Adenosine 5′-Monophosphate-Activated Protein Kinase-Mammalian Target of Rapamycin Cross Talk Regulates Brown Adipocyte Differentiation

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Brown adipose tissue (BAT) is considered of metabolic significance in mammalian physiology, because it plays an important role in regulating energy balance. Alterations in this tissue have been associated with obesity and type 2 diabetes. The molecular mechanisms modulating brown adipocyte differentiation are not fully understood. Using a murine brown preadipocyte cell line, primary cultures, and 3T3-L1 cells, we analyzed the contribution of various intracellular signaling pathways to adipogenic and thermogenic programs. Sequential activation of p38MAPK and LKB1-AMPK-tuberous sclerosis complex 2 (TSC2) as well as significant attenuation of ERK1/2 and mammalian target of rapamycin (mTOR)-p70 S6 kinase 1 (p70S6K1) activation was observed through the brown differentiation process. This study demonstrates a critical role for AMPK in controlling the mTOR-p70S6K1 signaling cascade in brown but not in 3T3-L1 adipocytes. We observed that mTOR activity is essential in the first stages of differentiation. Nevertheless, subsequent inhibition of this cascade by AMPK activation is also necessary at later stages. An *in vivo* study showed that prolonged 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR)-induced AMPK activation increases uncoupling protein 1 expression and induces an accumulation of brown adipocytes in white adipose tissue (WAT), as revealed by immunohistology. Moreover, the induction of brown adipogenesis in areas of white fat partially correlates with the body weight reduction detected in response to treatment with AICAR. Taken together, our study reveals that differentiation of brown adipocytes employs different signaling pathways from white adipocytes, with AMPK-mTOR cross talk a central mediator of this process. Promotion of BAT development in WAT by pharmacological activation of AMPK may have potential in treating obesity by acting on energy dissipation. **(*Endocrinology* 151: 980–992, 2010)**

Brown adipose tissue (BAT), a thermogenic organ, is present in most mammals and is responsible for cold-induced nonshivering thermogenesis (as reviewed in Refs. 1 and 2). The unique thermogenic capacity of BAT results from the expression of the uncoupling protein 1 (UCP1) in the mitochondrial inner membrane (3). Unlike white adipose tissue (WAT), BAT accumulates lipids as a source of fatty acids (FA) to be oxidized in mitochondria when thermogenesis is activated to produce heat (4). Furthermore, *de novo* lipid synthesis also occurs in BAT with insulin as an essential lipogenic regulator (5). Most fat depots can be characterized as either brown or white, but some brown fat cells can also be found dispersed through white fat depots in rodents and humans (6, 7). Until quite recently, BAT was thought to be of metabolic importance only in small mammals and infant humans. Recent studies using

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Abbreviations: ACC, Acetyl-coenzyme A carboxylase; AICAR, 5-aminoimidazole-4-carboxamide ribonucleoside; AMPK, AMP-activated protein kinase; aP2, adipocyte fatty acid-binding protein 2; BAT, brown adipose tissue; C/EBP, C/AAT enhancer binding protein; Cyt, cytochrome; FA, fatty acids; FAS, fatty acid synthase; FS, fetal serum; GLUT1, glucose transporter 1; HSL, hormone-sensitive lipase; IR, insulin receptor; IRS, IR substrate; LTAg, large T antigen; mTOR, mammalian target of rapamycin; PGC-1α, PPAR coactivator 1α; PI3K, phosphoinositide 3-kinase; PPAR, peroxisome proliferator-activated receptor; p70S6K, p70 S6 kinase 1; RXRα, retinoic X receptor-α; siRNA, small interfering RNA; TSC2, tuberous sclerosis complex 2; UCP1, uncoupling protein 1; WAT, white adipose tissue.

positron-emission tomography scanning, however, suggest that adult humans have several discrete areas of metabolically active BAT (8–10). In this regard, BAT may have a much more important role in human metabolism than was previously thought. Loss of BAT function is linked to obesity and metabolic disease (11), whereas experimental increases of BAT in animals have been associated with a lean and healthy phenotype (12, 13). Thus, promotion of BAT development in humans offers the possibility of increasing energy expenditure. Nevertheless, development of such therapeutics requires significant understanding of the molecular mechanisms controlling the formation of brown fat cells.

Brown adipocytes are derived from mesenchymal stem cells, and development of BAT occurs late in embryonic life (14). Although the brown adipogenesis program has not been as extensively studied as that of white, the process of differentiating into brown or white adipocytes appears to employ a similar canonical transcriptional pattern. Along these lines, several nuclear factors regulate some processes that are common to both cell types, including lipogenesis and insulin-dependent glucose transport (15). The most notable are the C/AAT enhancer binding protein (C/EBP) family members α, β, and δ and the peroxisome proliferator-activated receptor (PPAR)-γ, considered as a master regulator of the adipogenic process. Nevertheless, recent studies demonstrate that brown preadipocytes express myoblast markers, suggesting a distinct origin for BAT *vs.* WAT (16). It has been reported that transcription factors such as PRDM16 may control brown fat determination (17). Upstream signals regulating the expression and activation of these transcription factors during adipocyte differentiation are not fully understood. The phosphoinositide 3-kinase (PI3K) pathway appears to be required for the differentiation of 3T3-L1 adipocytes (18). In contrast, MAPK pathways differentially regulate adipogenesis. Although ERK1/2 displays both positive and negative effects through the process, the role of p38MAPK seems to be related to UCP1 expression in brown adipocytes (19), but is still controversial in white (as reviewed in Ref. 20). Several studies suggest an inhibitory role for the AMP-activated protein kinase (AMPK) in the adipogenic process (21, 22). Although AMPK was traditionally viewed as a sensor of the energetic status of the cell, recent discoveries revealed that tumor suppressor LKB1 and tuberous sclerosis complex 2 (TSC2) lie upstream and downstream of AMPK, respectively, indicating that this kinase may be equally important in the regulation of cell growth and proliferation. It is well known that growth factors inhibit TSC2 activity by an AKT-dependent mechanism. This inhibition results in positive signaling to the mammalian target of rapamycin (mTOR), stimulating protein synthesis and cell growth via multiple mechanisms, including phosphorylation of the protein kinase p70 S6 kinase 1 (p70S6K1) (23, 24). In contrast, the LKB1-AMPK cascade induces TSC2 phosphorylation at two sites distinct from the AKT sites, promoting TSC2 activation and consequently mTOR inhibition (25). More recently, it has also been reported that the Wnt pathway is able to inhibit TSC2 and stimulate mTOR (26). In this regard, Wnt activation negatively regulates adipogenesis, with β-catenin as a crucial regulator of this process (27).

Accordingly, we investigated the contribution of MAPKs, LKB1-AMPK, mTOR-p70S6K1, and Wnt signaling cascades to brown and white adipocyte differentiation. Our data identify AMPK as a key regulator of the brown differentiation program that controls the mTOR-p70S6K1 signaling pathway and UCP1 expression.

