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Thermogenically competent nonadrenergic recruitment in brown preadipocytes by a PPARγ agonist

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Petrovic N, Shabalina IG, Timmons JA, Cannon B, Nedergaard J. Thermogenically competent nonadrenergic recruitment in brown preadipocytes by a PPARγ agonist. *Am J Physiol Endocrinol Metab* 295: E287–E296, 2008. First published May 20, 2008; doi:10.1152/ajpendo.00035.2008.—Most physiologically induced examples of recruitment of brown adipose tissue (BAT) occur as a consequence of chronic sympathetic stimulation (norepinephrine release within the tissue). However, in some physiological contexts (e.g., prenatal and prehibernation recruitment), this pathway is functionally contraindicated. Thus a nonsympathetically mediated mechanism of BAT recruitment must exist. Here we have tested whether a PPARγ activation pathway could competently recruit BAT, independently of sympathetic stimulation. We continuously treated primary cultures of mouse brown (pre)adipocytes with the potent peroxisome proliferator-activated receptor-γ (PPARγ) agonist rosiglitazone. In rosiglitazone-treated cultures, morphological signs of adipose differentiation and expression levels of the general adipogenic marker aP2 were manifested much earlier than in control cultures. Importantly, in the presence of the PPARγ agonist the brown adipocyte phenotype was significantly enhanced: UCP1 was expressed even in the absence of norepinephrine, and PPARα expression and norepinephrine-induced PGC-1α mRNA levels were significantly increased. However, the augmented levels of PPARα could not explain the brown-fat promoting effect of rosiglitazone, as this effect was still evident in PPARα-null cells. In continuously rosiglitazone-treated brown adipocytes, mitochondriogenesis, an essential part of BAT recruitment, was significantly enhanced. Most importantly, these mitochondria were capable of thermogenesis, as rosiglitazone-treated brown adipocytes responded to the addition of norepinephrine with a large increase in oxygen consumption. This thermogenic response was not observable in rosiglitazone-treated brown adipocytes originating from UCP1-ablated mice; hence, it was UCP1 dependent. Thus the PPARγ pathway represents an alternative, potent, and fully competent mechanism for BAT recruitment, which may be the cellular explanation for the enigmatic recruitment in prehibernation and prenatal states.

brown fat; rosiglitazone; norepinephrine; mitochondria; thermogenesis; peroxisome proliferator-activated receptor-γ

THERMOGENESIS IN BROWN ADIPOSE TISSUE (BAT) is acutely controlled by the sympathetic nervous system through norepinephrine (NE) release within the tissue. Furthermore, most physiologically induced events of cellular recruitment taking place during cold acclimation and in diet-induced thermogenesis (enhanced cell proliferation, enhanced cell differentiation, and mitochondriogenesis) can be understood as occurring as a consequence of chronic sympathetic stimulation of the tissue (for review see Ref. 8).

However, a chronic sympathetic drive cannot explain all physiological conditions demonstrating enhanced recruitment of BAT. For instance, in precocial newborns, BAT is fully recruited already at birth (37, 40, 41). It is highly unlikely that an increased chronic sympathetic stimulation could drive BAT recruitment during intrauterine life at 37°C, because this would enhance the heat load of the mother. Thus this condition indicates the existence of an alternative, potent and fully competent nonadrenergic mechanism for recruitment of BAT. Also, the process of preparation for hibernation represents a recruitment process where the adrenergic pathway would be contraindicated, since during the time when BAT is recruited, i.e., in late summer, hibernators accumulate reserves of fat for the winter. Sympathetically activated BAT would utilize these winter reserves of fat. Recruitment of brown fat cells in certain hibernators has also been directly demonstrated to be nonadrenergic (5, 10). Thus a nonsympathetically mediated mechanism of UCP1 induction and/or recruitment of BAT must exist to explain these conditions of recruitment.

