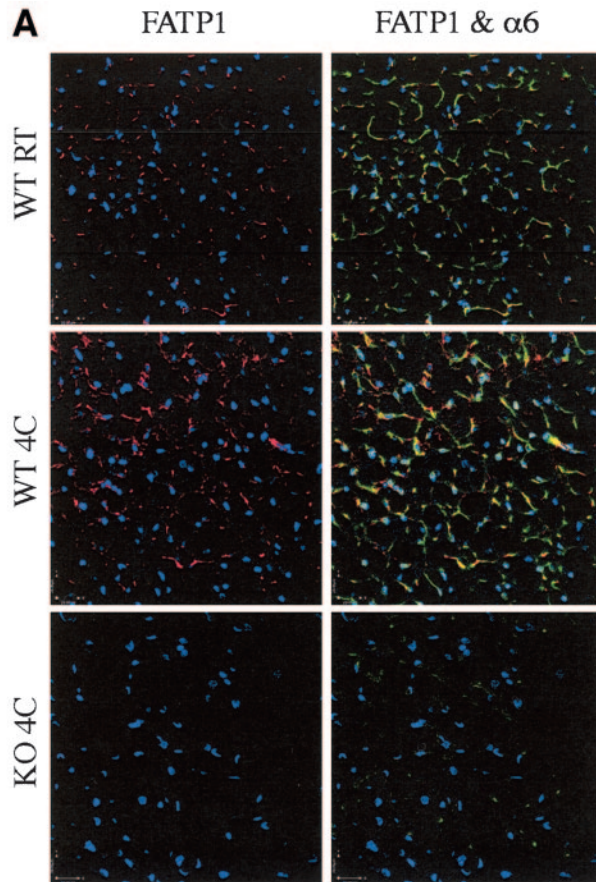


**FIG. 1. A:** The effect of cold treatment on the expression of FATPs in brown fat. Mice were exposed to 4°C for 12 h before their BATs were taken. a, no cold-exposed BAT; b, cold-exposed BAT; c, muscle lysate control; d, liver lysate control; e, lung lysate control; f, heart lysate control. **B:** Cold-induced gene expression of FATP1 and UCP-1 expression in brown fat tissues. Expression level was analyzed by densitometry and normalized to the expression of  $\beta$ -tubulin.

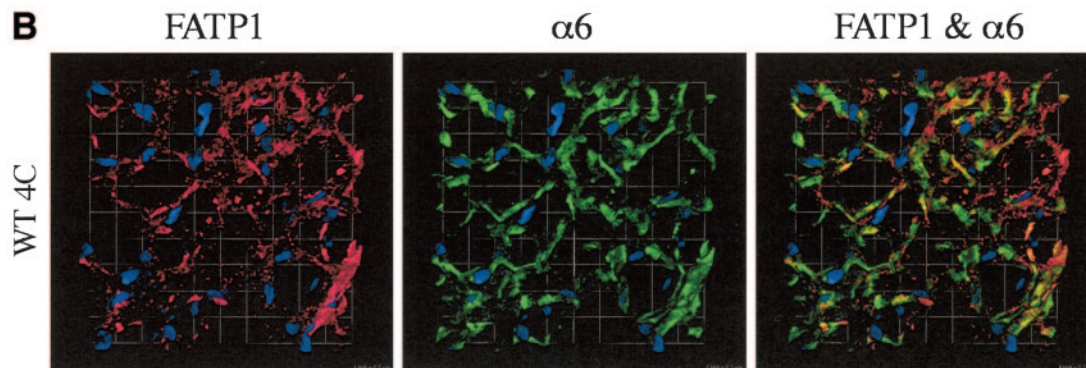
studies in WT C57B6 mice showed that BAT lipid droplet size increased after 6 h of cold exposure and reached a maximum after 12 h, followed by a marked decrease in size after 24 h of cold (supplemental Fig. 1 [available at <http://diabetes.diabetesjournals.org>]). Following a 12-h fasting/cold treatment, the adipocytes of wild-type mice were engorged with lipid droplets. In stark contrast, lipid droplets in the FATP1 KO BAT were unchanged or slightly reduced in size. To directly visualize lipid droplets, we stained BAT cryosections of FATP1-null and wild-type mice with the lipophilic BODIPY dye lipid probe followed by confocal microscopy (Fig. 3B). Three-dimensional reconstructions of the stained lipid droplets confirmed that FATP1-null brown adipocytes had smaller lipid droplets and that they failed to expand lipid droplet size in response to a cold challenge (Fig. 3B). Since this failure to expand the triglyceride pool size was indicative of a FFA uptake defect, we isolated brown adipocytes from FATP1 wild-type and KO animals housed for 12 h at ambient temperature or 4°C and measured FFA uptake directly using a FACS-based assay that we previously established for white adipocytes (24). Under basal conditions, FFA uptake by FATP1-null brown adipocytes was reduced by 35%. Importantly, under cold conditions, FFA uptake more than doubled in wild-type mice but remained unchanged in FATP1-null cells (Fig. 3C). This failure of FATP1-null BAT cells to respond to an adrenergic stimulus with increased FFA uptake was further illustrated by injecting FATP1 wild-type and KO animals with 0.5 mg/kg body wt norepinephrine intraperitoneally. Animals were killed 1 h after injection and brown adipocytes prepared. While the wild-type brown adipocytes responded with a robust 2.8-fold increase in FFA uptake, FATP1-null adipocytes were completely unaffected (Fig. 3C), indicating that cold-induced fatty acid uptake completely depends on FATP1 function. Importantly, induction of UCP-1 expression following cold exposure was unaffected by loss of FATP1 expression (Fig. 3D).