Materials and Methods

Materials

Dibutyryl cAMP, insulin, rosiglitazone, CL361243, and anti-β-actin antibody were from Sigma-Aldrich (St. Louis, MO). PD98059 and compound C were from Calbiochem-Novabiochem (La Jolla, CA). SB202190 and rapamycin were from Alexis (Lausen, Switzerland). Iodotubercidin was from Biomol Research Laboratories (Plymouth Meeting, PA). 5-Aminoimidazole-4-carboxamide ribonucleoside (AICAR) was purchased from Toronto Research Chemicals (Ontario, Canada). Culture media and sera were from Invitrogen (Paisley, UK). Autoradiographic films, 2-deoxy-D[1-^{3}H]glucose and 9,10-[^{3}H]palmitate were from GE Healthcare (Rainham, UK). Antibodies against glucose transporter 1 (GLUT1) and GLUT4 were from Chemicon (Temecula, CA). Hormone-sensitive lipase (HSL) and perilipins were from Abcam (Cambridge, UK); active β-catenin from Millipore (Billerica, MA); total and phosphorylated AMPKα(T172), (ERK)1/2(T202/Y204), mTOR(S2448), p38MAPK(T180/Y182), p70S6K1(T389), p70S6K1(T421/S424), TSC2(T1462), and glyceraldehyde-3-phosphate dehydrogenase from Cell Signaling (Beverly, MA); insulin receptor (IR) substrate 1 (IRS-1), IRS-2, and total and phosphorylated acetyl-coenzyme A carboxylase (ACC)(S79) from Upstate Biotechnology (Lake Placid, NY); C/EBPα and -β, IRβ, phosphorylated LKB1(S431) (sc-28465), PPAR coactivator 1α (PGC-1α) (sc-13067), PPARγ (sc-7196), retinoic X receptor α (RXRα) (sc-553), and UCP1 (sc-6529) from Santa Cruz Biotechnology (Palo Alto, CA); and total β-catenin and fatty acid synthase (FAS) from BD Transduction Laboratories (Lexington, KY). All other reagents used were of the purest grade available.

Cell culture and treatments

Fetal brown adipocytes were obtained from interscapular BAT of 20-d-old Wistar rat fetuses and isolated by collagenase dispersion as previously described (28). Isolated cells were plated in tissue culture dishes in MEM supplemented with 10% fetal serum (FS). Immortalization was performed as described (29). Briefly, fetal brown adipocytes were infected with the puromy-

cin-resistant retroviral vector pBabe encoding simian virus 40 large T antigen (LTAg) and selected with puromycin (0.5–1 mg/ml) for 1–2 wk. Several clones were obtained. The immortalized cell lines were maintained in DMEM supplemented with 10% FS and antibiotics, at 37 C and 5% CO_2. To induce differentiation, cells were cultured for 10 d in 10% FS-DMEM supplemented with 1 nM insulin, 3 μM rosiglitazone, 100 μM isobutylmethylxanthine, and 1 μM dexamethasone. 3T3-L1 preadipocytes were cultured in DMEM containing 10% FS. On d 0, cells were induced to differentiate by 50 nM insulin, 3 μM rosiglitazone, 0.5 mM isobutylmethylxanthine, and 1 μM dexamethasone. On d 2, the induction medium was substituted by maintenance medium, supplemented with 50 nM insulin and 3 μM rosiglitazone. Fresh maintenance medium was added every 3 d until d 10.

Analysis of intracellular lipids

Accumulation of triglycerides was visualized by staining the cells with Oil Red O or Nile Red dye (Sigma-Aldrich). Cells were fixed in formalin solution 10% (Sigma-Aldrich) for 30 min and stained either with Oil Red O for 45 min or with Nile Red for 10 min. After staining, dishes were washed and photographed. Oil Red O was quantified spectrophotometrically at 520 nm. Quantification of Nile Red was performed using the Tecan infinite 200 microplate reader (excitation 488 nm, emission 528 nm).

Cell size measurement

Cell size analysis was performed by flow cytometry. Cells were trypsinized, counted, washed with PBS, and fixed with cold ethanol (70%). Then they were resuspended in PBS (10^6 cells/ml), treated with ribonuclease (Roche Diagnostics, Indianapolis, IN) for 30 min at 37 C, and analyzed in the cytometer.

Glucose transport determination

Cells were cultured overnight in serum-free, low-glucose (1000 mg/liter glucose) DMEM supplemented with 0.2% (wt/vol) BSA and further stimulated or not for 30 min with insulin at different doses. Glucose uptake was measured during the last 10 min of culture by incorporation of 2-deoxy-D[1-^{3}H]glucose, as previously described (28). Results are expressed as the percentage of stimulation over basal (control = 100).

Determination of lipolytic rate

Rate of lipolysis was measured by following the rate of glycerol release from the cells. Adipocytes were cultured overnight in serum-free medium supplemented with 0.2% (wt/vol) BSA before stimulation. After washing, cells were incubated at 37 C for 3 h, and glycerol released was assayed by enzymatic method using a free-glycerol determination kit (Sigma-Aldrich). Results are expressed as percentage of stimulation over basal (control = 100).

Fatty acid oxidation

The oxidation capacity of 9,10-[^{3}H]palmitate was assessed by measuring ^3H_2O produced in the incubation medium. Excess of [^{3}H]palmitate was removed by precipitation, and the supernatants were extracted by addition of methanol/chloroform (2:1) and KCl/HCl (2 M) followed by centrifugation. An aliquot from the aqueous phase was taken for counting. Results are expressed as percentage of stimulation over basal (control = 100).

Immunoblot analysis

Cellular proteins were submitted to SDS-PAGE, transferred to Immobilon membranes, and blocked (28). Immunoreactive bands were visualized using the enhanced chemiluminescence (ECL-Plus) Western blot protocol (GE Healthcare).

Transient transfection with small interfering RNA (siRNA)

Rat mTOR, AMPKα1 and -α2, and control (scrambled) siRNAs were purchased from Dharmacon (Lafayette, CO) and transfected using Dharmafect 3 reagent according to manufacturer's instructions. Brown adipocytes were transfected with 100 nM control and either AMPKα1 or -α2 or mTOR siRNA. The following day, cells were induced to differentiate. After 72 h, lysates were collected and further analyzed to verify transfection efficiency. To ensure AMPK silencing through the differentiation process, brown adipocytes were transfected again with AMPKα1 and -α2 siRNAs after 72 h. Cells were harvested after 7 d.

Administration of AICAR *in vivo*

To study the effect of AICAR *in vivo*, 8-wk-old mice were treated with AICAR (0.5 mg/g body weight) dissolved in DMSO and NaCl 0.9% (1:2) (ip injection, volume of 300 μl/mouse) three times a week for 2 wk. Mice in the control group received 300 μl of vehicle. Five animals were used for each group. Body weight and food intake were measured periodically. In the pair-fed group, animals were fed the same amount of food as that consumed by the AICAR-treated animals over the preceding 24 h. This was divided into two meals and given at 0800 h and 1800 h to avoid long durations of fasting. After treatment, animals were killed and adipose tissues harvested and processed for subsequent analysis. The local ethics committee approved the study, and all procedures were performed in accordance with the Federation of European Laboratory Animal Science.

Histology and immunohistochemistry

Freshly isolated fat depots were fixed in 10% formalin and embedded in paraffin. Different sections (5 μm) were deparaffinized and rehydrated, and 10 mM sodium citrate (pH 6.0) was used for antigen retrieval. Immunohistochemical detection of UCP1 was performed as follows: 1) 3% hydrogen peroxide in methanol for 10 min, 2) incubation for 1 h at room temperature with the antiserum diluted 1:100, 3) staining procedure according to Dako (Carpinteria, CA) instructions, and 4) counterstain with hematoxylin and coverslip with DPX Mountant (Sigma-Aldrich). For each treatment, a negative control without the primary antibody was used. Different high-power fields from each different section were analyzed for each individual adipose depot. BAT was used as a positive control for UCP1 immunoreactivity. UCP-1 antibody was kindly provided by Dr. Jan Kopecky (Academy of Sciences of the Czech Republic, Prague, Czech Republic) (13).