Sympathetic (adrenergic) stimulation of brown adipocytes results in a very large increase in UCP1 expression (42). As UCP1 mRNA levels may be nearly undetectable in unstimulated cells, the relative NE-induced increase is difficult to determine, but quantitative PCR analysis indicates a nearly 1,000-fold rise in UCP1 levels within a few hours (unpublished observations). Nonsympathetically driven recruitment in brown fat cells must also include a similar activation of UCP1 gene expression. An induction of UCP1 gene expression has been observed in cultured brown adipocytes upon acute treatment with peroxisome proliferator-activated receptor-γ (PPARγ) ligands (2, 16, 25), but the increases reported as yet have been modest, a 5- to 10-fold increase in UCP1 mRNA level. However, although the PPARγ ligand-induced increases thus may be ~100-fold lower than those observed with adrenergic stimulation, the observations as such indicate the possibility that an endogenous activator of the PPARγ pathway could competently recruit brown fat cells without concomitant adrenergic stimulation. PPARγ is a central transcriptional regulator of differentiation of both brown and white adipose cells (48) and is absolutely required for BAT development (1, 12) as well as for survival of mature brown adipocytes (24, 26). Accordingly, in mice with impaired PPARγ function, brown adipocyte recruitment is impaired, and nonshivering thermogenic capacity after cold acclimation is decreased (21).

PPARγ is expressed not only in differentiated brown adipocytes but also in brown preadipocytes (33). Such primary

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cultures of brown adipocytes have been frequently used as an in vitro model system to recapitulate the process of differentiation in vivo. The cells do not express UCP1 in the absence of adrenergic stimulation (42). Thus the mere presence of PPARγ is insufficient in itself to activate UCP1 gene expression, but the absence of an endogenous ligand of PPARγ could conceivably explain the lack of UCP1 expression. We have therefore here continuously treated cultures of brown (pre)adipocytes with the potent PPARγ agonist rosiglitazone. This markedly accelerated the differentiation of the adipocytes. We found that the brown adipocytes in which PPARγ was constantly activated express UCP1 maximally. The resulting high levels of UCP1 protein were incorporated into mitochondria since the brown adipocytes become capable of responding to NE stimulation with a large increase in oxygen consumption: thermogenesis. Thus the presence of continuously active PPARγ was sufficient to enable thermogenically competent recruitment of brown adipocytes in primary culture.

MATERIALS AND METHODS

Animals, cell isolation, and cell culture. Male NMRI mice, purchased from a local supplier (B&K, Stockholm, Sweden), were used for the preparation of primary cultures of brown adipocytes, if not otherwise stated. Mice were kept at room temperature (~22 °C) for at least 24 h after arrival. At the age of 3–4 wk, mice were killed by CO_2, and the BAT was isolated from the interscapular, cervical, and auxiliary depots, principally as described by Rehnmark et al. (42). The pooled tissue pieces were minced in DMEM and transferred to a digestion solution with 0.2% (wt/vol) collagenase (type II; Sigma) in a buffer consisting of 0.1 M HEPES (pH 7.4), 123 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 4.5 mM glucose, and 1.5% (wt/vol) BSA. The digestion was performed for 30 min at 37°C with continuous vortex mixing. The cell suspension was filtered through a 250-μm pore-size nylon filter (Sintab, Oxie, Sweden) into sterile 15-ml tubes. The filtered suspension was kept on ice for 20 min to let the mature adipocytes float up. The top layer of the suspension was removed, and the rest of the suspension was filtered through a 25-μm pore-size nylon filter (Sintab) and centrifuged at 700 *g* for 10 min, to pellet preadipocytes. The pellet was resuspended in 10 ml of DMEM and centrifuged at 700 *g* for 10 min. The pellet was then suspended in culture medium (0.5 ml/animal). The cells were cultured in six-well plates (10 cm^2/well; Corning; 12-well plates for cAMP determination); 1.8 ml of culture medium were added to each well before 0.2 ml of cell suspension were added. The culture medium was DMEM with 10% (vol/vol) newborn calf serum (Invitrogen or Hyclone), 2.4 nM insulin, 25 μg/ml sodium ascorbate, 10 mM HEPES, 4 mM glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin and supplemented or not (as indicated) with 1 μM rosiglitazone maleate (Alexis Biochemicals). The cells were grown at 37°C in an atmosphere of 8% CO_2 in air with 80% humidity. The cells were washed in DMEM, and the medium was changed on the first day and then every second day. The new medium was prewarmed to 37°C before being changed. The medium was not changed on the day the cells were harvested. The experiments were performed on different days of culture, as indicated in each individual experiment.

Analysis of mRNA levels. After the experiments, the medium was discarded and the cells were harvested from each well with 1 ml Ultraspec (Biotecx Laboratories, Houston, TX), as described in the manufacturer's protocol. The RNA obtained was examined by Northern blotting, principally as described previously (33), but the gels were run for 5 to 6 h. The membranes were probed consecutively for aP2, PPARγ, PGC-1α, UCP1 and PPARα mRNAs, and 18S rRNA after being stripped in-between by repeated washing with boiling 0.2% (wt/vol) SDS.