**FATP1-null animals fail to defend their core body temperature.** If the uptake of external fatty acids plays a significant role in the process of  $\beta$ -oxidation-driven ATP uncoupling and heat production by BAT, FATP1 KO mice should show impaired cold tolerance based on the reduced FFA uptake rates we observed. To address this question, we used direct calorimetry to determine core body temperatures of FATP1 wild-type and KO mice during a 24-h 4°C cold challenge in the presence or absence of food. Starting core body temperatures of FATP1 wild-type and KO mice were undistinguishable. However, under fasting conditions, we found that the core body temperature of FATP1-null mice fell precipitously to 23°C after a 24-h cold exposure, whereas sex- and age-matched wild-type littermates were able to maintain a significantly higher body temperature (30°C;  $P < 0.05$ , Student's *t* test) (Fig. 4A). When the mice were allowed free access to food during the cold exposure, temperature loss was decreased in both FATP1 wild-type and KO mice, and the two groups behaved more similarly during the initial phase of cold adaptation (Fig. 4A). However, after prolonged cold exposure (12- and 24-h time points), FATP1 KO mice were less efficient at defending their body temperature (12 h: 33.7 vs. 31.53°C; 24 h: 32.6 vs. 29.0°C) as shown in Fig. 4A.

Since FATP1 is expressed in other tissues besides BAT, most notably in muscle and WAT, the observed cold intolerance of FATP1 KO mice might potentially have been due to decreased release of FFA from WAT in response to adrenergic stimulation. This is either because the WAT depots are smaller or efflux of FFAs from WAT might be impaired. Although we have previously shown that FATP1 does not mediate efflux of fatty acids and that adipose depots of FATP1 KO animals fed a standard diet are morphologically normal (24), we wanted to directly exclude the possibility of decreased FFA mobilization. To this end, we determined serum FFA concentrations of FATP1 wild-type and KO animals during a 24-h cold exposure in the absence of food. In wild-type animals, serum FFAs quickly rose to 0.73 mmol/l after the first 6 h