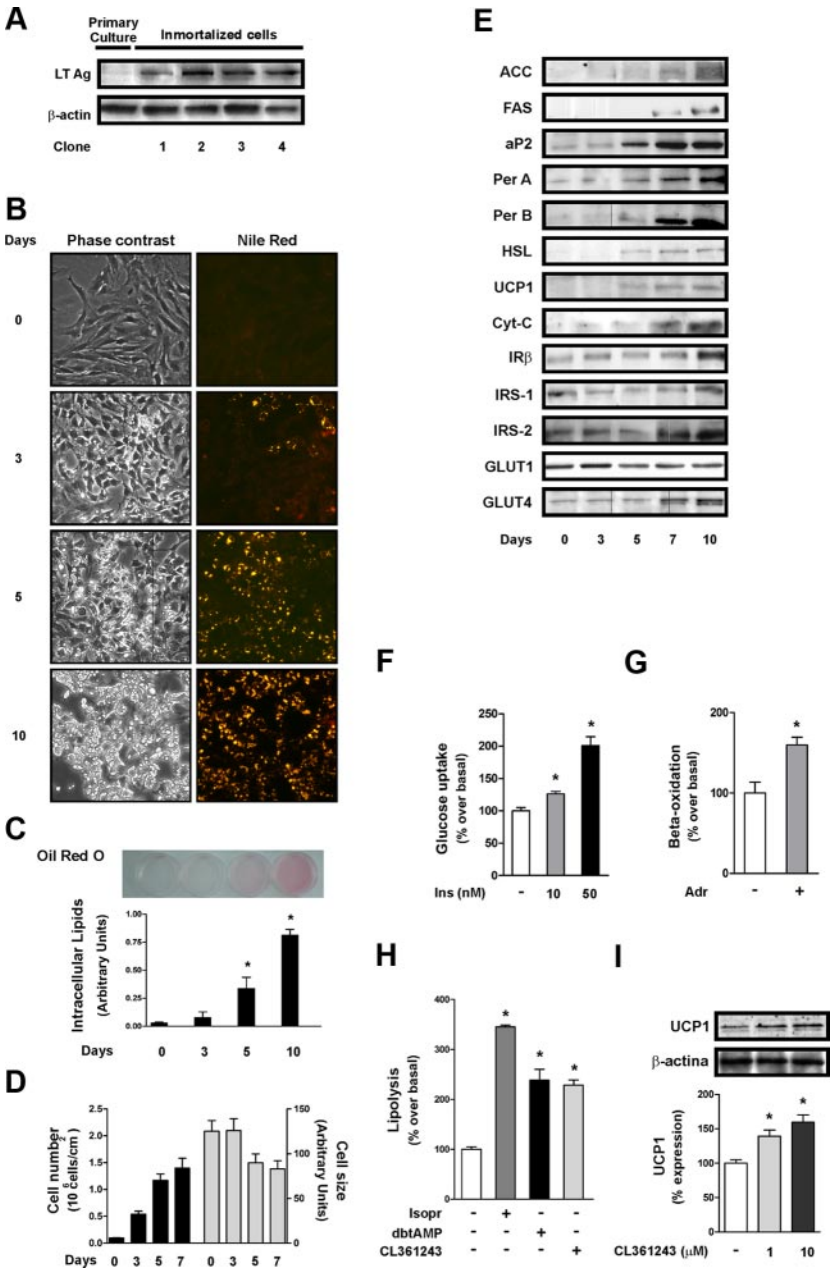

FIG. 1. Immortalized brown preadipocyte cell lines displayed high capacity to undergo differentiation. A, Four clones were analyzed by Western blot to examine LTAg expression after the immortalization process of primary brown adipocytes. B and C, Adipocyte differentiation was induced by culturing cells in differentiation medium as described in *Materials and Methods*, and intracellular lipids accumulation was monitored by Nile Red (B) and Oil Red O staining (C). After staining, dishes were photographed and quantified. Results are represented as arbitrary units. D, Cell number (*black bars*) and cell size (*gray bars*) were determined by flow cytometry. E, Analysis of ACC, FAS, aP2, perilipin (Per) A and B, HSL, UCP1, Cyt-C, IRβ, IRS-1, IRS-2, GLUT1, and GLUT4 was performed by Western blot. Representative immunoblots of three independent experiments are shown. Glucose and lipid metabolism were also studied. Brown adipocytes were differentiated for 10 d and cultured overnight in serum-free medium. F, Cells were stimulated or not with several doses of insulin (Ins) (1, 10, and 50 nM) for 30 min, and glucose uptake was measured. G, β-Oxidation was analyzed after 30 min of 100 nM adrenaline (Adr) stimulation. H, Cells were cultured for 3 h in the presence of 10 μM isoproterenol (Isopr) as β-adrenergic agonist, 1 mM dibutyryl cAMP (cAMP analog), and for 6 h in the presence of 1 μM CL361243 (β3-adrenergic agonist). Lipolysis was measured at the end of the culture period. I, Analysis of UCP1 expression was performed by Western blot after treating the cells in the absence or in the presence of 1–10 μM CL361243 for 18 h. Representative immunoblots and densitometric analysis of two independent experiments are shown. Results are expressed as percentage over control. Statistical significance was established. *, Differences between values *vs.* control.

Data analysis

Results are means ± SE from four to 10 independent experiments. Statistical significance was tested with the unpaired Student's *t* test or with the one-way ANOVA followed by the protected least-significant difference test. P values < 0.01 were considered significant.

Results

Protein expression profile and typical metabolic functions of BAT throughout the differentiation process

Primary cultures of brown adipocytes from rat fetuses were immortalized by infection with a retroviral vector encoding LTAg and selected by puromycin resistance. Fetuses were used because the retroviral infection required proliferating cells. Four clones were obtained and analyzed for LTAg expression, as shown in Fig. 1A. Brown adipocyte differentiation was induced by treating preconfluent cells with a differentiation medium as described in *Materials and Methods*. The fat-specific dyes Nile Red (Fig. 1B) and Oil Red O (Fig. 1C) were used to monitor lipid accumulation. No significant phenotypic differences (detected by the ability or not to accumulate lipids) among the four clones were observed (data not shown). Cell proliferation and cell size were analyzed by flow cytometry (Fig. 1D), and a transient increase in cell number was detected early in the differentiation process. A decrease in cell size was also detected in later stages. Accordingly, an increase in G0/G1 cells (81 *vs.* 66%) and a significant decrease in S+G2/M cells (18 *vs.* 30%) were observed in 7-d adipocytes. Next, we characterized the protein expression pattern through the differentiation process (Fig. 1E). An increase in proteins involved in lipid metabolism, such as ACC, FAS, adipocyte fatty acid-binding protein 2 (aP2), perilipins, and HSL, was observed on d 7 and 10 of differentiation. Expression of the tissue-specific thermogenic marker UCP1 and

cytochrome (Cyt)-C, a mitochondrial marker, were also induced at these stages. When examining the main elements of the insulin-signaling cascade, expression of the IRβ chain and IRS-1 and -2 increased during the differentiation process. Regarding proteins involved in glucose uptake, a decrease in GLUT1 protein content was found on differentiated adipocytes; meanwhile, insulin-regulated GLUT4 expression was induced. In addition, representative typical BAT functions concerning glucose and lipid metabolism were analyzed in mature adipocytes. Glucose uptake was dose-dependently stimulated by insulin (Fig. 1F), and differentiated adipocytes displayed sensitivity to adrenergic stimulation on β-oxidation (Fig. 1G) as well as on lipolysis (Fig. 1H). Regulation of UCP1 expression was confirmed in Fig. 1I, suggesting these cells may respond to β-adrenergic agonists enhancing thermogenic function.