Analysis of protein levels. Brown adipocytes were washed twice in ice-cold PBS and then harvested in a modified RIPA buffer {50 mM Tris·HCl, pH 7.4, 1% Triton X-100, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF [protease inhibitor cocktail (Complete-Mini, Roche Diagnostics)], 1 mM Na_3VO_4, and 1 mM NaF}. Cells were lysed on ice for 15 min and then centrifuged at 14,000 *g* for 15 min. The concentration of proteins in the supernatant was determined using the method of Lowry. An equal volume of reducing sample buffer [62.5 mM Tris·HCl, pH 6.8, 2% (wt/vol) SDS, 10% (vol/vol) glycerol, 100 mM dithiothreitol, and 0.1% (wt/vol) bromphenol blue] was added to each sample. Proteins were separated by SDS-PAGE in ordinary 12% polyacrylamide gel (acrylamide/bis-acrylamide = 37.5/1) or, where indicated, in highly porous 12% polyacrylamide gel (acrylamide/bis-acrylamide = 175/1) with high-resolution capacity. Proteins were transferred to polyvinylidene difluoride membranes (GE Healthcare Life Sciences) in 48 mM Tris·HCl, 39 mM glycine, 0.037 (wt/vol) SDS, and 15% (vol/vol) methanol using a semi-dry electrophoretic transfer cell (Bio-Rad Trans-Blot SD; Bio-Rad Laboratories) at 1.2 mA/cm^2 for 90 min. After transfer, the membrane was stained with Ponceau S for examination of equal loading of proteins. After being washed, the membrane was blocked in 5% milk in Tris-buffered saline-Tween for 1 h at room temperature and probed with the indicated antibodies overnight at 4°C. The immunoblot was visualized with appropriate horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence (ECL kit, GE Healthcare Life Sciences) in a charge-coupled device camera (Fuji Film).

Antibodies used were as follows: PPAR antibody (Santa Cruz Laboratories, sc-7273) diluted 1:1,000, which reacts with PPARα, PPARβ/δ, and PPARγ; UCP1 antibody (rabbit polyclonal, raised against COOH-terminal decapeptide), diluted 1:3,000; COX4 antibody (Santa Cruz Laboratories, sc-58348), diluted 1:1,000; and CPT-1M antibody (Nordic BioSite, C1385-45), diluted 1:1,000.

cAMP determinations. Brown adipocytes were cultured in 12-well plates for 7 days. Control and continuously rosiglitazone-treated cells were treated for 10 min with 1 μM NE or water. Control cells were also treated for 10 min with 1 μM rosiglitazone or vehicle. After 10 min, the medium was removed, 0.25 ml of 75% ethanol were added to each well, and the cells were scraped off. The wells were washed with 0.25 ml of 75% ethanol, and the combined suspensions were dried in a Speedvac centrifuge. The dried samples were dissolved in 100 μl of the buffer 1 provided with the cAMP (^3H) assay system from GE Healthcare (TRK 432), sonicated briefly, and centrifuged at 14,000 rpm for 10 min. Two 25 μl-aliquots of the supernatant were analyzed for every sample according to the description in the assay system; for every treatment, duplicate wells were used.

Analysis of PPAR by immunocytochemistry. Brown adipocytes were cultured as described above except that 18 × 18-mm coverslips were placed in the wells. Cells were cultured for 7 days in the absence or presence of 1 μM rosiglitazone. The cells were then washed twice with PBS and fixed with 3% paraformaldehyde in PBS for 20 min at room temperature. Cells were washed three times with PBS and then exposed to 5% glycine in PBS to quench unspecific fluorescence. Cells were then washed three times with PBS and permeabilized with 0.5% Triton X-100 in water for 15 min at room temperature. Then, the cells were washed three times with PBS and blocked with 8% BSA in PBS for 1 h at room temperature. Cells were washed three times with PBS and incubated with 1:300 diluted anti-PPAR antibody in 4% BSA in PBS overnight at 4°C. Cells were washed three times with PBS and incubated with anti-mouse-AlexaFluor 488-labeled secondary antibody (Molecular Probes), diluted 1:1,000 in 4% BSA in PBS for 1 h at room temperature. Finally, coverslips were mounted on microscopic slides with ProLong Gold antifade reagent (Molecular Probes). The cells were examined with a Zeiss fluorescence microscope.