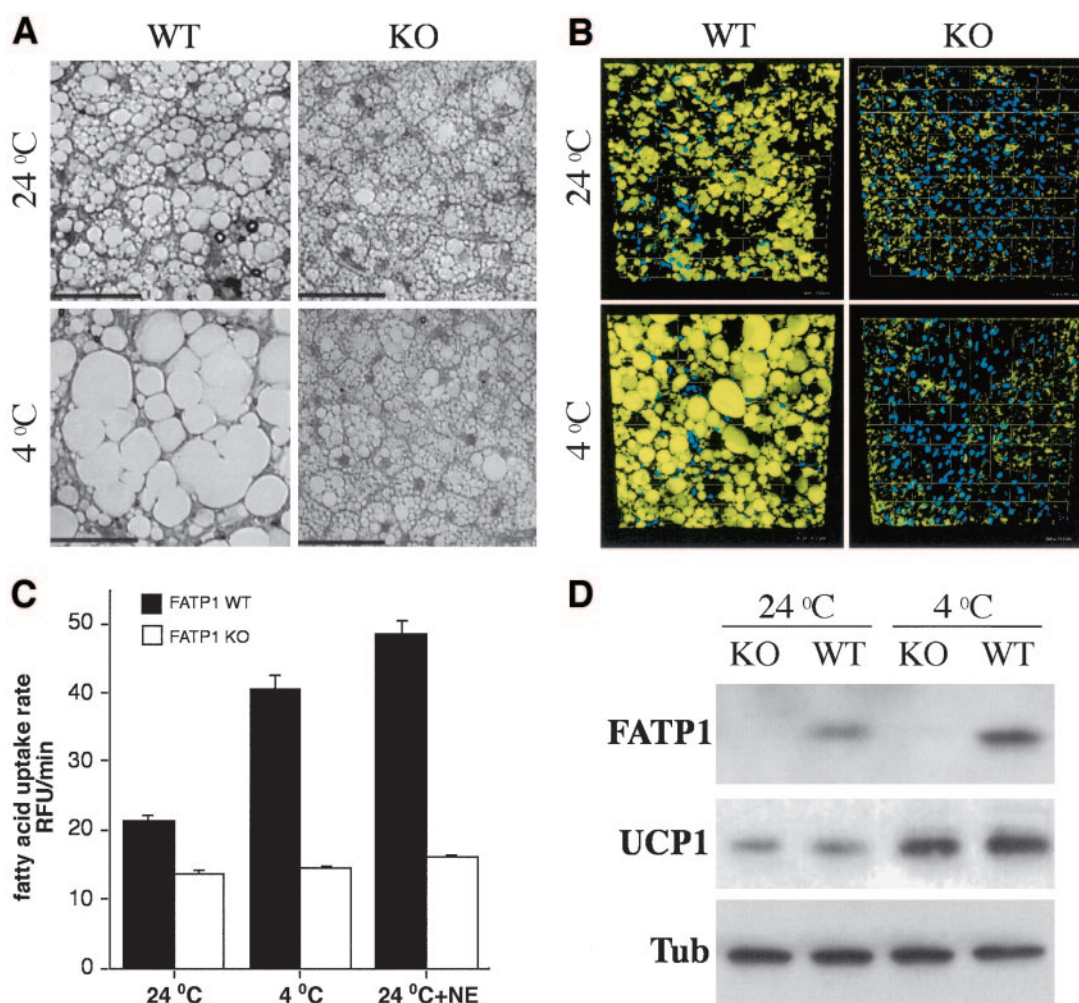


**FIG. 2.A:** Localization of FATP1 in brown fat. Frozen brown fat sections were paraformaldehyde fixed and incubated with anti-FATP1 antibody, followed by Cy5-tagged goat anti-rabbit secondary antibody, and analyzed by immunofluorescence microscopy. **B:** Three-dimensional surface projects of FATP1 (red), integrin  $\alpha 6$  (green), as well as their superimposition (right panels) of 15- $\mu\text{m}$  sections of BAT from wild-type (WT) mice at 4°C. Nuclei were stained with DAPI (4',6-diamidino-2-phenylindole) (blue).



of cold exposure and plateaued at this level (Fig. 4B). Serum FFA levels in FATP1 KO animals showed an equally rapid cold response, indicating that WAT lipolysis and FFA efflux are normal. In fact, serum FFA levels in the FATP1 KO mice continued to rise until reaching 0.9 mmol/l after a 24-h cold exposure (Fig. 4B), which is in line with the hypothesis that FFA uptake and consumption by BAT is impaired. To determine whether loss of FATP1 would also impair the initial thermogenic response to  $\beta$ -AR stimulation, male FATP1 wild-type and KO mice were injected with the  $\beta 3$  selective agonist CL316,243 at 1 mg/kg. Using metabolic cages, we recorded  $\text{O}_2$  consumption,  $\text{CO}_2$  production, and locomotion at basal state (without access to food) and over 1 h following CL316,243 injection. Data points at rest were used to calculate average  $\text{V}_{\text{O}_2}$ ,  $\text{V}_{\text{CO}_2}$ , and heat production rates (Fig. 4C). Wild-type mice responded to CL316,243 injection with a significant burst in metabolic activity, which was significantly reduced in FATP1 KO

animals (Fig. 4C), indicating that FATP1 is not only required for thermogenesis following prolonged cold exposure but also following acute  $\beta$ -adrenergic stimulation. **FATP1 is induced during brown adipocyte differentiation and facilitates FFA uptake following adrenergic stimulation.** While our results from isolated brown adipocytes demonstrated that FATP1 is required for norepinephrine-induced FFA uptake (Fig. 3C), these primary cells are difficult to manipulate. Thus, we took advantage of a pre-brown adipocyte cell line, HIB-1B, which can be readily differentiated into brown adipocyte-like cells (27), to further investigate the connection between adrenergic stimulation, altered FFA uptake, and FATP1 expression. HIB-1B preadipocytes showed a spindle-shaped morphology similar to fibroblast. Following differentiation with insulin, T3, indomethacin, isobutylmethlxanthine, and dexamethasone, confluent cells became smaller, rounded up, and accumulated fat. The differentiation process was