The brown adipocyte differentiation program is temporally controlled by MAPKs, LKB1-AMPK, and TSC2-mTOR-p70S6K1 intracellular signaling pathways

Adipocyte differentiation is under complex transcriptional control and requires timed and regulated activation of various molecular events. First, we confirmed the expression of various transcription factors through adipogenesis. As shown in Fig. 2A, C/EBPβ protein content increased transiently at d 3, and C/EBPα expression was subsequently induced. Additionally, we detected an increase in PPARγ expression, as well as its partner RXRα, on d 3 of differentiation, which was sustained during the culture period. We also confirmed the expression of PGC-1α, a transcription factor expressed in tissues with high energy demand such as brown fat, whose activity is particularly regulated. Furthermore, we explored β-catenin

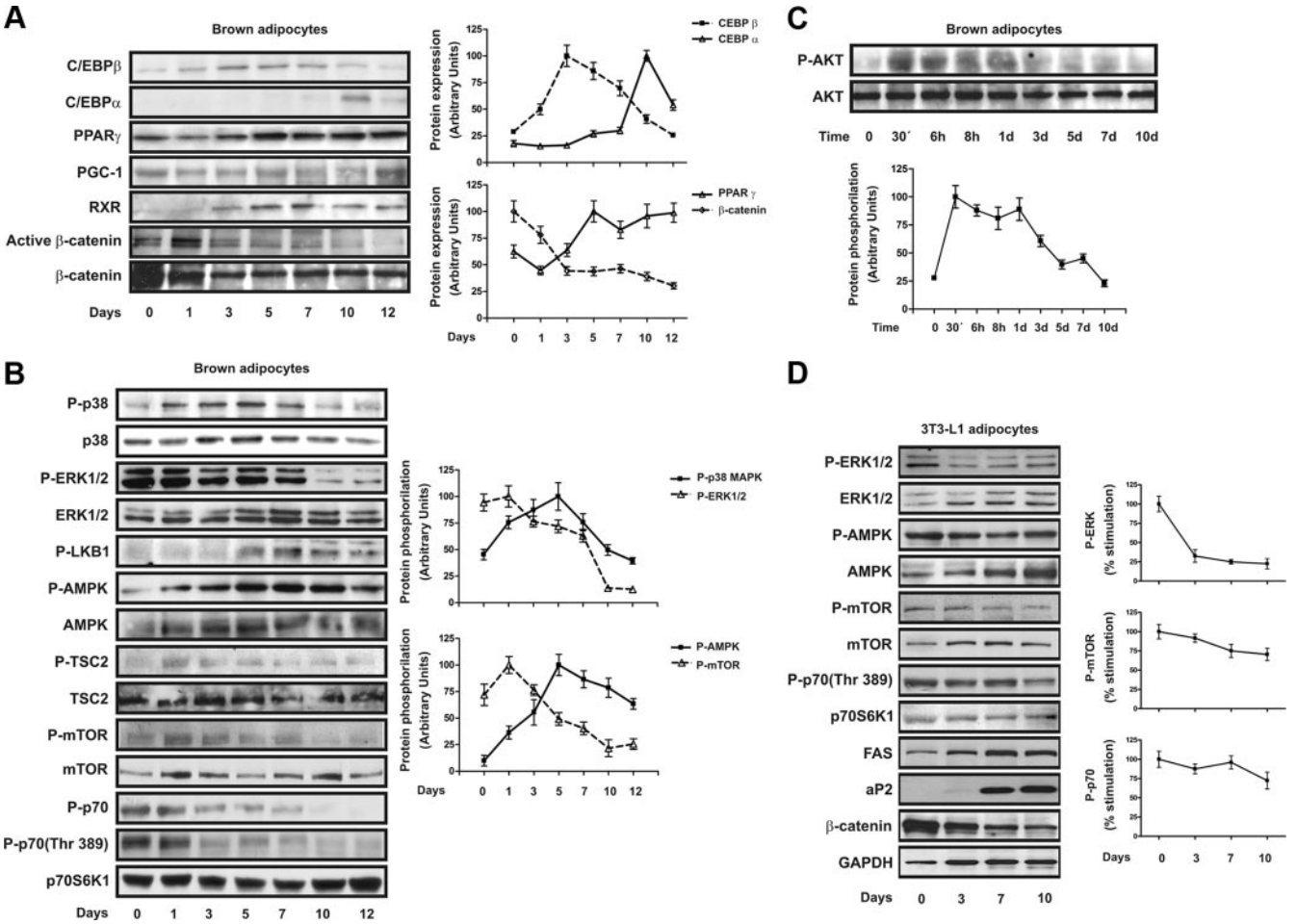

FIG. 2. Kinases involved in differentiation of brown and white adipocytes. Cells were differentiated and collected at several days through the differentiation process. A, Protein extracts from brown adipocytes were analyzed by Western blot with the corresponding antibodies against C/EBPα and -β, PPARγ, PGC-1α, RXRα, and active and total β-catenin. Cell lysates from brown adipocytes (B) and 3T3-L1 (D) were analyzed by Western blot with the corresponding antibodies against phosphorylated and total p38MAPK, ERK1/2, LKB1, AMPK, TSC2, mTOR, p70S6K1, and β-catenin. Differentiation of 3T3-L1 cells was sustained by analysis of FAS and aP2 expression. C, Cells were collected at the indicated times, and expression of phosphorylated and total AKT was analyzed by Western blot. Representative immunoblots and densitometric analysis of three independent experiments are shown.

regulation as the main component of the canonical Wnt signaling pathway, recently discovered to be involved in brown adipocyte differentiation (30). We observed a rapid and transient activation of this pathway early in the culture period followed by suppression of β-catenin expression throughout the differentiation process.

Because specific intracellular signaling pathways control the differentiation process, we next studied the main signaling cascades in brown adipocytes. As depicted in Fig. 2B, phosphorylation of p38MAPK was transiently increased, whereas ERK1/2 phosphorylation decreased throughout the differentiation process. High phosphorylation of AKT (Fig. 2C) caused by the addition of differentiation medium was also detected at the beginning of the process. Moreover, LKB1 and AMPK phosphorylation peaked between d 3 and 7 after brown adipogenesis induction (Fig. 2B). A subsequent decrease in the inactive phosphorylated TSC2 form, concurrent with the inactivation of the mTOR-p70S6K1 signaling pathway, was also detected. This inactivation was verified by examining not only p70S6K1 activating phosphorylation sites (Thr421/Ser424) but also the mTOR direct phosphorylation site (Thr389). No significant changes in the total expression of these kinases were detected throughout the culture period.

To assign this behavior to the differentiation process rather than to the immortalization protocol, these observations were confirmed in primary brown adipocytes. Differentiation was monitored by analysis of lipid accumulation (Supplemental Fig. 1A published on The Endocrine Society's Journals Online web site at http://endo.endojournals.org) and brown fat cell-specific markers expression (Supplemental Fig. 1B). In concordance with data obtained in the immortalized preadipocyte cell line (Fig. 2B), sequential activation of p38MAPK and AMPK, as well as significant attenuation of activation of ERK1/2 and mTOR-p70S6K1 signaling pathways, were observed in primary brown cells induced to differentiate (Supplemental Fig. 1B).

A similar analysis was performed on 3T3-L1 cells. As shown in Fig. 2D, significant attenuation of the activation of ERK1/2 as well as degradation of β-catenin was also observed throughout the white differentiation process. Although a weak inactivation of mTOR was observed at later stages of culture period, neither AMPK activation nor significant attenuation of phosphorylated p70S6K were detected, suggesting the differentiation of brown adipocytes may employ different intracellular signaling pathways than white adipocytes.

To explore the contribution of these pathways to the brown differentiation process, we blocked these signaling cascades with chemical inhibitors (31) and siRNA technology. As shown in Fig. 3A, inhibition of p38MAPK (with SB202190), ERK1/2 (with PD98059) or PI3K (with wortmannin) did not significantly preclude the differentiation process, although it may have affected cell proliferation. In contrast, brown preadipocytes treated with rapamycin, an inhibitor of the mTOR-p70S6K1 pathway, showed a differentiation-defective phenotype. The contribution of AMPK to brown adipocyte differentiation was explored by treating the cells with compound C as a direct inhibitor of AMPK (32) and with iodotubercidin, an indirect inhibitor of AMPK because it impairs AMP synthesis by inhibiting adenosine kinase (33). In this regard, both treatments precluded brown adipogenesis. In fact, chronic treatment with compound C was toxic for brown adipocytes, so iodotubercidin was selected for subsequent experiments. The importance of AMPK and mTOR-p70S6K1 in brown adipocyte differentiation was confirmed using siRNAs. As shown in Fig. 3B, mTOR as well as AMPK protein knockdown (45 and 70%, respectively) significantly blocked brown adipogenesis.