Analysis of mitochondrial content by MitoTracker Green staining. Brown adipocytes were cultured on coverslips (as described above) for 7 days. The cells were then washed twice with PBS and fixed with

3% paraformaldehyde in PBS for 20 min at room temperature. Cells were washed three times with PBS and then exposed to 5% glycine in PBS to quench unspecific fluorescence. Cells were then washed three times with PBS and incubated with 50 nM MitoTracker Green (Molecular probes) in PBS for 20 min. After being washed with PBS, coverslips were mounted on microscopic slides with ProLong Gold antifade reagent (Molecular Probes). The cells were examined with a Zeiss fluorescence microscope.

Oxygen consumption. Brown adipocytes were cultured in 6-well plates for 7 days. Then, the cells in four wells were simultaneously trypsinized for 3–5 min, pooled, and centrifuged for 2 min at 700 *g*. The cells were resuspended in 1.4 ml of culture medium (see above) supplemented with 4% fatty acid-free BSA. Oxygen consumption rates of brown adipocytes were monitored with a Clark-type oxygen electrode (Yellow Springs Instrument). Approximately 1.2 ml of cell suspension were added to a magnetically stirred oxygen electrode chamber thermostated to 37°C. The chamber was closed, and the cells were incubated for 3–4 min to determine the basal respiratory rate. Then NE was added (at 1 μM final concentration) with a Hamilton syringe through a small hole in the cover of the chamber. The output signal from the oxygen electrode amplifier was electronically time-differentiated and collected every 0.5 s by a Power-Lab/ADInstrument (application program Chart, version 4.1.1.). The Chart data files were transferred to the KaleidaGraph MacIntosh application and converted to absolute values based on an oxygen content of 217 nmol of O_2/1 ml of water, on the calibrated electronic differentiation constants, and on the amount of protein used (2 parallel wells of brown adipocytes on the same plate were used for determination of protein amount). For calculation of stable oxygen consumption rates, the mean values during 1 min were obtained from these recordings.

RESULTS AND DISCUSSION

In the present study, we examined recruitment of brown adipocytes driven by nonsympathetically mediated processes. We chronically treated brown (pre)adipocytes with the PPARγ agonist rosiglitazone and examined the parameters of brown adipocyte differentiation.

Chronic rosiglitazone treatment accelerates and augments differentiation of brown adipocytes in primary culture. Brown preadipocytes have the ability to grow and differentiate in culture in a spontaneous but highly reproducible way (42). When grown under control conditions, the fibroblast-like preadipocytes proliferate until they are confluent (at *days 4–5*) and then differentiate to become mature, lipid-laden brown adipocytes. On *day 7*, ~80–90% of cells visibly represent mature brown adipocytes (Fig. 1*A*), based on lipid accumulation.

In cultures continuously treated with the PPARγ-agonist rosiglitazone from the time of plating, morphological signs of adipose conversion were observed already on *days 3* and *4*; on *day* 7, nearly 100% of the cells visibly represented mature adipocytes (Fig. 1*A*). As seen, compared with adipocytes differentiated under control conditions, brown adipocytes grown in the presence of PPARγ agonists were larger, with more pronounced lipid droplets.

To characterize the differentiation state of brown adipocytes at the molecular level, Northern blot analysis was performed to examine the expression of general adipogenic genes (aP2 and PPARγ; Fig. 1, *B–D*), as well as brown (as opposed to white)-adipocyte-related genes (PGC-1α, UCP1, and PPARα; Figs. 2 and 3, *A–C*) during the differentiation of control and rosiglitazone-treated cultures. Although these latter genes have also been shown to increase in traditional white-fat depots after chronic PPARγ agonist treatment in vivo (44), their mRNA levels are more than an order of magnitude lower in white fat than in brown fat (and may represent the presence of brown adipocytes in these depots; Ref. 22).

During conversion of brown preadipocytes into mature adipocytes in the absence of any treatment, the expression of the adipocyte marker gene aP2 gradually increased, reaching a maximum on *day 6* (Fig. 1*C*). The chronic presence of the PPARγ agonist in the brown preadipocyte cultures significantly increased the aP2 mRNA levels (Fig. 1*C*), much above control levels already at *day 4*, in accordance with aP2 being a PPARγ-target gene.