**FIG. 3.** Histology of brown fat shows lipid accumulation in adipocytes. **A:** Interscapular brown fat was removed from 8-week-old FATP1 KO and wild-type (WT) littermates. Paraffin-embedded sections were stained with hematoxylin and eosin. **B:** Three-dimensional reconstructions of frozen BAT section from FATP1 wild-type and KO mice stained with BODIPY fluorophore 493/503 for neutral lipids (yellow) and DAPI for nuclei (blue). One grid corresponds to 23  $\mu\text{mol/l}$ . **C:** Uptake of a fluorescently labeled long-chain fatty acid by primary brown adipocytes from FATP1 wild-type and KO animals following cold or 0.5 mg/kg body wt of norepinephrine (NE) treatment. **D:** Western blot analysis of FATP1 and UCP-1. The expression of the UCP-1 increase after exposure to cold in both wild-type and FATP1-null BAT pads. Level of  $\beta$ -tubulin (Tub) in BAT pads were used as a loading control.

paralleled by increased protein levels of UCP-1 and FATP1 (Fig. 5A). Both UCP-1 and FATP1 expression could be further stimulated by incubation of these differentiated cells with 10  $\mu\text{mol/l}$  isoproterenol (Fig. 5A). Using a real-time assay for fatty acid uptake (30), we were able to determine the initial uptake kinetics for isoproterenol-stimulated and unstimulated HIB-1B adipocytes. While uptake by unstimulated cells was linear, stimulated uptake was significantly faster and showed complex uptake kinetics (Fig. 5B). We utilized this assay to compare the effects of duration and concentration of the isoproterenol stimulus (Fig. 5C). Surprisingly, the isoproterenol effect was very rapid, with an optimum between 30 and 60 min. Since we had previously found that FATP1 in WAT could increase FFA uptake by dynamic translocation to plasma membrane, we wanted to determine whether the  $\beta$ -AR-induced FFA uptake in BAT was mainly due to transcriptional or posttranslational regulation. To this end, we inhibited transcription with 1  $\mu\text{mol/l}$   $\alpha$ -amanitin and translation with 1  $\mu\text{mol/l}$  NSC119889, corresponding to 10-fold half-maximal inhibitory concentration for each compound, while stimulating differentiated HIB-1B cells with 10  $\mu\text{mol/l}$  isoproterenol for 1 h. Transcriptional inhibition

reduced the isoproterenol effect by 70%, while targeting translation diminished the increase in FFA uptake by 80% (Fig. 5D). We expanded this pharmacological approach for an initial characterization of the signaling pathway leading from  $\beta$ -AR stimulation to increased FATP1 expression. Not surprisingly, inhibition of  $G_s$  with 2 nmol/l pertussis toxin resulted in an 80% reduction of the isoproterenol effect, while coincubation of isoproterenol with 10 nmol/l PKA inhibitor (H89) resulted in a 76% reduction (Fig. 5D). Signaling through the MAPK cascade has recently been shown to be required, together with PKA activation, for the induction of UCP-1 by adrenergic agonists (9). Interestingly, MAPK signaling seems also to be required for increased FFA uptake, as the 10 nmol/l MEK (MAPK/extracellular signal-regulated kinase) II inhibitor (U0126) reduced the isoproterenol effect to a similar extent (78%) as the PKA inhibitor (Fig. 5D). Throughout these experiments, FATP1 expression closely followed the observed uptake rates (Fig. 5D).

To functionally link isoproterenol-stimulated fatty acid uptake and FATP1 expression in HIB-1B cells, we created stable clones expressing FATP1-specific shRNAs (HIB-1.5) or scrambled shRNAs (HIB-SCR) as controls. In differen-