We also analyzed the contribution of these signaling pathways to white adipogenesis (Fig. 3C). Rapamycin reduced the differentiated phenotype in 3T3-L1 cells, as previously described (34), although to a lesser extent than in brown adipocytes (Fig. 3A). However, AMPK inhibition by iodotubercidin did not significantly impair white adipocyte differentiation. Based on these results, we decided to focus our studies on the role of AMPK and mTOR-p70S6K1 in brown adipocyte differentiation.

Regulation of the mTOR-p70S6K1 signaling pathway by AMPK activation is essential for brown adipocyte differentiation

Despite the negative AMPK effects described in white adipocyte differentiation (21, 22), this study reveals AMPK activation to be an essential event in the brown differentiation process and suggests a critical role for the mTOR cascade. To further analyze whether the activity of these signaling pathways was temporally regulated, rapamycin and iodotubercidin were added from d 0, 5, or 7 until the end of differentiation (10 d). As shown in Fig. 4A, only inhibition of mTOR signaling early in differentiation blocked brown adipogenesis. A decrease in cell number was also detected (Fig. 4B), suggesting that mTOR activation is essential during the early proliferative step of brown adipocyte differentiation. These effects were accompanied by a decrease in thermogenic (UCP1) and adipogenic (FAS) markers (Fig. 4C). In contrast, inhibition of AMPK induced different effects depending on the stage of differentiation. As shown in Fig. 4A, the addition of iodotubercidin from d 0 or 5 until the end of the process precluded brown adipocyte differentiation (Fig. 4A), also confirmed by UCP1 and FAS expression (Fig. 4D). In con-

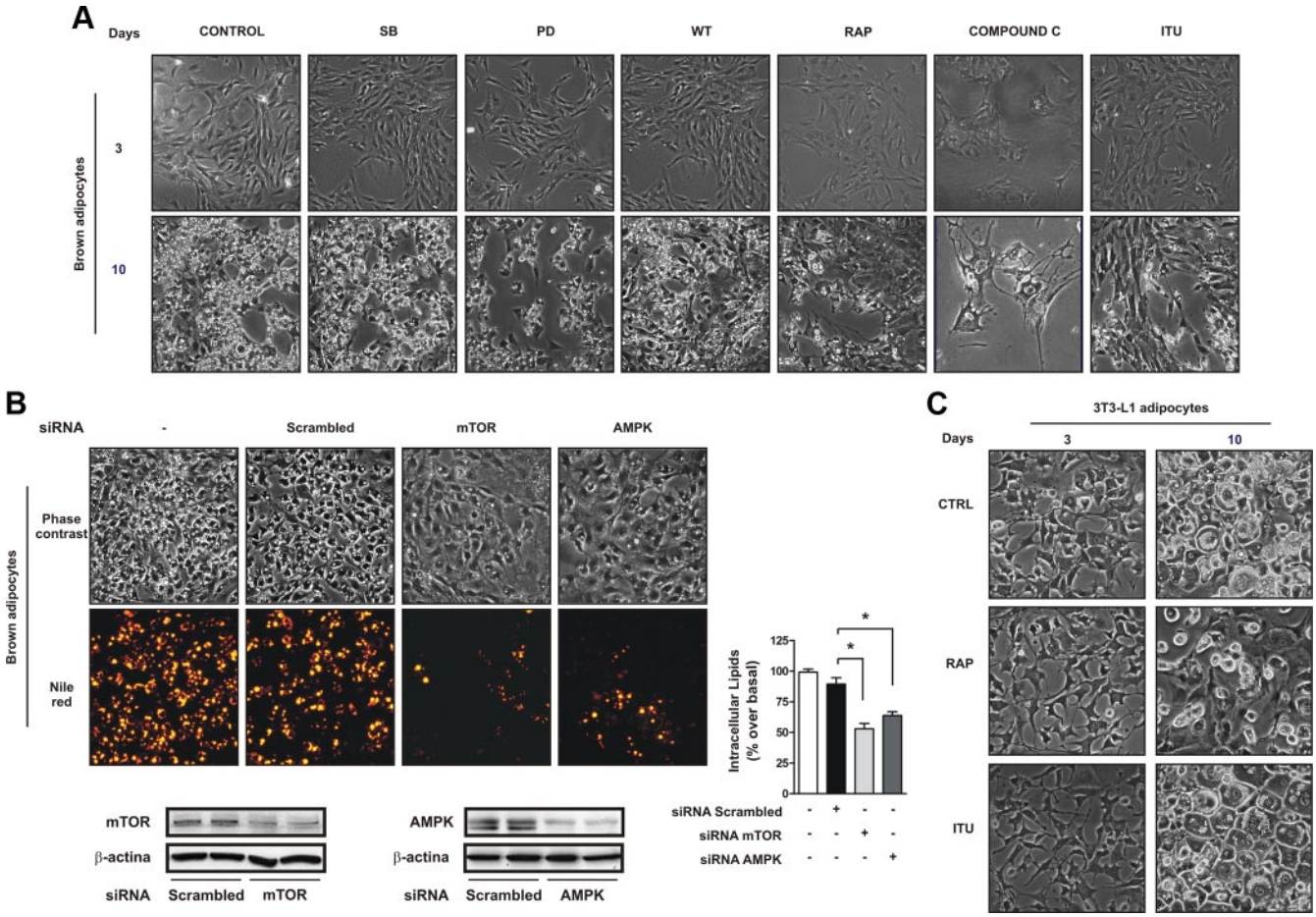

FIG. 3. Effect of mTOR and AMPK inhibition on brown and 3T3-L1 adipocyte differentiation. A, Brown adipocytes were maintained in differentiation medium in the absence or presence of different chemical inhibitors for 10 d. The compounds used were 10 μM SB202190 (SB) as p38MAPK inhibitor, 20 μM PD98059 (PD) as MEK1 inhibitor, 40 nM wortmannin (WT) as PI3K inhibitor, 27 nM rapamycin (RAP) as mTOR inhibitor, and 20 μM compound C (CC) and 0.2 μM iodotubercidin (ITU) as direct and indirect AMPK inhibitors, respectively. Inhibitors were added every 48 h during the experiment. B, Brown adipocytes were transfected with 100 nM scrambled siRNA and 100 nM siRNA against either mTOR or AMPKα1/α2 and induced to differentiate as described in *Materials and Methods*. Intracellular lipids accumulation was monitored and quantified by Nile Red (*upper panel*). Knockdown protein expression of mTOR and AMPK was confirmed by Western blot (*lower panel*). C, 3T3-L1 cells were cultured in the absence or presence of RAP and ITU as described in A. Phase-contrast images (magnification, ×20) of the cells were taken after the treatments.

trast, AMPK inhibition in the latest stages (from d 7–10) increased lipid accumulation (Fig. 4A). No significant differences in cell number were detected (Fig. 4B), suggesting that AMPK activation is not involved in the proliferative step.

Possible cross talk between the main signaling pathways regulating brown adipocyte differentiation was also explored. We observed that mTOR inhibition by rapamycin precluded ERK1/2 inactivation and β-catenin degradation throughout the differentiation process (Fig. 4E). On the other hand, inhibition of AMPK by iodotubercidin delayed mTOR-p70S6K1 cascade inactivation (Fig. 4F). In consequence, and supporting the data observed in Fig. 4E, ERK inactivation and β-catenin degradation appeared in the opposite manner. The effect of the AMPK activator AICAR on the differentiation process was also tested. Nevertheless, daily treatment with this compound was toxic for brown cells, most likely because it decreases intracellular pH (35) but inhibited adipocyte differentiation in 3T3-L1, as previously described (22) (Fig. 5).