PPARγ itself may also be considered as an adipocyte marker, although PPARγ mRNA is expressed not only in mature brown adipocytes but already in brown preadipocytes (Fig. 1*D*; Ref. 33), indicating that the fibroblast-like undifferentiated precursor cells are already determined for their adipocyte destiny. The chronic presence of rosiglitazone in the

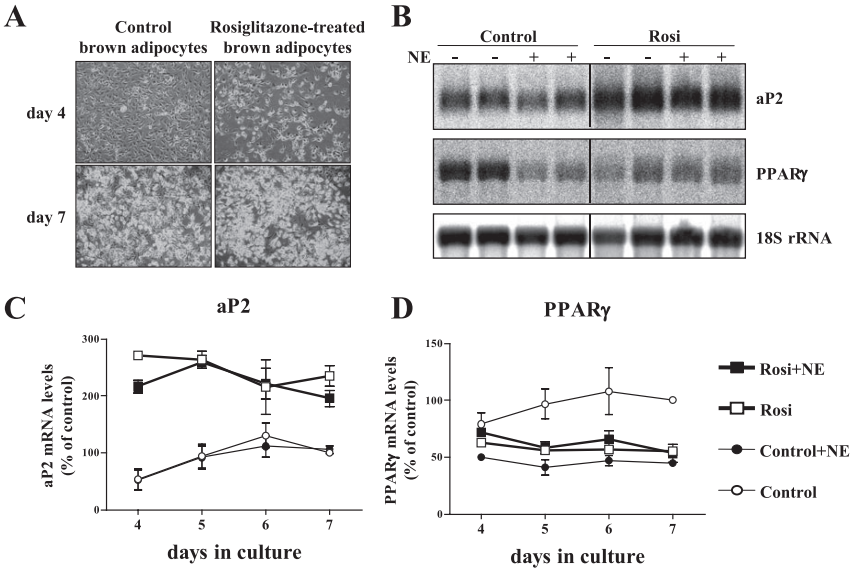

Fig. 1. Morphology and expression levels of aP2 and peroxisome proliferator-activated receptor-γ (PPARγ) during spontaneous and rosiglitazone-stimulated differentiation of brown adipocytes. Primary cultures of brown adipocytes were grown for the indicated number of days in the absence (control) or presence of 1 μM rosiglitazone. Where indicated, 1 μM norepinephrine (NE) had been added 2 h before harvest. *A*: cells were examined under phase-contrast microscope (Olympus) and photographed by an Olympus digital camera after 4 or 7 days in culture. *B*: representative Northern blot, *day 5* of culture. Total RNA (10 μg) was used for each lane, and the blot was hybridized with the aP2, PPARγ, and 18S rRNA probes. aP2 (*C*) and PPARγ (*D*) mRNA levels during spontaneous and rosiglitazone-stimulated differentiation are shown. The aP2 and PPARγ mRNA levels were normalized to the 18S rRNA levels in each sample. The points are means ± SE of 3 (*days 5*, *6*, and *7*) or 2 (*day 4*) independent experiments, each performed in duplicate. The control level at *day 7* was set in each experiment to 100%, and the aP2 and PPARγ mRNA levels on the other days were expressed relative to this value in each individual experiment.