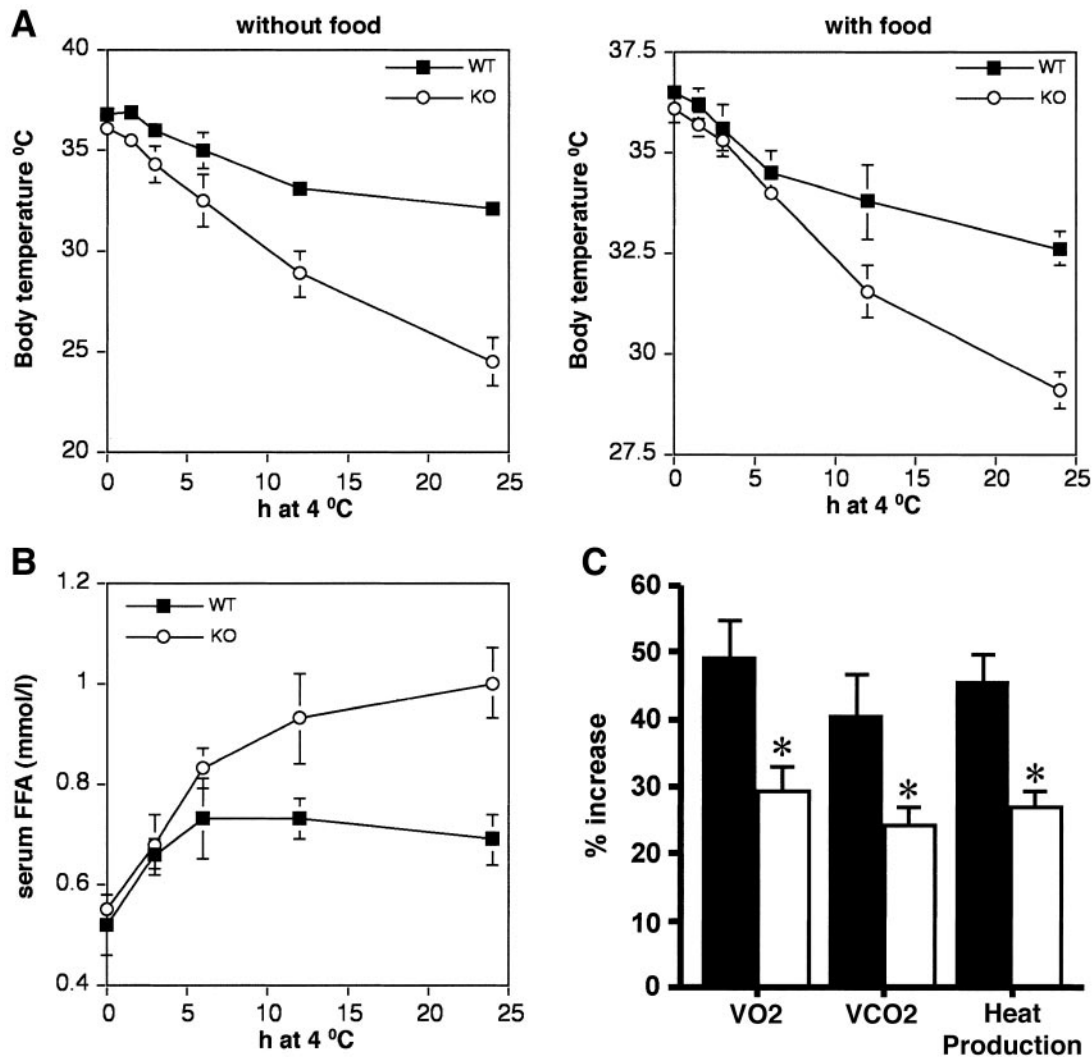


FIG. 4. FATP-null mice are unable to maintain core body temperature during fasting and cold exposure. *A*: Colonic temperature was recorded at 1, 3, 6, 12, and 24 h of fasting mice in cold treatment; *right panel* is the reading of fed mice in cold treatment. *B*: Levels of serum nonesterified fatty acids in both wild-type (WT) and FATP1-null mice in response to the cold exposure. *C*: Indirect calorimetry comparing the increases in O<sub>2</sub> consumption and CO<sub>2</sub> production rates during a 1-h phase, following injection of the  $\beta$ -AR agonist CL316,243 in FATP1 KO mice ( $\square$ ) and wild-type littermates ( $\blacksquare$ ). \* $P < 0.05$  in paired Student's *t* test.