Thus, these data suggest that activation of the mTOR-P70S6K1 signaling pathway at early stages is essential for brown adipocyte differentiation. The activation of AMPK at later stages, also necessary for brown adipogenesis, would be involved in mTOR-P70S6K1 signaling cascade inactivation.

Chronic AMPK activation *in vivo* induces brown adipocyte accumulation within WAT

Recent studies indicate that AMPK may remodel adipocyte metabolism by preventing triacylglicerides storage and by activating pathways that promote energy dissipation within the adipocyte (36). We performed an *in vivo* study to further analyze the potential role of AMPK in

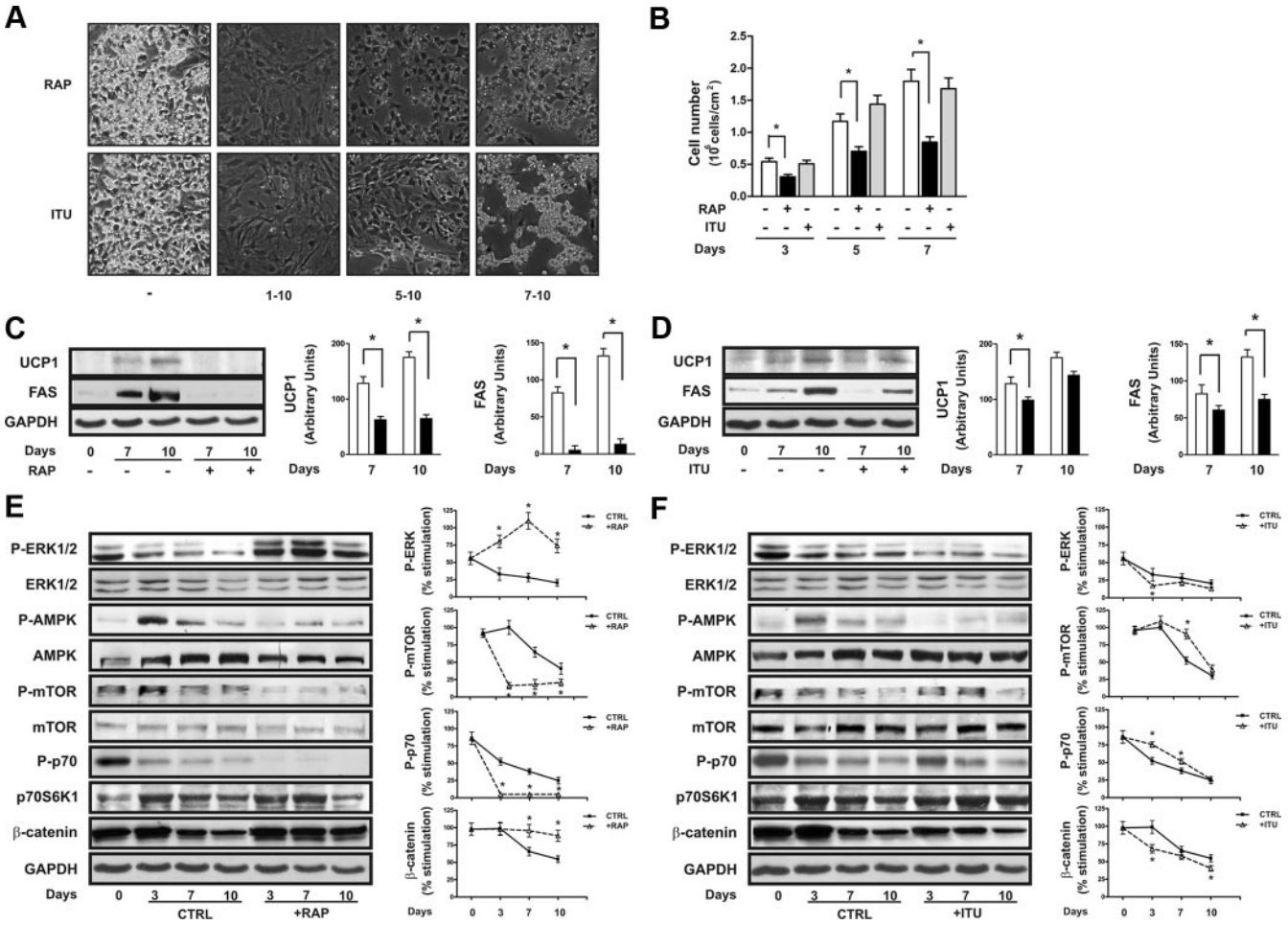

FIG. 4. Activation of mTOR-p70S6K1 signaling pathway at early stages, followed by AMPK activation and mTOR-p70S6K1 inactivation at late stages, are essential for brown adipocyte differentiation. A, Brown adipocytes were differentiated in the absence or presence of either 27 nM rapamycin (RAP) or 0.2 μM iodotubercidin (ITU) added from the first, fifth, or seventh day of differentiation. Phase-contrast images (magnification, ×20) of the cells were taken after 10 d culture. B, Cells were counted throughout the differentiation process in the absence or presence of RAP or ITU. Protein extracts were analyzed by Western blot with the corresponding antibodies against FAS and UCP1 (C and D) and phosphorylated and total AMPK, mTOR, p70S6K1, ERK1/2, β-catenin, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (E and F). Inhibitors were added every 48 h during the experiment. Representative immunoblots and densitometric analysis of three independent experiments are shown. Statistical significance was established. *, Differences between cells differentiated in the presence of the inhibitors (*black bars*) *vs.* control cells (*white bars*).

brown adipocyte differentiation and its physiological significance. For this purpose, mice were chronically treated with AICAR. Body weight (Fig. 6A) and food intake (Fig. 6B) of mice injected with this AMPK activator were reduced after 2 wk treatment, as compared with vehicle-treated mice. In the pair-fed group, which was fed the same amount of food as the AICAR-treated group (Fig. 6B), a reduction in body weight was also observed, although to a lesser extent (Fig. 6A). As shown in Fig. 6C, chronic exposure to AICAR increased AMPK and ACC phosphorylation. Moreover, a significant increase in Cyt-C expression was detected in BAT and WAT of treated mice. Surprisingly, UCP1 protein levels were up-regulated only in WAT. No significant differences were detected in FAS expression. Hence, AMPK may play a positive role in mitochondrial biogenesis and thermogenesis, most likely by acting in brown preadipocytes dispersed within WAT. To test this hypothesis, a UCP1 immunohistochemical analysis was performed. According to UCP1 protein expression (Fig. 6C), no significant differences in UCP1 staining were observed in BAT between vehicle- and AICAR-treated animals (Fig. 6D). On the other hand, accumulation of brown adipocytes was found in epididymal white fat depots (Fig. 6E), as evidenced by UCP1-positive staining. Our results suggest that brown adipocyte-like cells present in WAT and the brown adipocytes constituting BAT are subject to different control systems.

Discussion

BAT depots decrease significantly in size as humans mature, existing in adults within small, defined locations throughout the body as well as distributed as small pock-

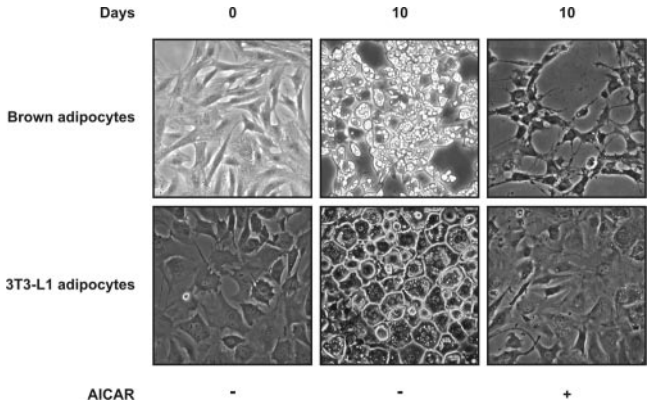

FIG. 5. AICAR-induced AMPK activation in brown and white adipocyte differentiation. Brown and 3T3-L1 adipocytes were maintained in differentiation medium in the absence or presence of 0.25 mM AICAR (added every 48 h) for 10 d. Phase-contrast images (magnification, ×20) of the cells were taken after the treatment.

ets within WAT depots (2). In this regard, the development of therapeutics to enhance the brown phenotype remaining in WAT depots might represent a potential strategy to combat obesity-associated disorders. Understanding the molecular regulation of brown adipocyte differentiation is required for such therapeutics. In this study, we explored the intracellular signaling pathways controlling brown *vs.* white adipocyte differentiation. We detected activation of ERK1/2 and mTOR-p70S6K1 signaling cascades in the early proliferative step followed by subsequent down-regulation through brown adipogenesis. We also observed that this inactivation overlapped with p38MAPK and LKB1-AMPK signaling cascades activation. Silencing these intracellular pathways with either chemical inhibitors or siRNA technology revealed a critical role for AMPK controlling mTOR-p70S6K1 cascade in brown but not white adipocytes. Furthermore, we provide evidence for the first time that chronic AMPK activation *in vivo* may increase the presence of brown adipocytes within WAT depots.

Significant attenuation of activation of ERK1/2 was detected throughout brown and white differentiation. In this regard, the function of ERK in adipogenesis has been hypothesized to be temporally regulated; early on, ERK must be activated for a proliferative stage, although later, it must be shut off (37). ERK1/2 have been described as essential intermediates for the IGF-I/insulin-induced mitogenesis but may have a negative role in the regulation of adipocytic and thermogenic differentiation in brown adipocytes (38). In addition, we observed that cell proliferation, but not brown adipocyte differentiation, was impaired by blocking ERK1/2 activation. These data suggest that brown preadipocytes could differentiate into mature adipocytes without clonal expansion as previously reported in 3T3-L1 adipocytic differentiation (39).

On the other hand, our study demonstrated that early activation of the mTOR-p70S6K1 signaling pathway is an absolute requirement to reach a fully differentiated brown phenotype. However, subsequent down-regulation of this pathway also seems important. Here, we demonstrated that inhibition of mTOR decreased cell proliferation and precluded differentiation. These negative regulatory effects on brown adipogenesis may be exerted through a mechanism that depends on the control of cell growth, in a similar way as described in human preadipocytes (40). No significant attenuation of mTOR-p70S6K was detected through 3T3-L1 differentiation. Nevertheless, rapamycin also precluded the adipogenic program because constitutive activation of mTOR has been reported to be required for the execution of adipogenesis in 3T3-L1 (34, 41). Moreover, even though it has been reported that ERK1/2 and Wnt pathways positively regulate mTOR activity (26, 42), our results suggest negative feedback regulation between these cascades. Along these lines, MAPK activation induced by rapamycin has recently been described in both normal and cancer cells (43). Moreover, we show that rapamycin activated the Wnt pathway by delaying β-catenin degradation in what may represent a novel mTOR-Wnt feedback loop.

Down-regulation of the mTOR-p70S6K1 signaling pathway is consistent with LKB1-AMPK activation, also necessary for brown adipocyte differentiation. In this regard, inhibition of AMPK blocked brown but not white adipocyte differentiation. In fact, in white adipocytes, AMPK is widely accepted as a negative regulator of adipogenesis (21, 22, 44), even though a prevention of clonal expansion in 3T3-L1 preadipocytes by AMPK inhibitors has also been described (45). In contrast, treatment of 7-d mature brown adipocytes with iodotubercidin exerted a positive effect on lipid accumulation, in a similar way as has been observed in white adipocytes (36). All together, these data suggest a possible role for AMPK in controlling lipolytic-lipogenic balance in brown fat.

Furthermore, our data confirm that inactivation of mTOR throughout the differentiation process is dependent on AMPK activation. In fact, AMPK has been found to suppress mTOR signaling by growth factors and amino acids (46, 47) via phosphorylation of TSC2 (25). At the beginning of the process, we observed transitory phosphorylation of TSC2 at the Thr1462 residue, probably induced by an AKT-dependent mechanism, resulting in TSC2 inhibition and consequently in mTOR-dependent p70S6K1 activation, as described elsewhere (48, 49). However, once the LKB1-AMPK cascade is activated through brown adipogenesis, a decrease in the inactive form of TSC2 is detected, probably induced by phosphorylation at two different sites from the AKT phosphorylat-