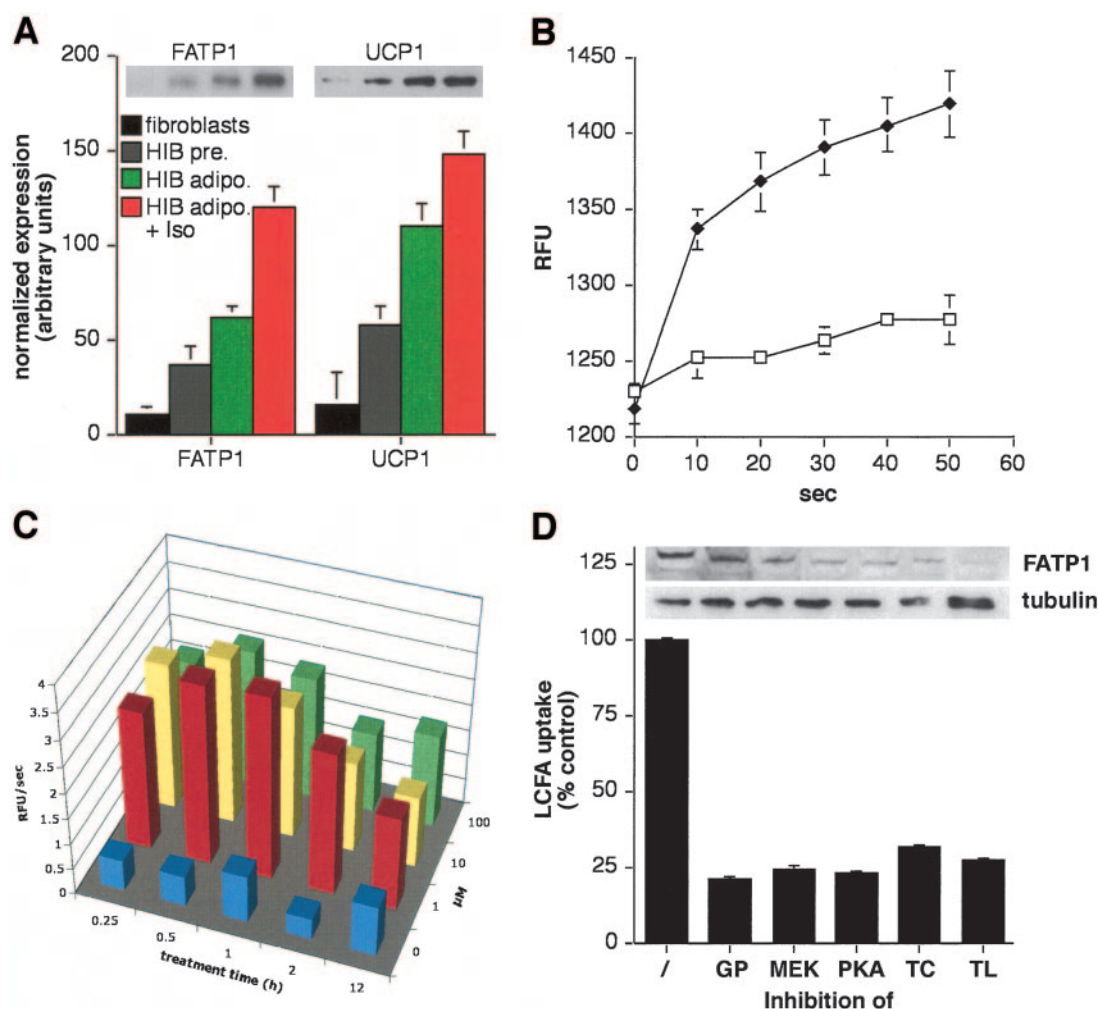
tiated HIB-1B cells, 10  $\mu$ mol/l isoproterenol and supra-physiological concentrations at 50 and 100 nmol/l insulin, but not 1 nmol/l insulin, stimulated both FATP1 expression and fatty acid uptake following a 1-h incubation (Fig. 6). Addition of both 100 nmol/l insulin and 10  $\mu$ mol/l isoproterenol did not have an additive effect but rather suppressed FATP1 expression and fatty acid uptake below the levels observed for the individual treatments (Fig. 6). In HIB-SCR cells, the same pattern of FATP1 and fatty acid uptake induction was observed, albeit at a slightly lower magnitude, possibly as a result of the G418 selection. In contrast, HIB-1.5 cells failed to increase FATP1 expression in response to insulin and isoproterenol and, subsequently, showed a lack of increased fatty acid uptake (Fig. 6). This demonstrates that both the insulin and isoproterenol effects on fatty acid uptake by BAT-like cells completely depend on FATP1 induction.

## DISCUSSION

While it was known that adrenergic stimulation of BAT and brown adipocytes not only enhanced glucose uptake (14,15,33,34) and  $\beta$ -oxidation rates (35,36), we found that

it also increases the cellular uptake of FFAs by this tissue. This is in clear contrast to WAT, where the same signal leads to a net efflux of fatty acids (37,38). We propose that the molecular mechanism for this previously unappreciated effect lies in the induction of FATP1 expression in BAT in response to  $\beta$ -AR stimulation, since FATP1 KO mice have normal UCP-1 expression but reduced FFA uptake rates in BAT. Further, knock down of FATP1 in HIB-1B cells abrogates the effects of isoproterenol on FFA uptake. Increased FATP1 activity is mainly due to increased protein levels, which rise quickly after cold or isoproterenol stimulation. Since the effect on fatty acid uptake was very rapid (30–60 min), other FATP1-dependent activation mechanisms may also be responsible. Additional mechanisms could include FATP1 translocation, particularly since insulin-induced translocation of FATP1 in WAT and skeletal muscle has been shown to mediate the postprandial uptake of dietary lipids into these tissues (24). Clearly, more detailed investigations of the mechanism by which FATP1 increases BAT FFA uptake will be required and are currently underway.

Loss of FATP1 in WAT had no effect on mobilization of

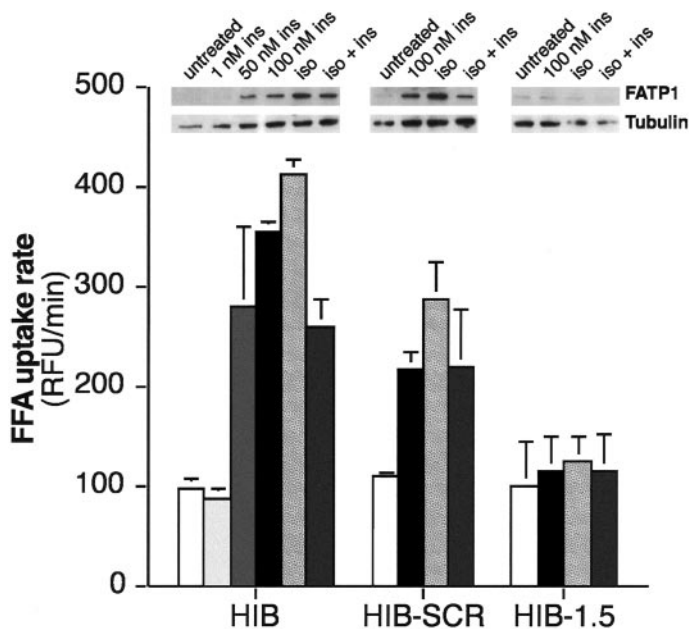


**FIG. 5.** Effect of isoproterenol on long-chain fatty acid uptake. **A:** Expression of FATP1 and UCP-1 in cultured cells. Fibroblasts 3T3-L1 (black bar), HIB-1B preadipocytes (gray bar), HIB-1B adipocytes (green bar), and HIB-1B adipocytes + isoproterenol are indicated. Isoproterenol (10  $\mu\text{mol/l}$ ) was added into the medium 1 h before harvesting the cells for Western blot. Level of expression was analyzed by densitometry and normalized to the expression of  $\beta$ -tubulin. **B:** Comparison of kinetic readings of long-chain fatty acid uptake by HIB adipocytes.  $\square$ , untreated cells;  $\blacklozenge$ , 10  $\mu\text{mol/l}$  isoproterenol treated cells. **C:** Effects of duration and dose of isoproterenol stimulus on initial uptake of long-chain fatty acid in HIB adipocytes. **D:** Fatty acid uptake 1 h after a 10  $\mu\text{mol/l}$  isoproterenol stimulation and FATP1 expression. /, control; GP, Gs inhibitor (2 nmol/l pertussis toxin); MEK, MEK II inhibitor (10 nmol/l U0126); PKA, PKA inhibitor (10 nmol/l H89); TC, transcription inhibitor (1  $\mu\text{mol/l}$   $\alpha$ -amanitin); TL, translation inhibitor (1  $\mu\text{mol/l}$  NSC119889).

FFA from this tissue in response to cold stimulus, as FATP1-null mice have increased rather than reduced serum FFA levels, and FATP1 seems to not be involved in FFA efflux (24). This is in contrast to another model of impaired nonshivering thermogenesis, the caveolin-1 KO mice (39). Loss of caveolin-1 results in a loss of lipolytic activity, both in BAT and WAT, leading to lower serum FFA levels and increased BAT triglyceride accumulation (39). While isoproterenol-stimulated FFA uptake was completely dependent on FATP1, basal uptake was less affected by FATP1 loss and may be mediated by CD36 or FATP4. However, this hypothesis has not been tested in vivo, as no defects in thermogenesis have been reported for CD36-null mice (40), and homozygote loss of FATP4 leads to embryonic (41) or neonatal (42) lethality.

FATP1 KO mice display defective thermogenesis when exposed to a cold challenge, which is most likely a result of impaired FFA uptake by BAT. Another compounding factor may be that FATP1-null mice have reduced epididymal fat stores (24) and potentially decreased cold insulation. Interestingly, temperature loss in FATP1 KO mice is more prominent following prolonged cold exposure,

which is in line with the notion that initial thermogenesis in FATP1-null BAT relies on intracellular lipid stores from basal FFA uptake and de novo synthesis. Once internal stores are depleted,  $\beta$ -oxidation rates are depressed and thermogenesis is less efficient despite normal UCP-1 levels. However, one has to note that FATP1 is not irrelevant during the initial phase of thermogenesis, as FATP1 KO animals had markedly decreased rates of  $\text{O}_2$  consumption and  $\text{CO}_2$  production immediately following injection of CL316,243. While other groups have found that BAT lipid droplets are unchanged after a 6-h cold exposure, but significantly decreased after 72 h (43), we found that droplet size increased after 6 h of cold exposure and reached a maximum after 12 h, followed by a marked decrease in size after 24 h of cold (supplemental Fig. 1). Our interpretation of this data are that during the initial phase of cold exposure, serum FFA levels increase rapidly due to increased lipolysis in WAT and increased FATP1-mediated uptake of serum FFAs by BAT. This increase exceeds the "burn rate" of fat in BAT, leading to a net accumulation of lipids. Following prolonged cold exposure, WAT depots are starting to be depleted, causing a