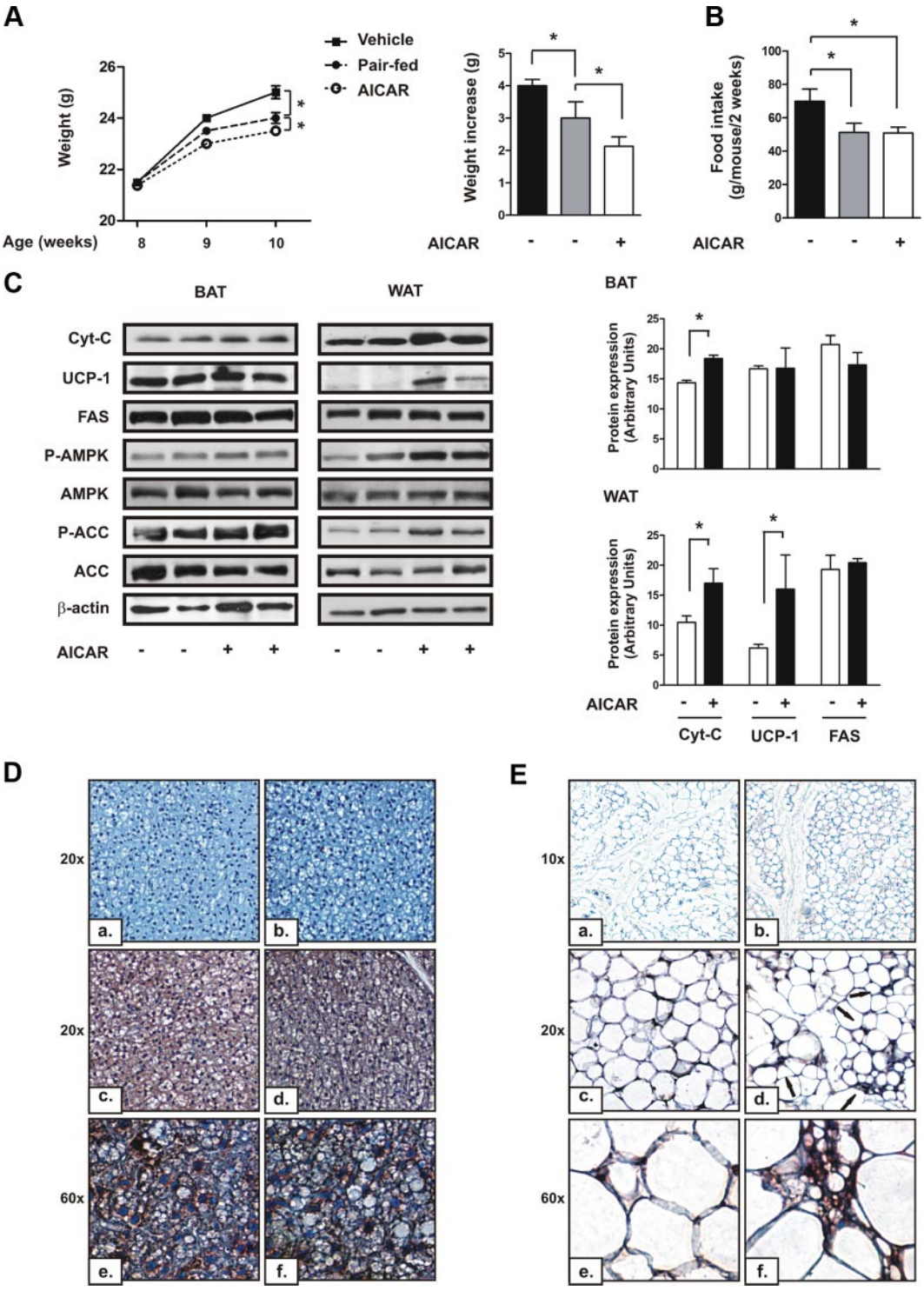

FIG. 6. Effects of prolonged AICAR-induced AMPK activation *in vivo*. C57BL6 mice were treated with AICAR (*white bars*) or vehicle (*black bars*) for 2 wk ip as described in *Materials and Methods*. In the pair-fed group (*gray bars*), animals were fed the same amount of food as that consumed by the AICAR-treated animals over the preceding 24 h. A and B, Body weight (A) and food intake (B) of these mice were measured periodically. C, After 2 wk, total protein content from epididymal WAT and BAT was isolated, and the expression of various key adipogenic and thermogenic markers, including Cyt-C, UCP1, and FAS as well as total and phosphorylated AMPK, ACC, and β-actin, was examined by immunoblot analysis. Results are shown as mean ± SD of five mice. *, Differences between untreated and treated mice. D, Histological analysis of paraffin-embedded BAT stained with UCP1 antibody and hematoxylin. Photomicrographs of sections from vehicle-treated (c and e) and AICAR-treated mice (d and f) mice at the indicated magnifications are shown. A negative control was prepared without the primary antibody for vehicle (a) and treated (b) mice. E, Immunolocalization of brown adipocytes (shown by *arrows*) in epididymal WAT using UCP1 antibody and hematoxylin counterstaining in vehicle-treated (a, c, and e) and AICAR-treated (b, d, and f) mice. Representative photomicrographs of different magnifications are shown.

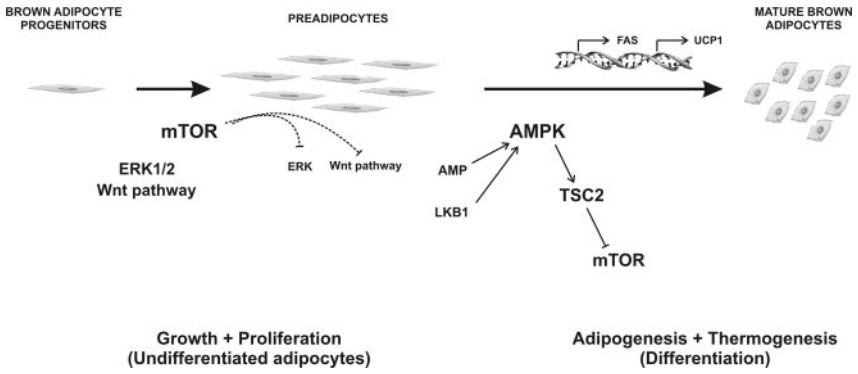

FIG. 7. Cross talk between AMPK and mTOR-p70S6K1 signaling pathways occurs during brown adipocyte differentiation. Activation of mTOR-p70S6K1 signaling pathway in the early proliferative step is an absolute requirement to reach a fully brown differentiated phenotype and might be related to cell growth and proliferation processes. Moreover, a cross talk mTOR-ERK1/2 and -Wnt pathway is described in this study. In this regard, mTOR would be controlling ERK1/2 inactivation and β-catenin degradation through the differentiation process. On the other hand, further activation of AMPK is also required and may be involved in the inhibition of mTOR-p70S6K1 signaling cascade later in the differentiation process, via TSC2 activation, as well as in the expression of thermogenic markers.

ing sites, as previously described (25, 50). Chemical suppression of AMPK activation confirmed this cross talk in the brown adipocyte differentiation program. Thus, mTOR-p70S6K1 inactivation detected throughout brown differentiation was delayed, and ERK1/2 inactivation and β-catenin degradation were detected at an earlier time in cells treated with an AMPK inhibitor. The molecular mechanisms by which AMPK is activated during brown adipocyte differentiation are currently under study. A change in energy status might be an appropriate candidate because inhibition of AMP synthesis precluded brown adipocyte differentiation. Moreover, activation of AMPK by the acquisition of thermogenic properties should not be ruled out. In this regard, the inhibition of AMPK might preclude cell energetic adaptation, thus impairing the brown adipocyte differentiation process.

Finally, an *in vivo* study was conducted to analyze the physiological significance of AMPK activation in brown fat development. We observed that mice chronically treated with AICAR showed reduced body weight and food intake, as previously observed (51). The pair-fed group also showed body weight reductions although to a lesser extent than AICAR-treated mice. AMPK activation increased Cyt-C expression in both BAT and WAT, whereas an increase in UCP1 protein levels was detected only in WAT. Along these lines, it has been reported that AMPK activation induces PGC-1α expression, which regulates UCP1, in WAT of obese mice (52). Moreover, it caused up-regulation of mitochondrial enzymes (53) and stimulated both fatty acid uptake and oxidation in muscle (54). Thus, the opposite effect of AMPK on brown and white adipocytes could be explained by the fact that brown adipocytes specialize in lipid catabolism rather than storage, much like oxidative skeletal muscle tissue. Currently, new data indicate that brown fat progenitors express myoblast markers and are not involved in white adipogenesis (16, 17). No significant differences were observed in FAS expression either in BAT or in WAT of AICAR-treated mice. However, an increase in ACC phosphorylation, in agreement with AMPK activation, was detected. This study demonstrates for the first time that chronic AMPK activation up-regulates UCP1 expression, supporting the hypothesis that AMPK is a key regulator in brown adipocyte differentiation. Nevertheless, only brown preadipocytes within white fat depots, not mature cells in BAT, seem to be sensitive to AICAR-induced UCP1 expression. Nevertheless, we cannot rule out an additional effect of AMPK activation on UCP1 activity in mature brown adipocytes. Most importantly, we demonstrate by UCP1 immunohistochemical analysis the appearance of a small number of brown adipocytes within white fat depots of treated mice. A similar accumulation of brown adipocytes was observed after cold exposure (55) or treatment with β3-adrenergic agonists (56). However, neither the mechanisms controlling the induction nor its physiological significance to body weight regulation are well understood. Our results indicate that AMPK might play an important role in the appearance of brown adipocytes in white fat. The reduction of body weight observed in AICAR-treated mice could be mainly due to the decrease in food intake, because the pair-fed group also showed a reduction in body weight. However, the contribution of enhanced energy expenditure as a consequence of brown phenotype increase cannot be ruled out. These data suggest that AMPK activators may have antiobesity effects and corroborate previous findings about the involvement of AMPK activation in remodeling adipocyte metabolism by up-regulating pathways that favor energy dissipation *vs.* lipid storage in WAT (36, 52). In summary, our study reveals for the first time the negative cross talk between AMPK and mTOR throughout the brown differentiation program. As illustrated in Fig. 7, temporal control of these cascades is required, with mTOR activation at the beginning of the process essential for brown differentiation. Later AMPK activation would be also crucial for switching off mTOR signaling as well as for inducing the expression of thermogenic markers. The requirement of AMPK activation for brown adipogenesis indicates a different role for AMPK in brown and white adipocytes. Taken together, our findings support the hypothesis of

differential regulation mechanisms for brown and white differentiation programs. Moreover, this study provides further insight into the use of AMPK activators to enhance the brown phenotype remaining in WAT as a strategy to combat obesity-associated disorders.

Acknowledgments

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