**FIG. 6.** Effects of FATP1 RNA interference (RNAi) on FATP1 expression and fatty acid uptake in HIB-1B cells. *Upper panel:* Expression of FATP1 was analyzed by Western blot analysis in HIB, HIB-SCR (scrambled RNAi), and HIB-1.5 (FATP1 RNAi) cell lines. These cells were incubated with solvent (basal), 1 nmol/l insulin, 50 nmol/l insulin, 100 nmol/l insulin (ins), 10  $\mu$ mol/l isoproterenol (iso), and both (iso+ins) for 1 h. *Lower panel:* Stimulation of adrenergic agonist and insulin induce increased uptake fatty acid in HIB cells, and this effect was suppressed by RNA interference in the stable cell line of FATP1 RNAi. HIB cell lines were incubated in the presence of insulin, isoproterenol, or both at indicated concentration for 1 h and then assayed for fatty acid transport into the cells as described in RESEARCH DESIGN AND METHODS. Data are representative of three independent experiments.

drop in serum FFA levels (Fig. 4B), and tip the balance in BAT toward decreasing lipid stores. The time this switch, from increasing to decreasing lipid droplets, occurs may critically depend on the WAT mass of the mouse and its age, sex, and possibly other currently unknown factors. This notion would also explain why lipid stores in the age- and sex-matched FATP1-null BAT shrank, rather than expanded, in response to a prolonged cold challenge. However, since fatty acids are required for UCP-1 function (44,45), uncoupling may also be impaired as a result of diminished intracellular FFA levels.

Despite their defects in adaptive thermogenesis, FATP1 KO mice are not obese but rather protected from high-fat diet-induced weight gain and insulin desensitization (24). Interestingly, mice lacking noradrenalin and adrenaline or UCP-1 are also cold intolerant but not obese, which has been ascribed to an increase in their basal metabolic rate (8) and increased UCP-2 expression (7), respectively.

Using HIB-1B cells, we were able to reproduce our key findings that  $\beta$ -AR stimulation induces FATP1 expression and fatty acid uptake and that the latter process completely relies on FATP1 function. Since supraphysiological levels of insulin had been reported to induce UCP-1 expression (46,47), we want to reveal if the same would hold true for FATP1 regulation. Indeed, high levels of insulin (50–100 nmol/l) were able to stimulate FFA uptake in HIB-1B cells. As with isoproterenol, the insulin effect completely relied on the induction of FATP1 to facilitate the increased uptake rates. While both insulin and adrenergic signals resulted in upregulation of FATP1 protein levels, their effects were not additive but rather interfered

with each other, possibly by insulin disrupting  $\beta$ -AR signaling to PKA (48). Similarly, UCP-1 has also been shown to be differentially regulated by insulin and  $\beta$ -AR (47). Further similarities in the regulation of the FATP1 and UCP-1 include a peroxisome proliferator-activated receptor binding site (49,50) in their promoter regions and the requirement for PKA and MAPK signaling in conjunction with promoter expression (9) (Fig. 5D). Clearly, a more detailed analysis of the FATP1 promoter is warranted to explain the rapid induction of FATP1 protein by adrenergic stimuli and high insulin, particularly since insulin reportedly suppresses FATP1 mRNA levels (51).

Taken together, our observations highlight the role of exogenous FFA uptake by BAT from circulation for heat production and establish FATP1 KO mice as a new model for deficient nonshivering thermogenesis.

#### ACKNOWLEDGMENTS

This work was supported by grants to A.S. from the National Institutes of Health (NIH), National Institute of Diabetes and Digestive and Kidney Diseases (NIH DK066336-01), and a Career Development Award from the American Diabetes Association.

We thank Dr. Bruce Spiegelman for providing the HIB-1B cell line and Dr. Febbraio for providing the mouse CD36 antibody. We also thank the staff of the Stanford Imaging Center and the Palo Alto Medical Foundation Research Institute (PAMFRI) animal facility for their support and advice and Rosemary Grammer for proofreading the manuscript.

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