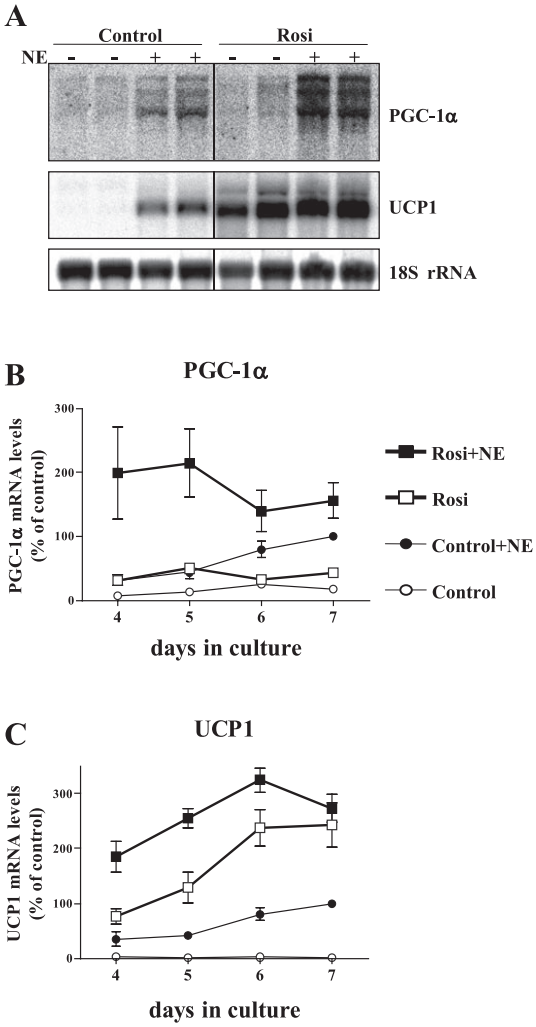

Fig. 2. Expression levels of PGC-1α and UCP1 during spontaneous and rosiglitazone-stimulated differentiation of brown adipocytes. Cells as in Fig. 1 were analyzed. *A*: representative Northern blot, *day 5* of culture. Total RNA (10 μg) was used for each lane, and the blot was hybridized with the PGC-1α UCP1 and 18S rRNA probes. PGC-1α (*B*) and UCP1 (*C*) mRNA levels during spontaneous and rosiglitazone-stimulated differentiation are shown. The PGC-1α and UCP1 mRNA levels were normalized to the 18S rRNA levels in each sample. The points are means ± SE of 3 (*days 5*, *6*, and *7*) or 2 (*day 4*) independent experiments, each performed in duplicate. The control NE-induced level at *day 7* was set in each experiment to 100%, and the PGC-1α and UCP1 mRNA levels on the other days were expressed relative to this value in each individual experiment.

cultures downregulated PPARγ mRNA expression (Fig. 1*D*), principally as expected (23, 49).

The sympathetic neurotransmitter NE is both the most important and most well-studied factor that influences the brown adipocyte (8). Therefore, cultures grown as above for different numbers of days in the absence or presence of rosiglitazone were acutely treated with 1 μM NE, 2 h before harvest. NE treatment did not significantly influence the mRNA levels of aP2, either in control or in rosiglitazone-treated cells (Fig. 1*C*). As shown in Fig. 1*D*, PPARγ mRNA levels were markedly decreased upon NE-treatment of control cultures. The NE-induced downregulation of PPARγ mRNA levels was observed in proliferative (*days 4* and *5*), as well as in differentiating (*days 6* and *7*) cells (Fig. 1*D*; Ref. 33); thus there was no switch in the qualitative response between preadipocytes and mature adipocytes concerning adrenergic regulation of PPARγ mRNA levels. In chronic rosiglitazone-treated cells, NE did not influence PPARγ mRNA levels. It is notable that the PPARγ mRNA levels were downregulated to similar, stable levels (~50% of the maximal expression level) by either the PPARγ agonist or NE (or both). The maintenance of these PPARγ mRNA levels is probably necessary to sustain survival of brown adipocytes in culture (24, 26).

In contrast to the general adipogenic markers aP2 and PPARγ, the brown adipocyte-related gene PGC-1α and the brown adipocyte-specific marker UCP1 were barely or not expressed in control brown adipocytes (Fig. 2, *B* and *C*), as reported earlier for UCP1 (42). As expected, in the control cultures, acute NE stimulation markedly induced expression of both PGC-1α and UCP1 (Fig. 2, *B* and *C*). The NE-induced mRNA levels of PGC-1α and UCP1 progressively increased during differentiation.

However, these brown-fat characteristics were much promoted when the cells were grown in the presence of rosiglitazone. As shown in Fig. 2*B*, the PGC-1α mRNA levels were somewhat augmented by chronic rosiglitazone treatment. Acute NE stimulation of these rosiglitazone-treated cultures caused a significant increment of PGC-1α expression (Fig. 2*C*).

The most striking feature of brown adipocytes continuously treated with rosiglitazone was, however, that they expressed UCP1 in the absence of NE stimulation, at levels several times higher than the corresponding levels induced by acute NE (Fig. 2*C*). Concurrent NE treatment also further increased UCP1 expression, but in *day 7* cells, the UCP1 mRNA level could not be further enhanced by NE (Fig. 2*C*); i.e., the chronic presence of rosiglitazone was sufficient to induce full differentiation of the cells in this respect in the absence of any adrenergic stimulation. There is thus convergence of the adrenergic and the PPARγ stimulation at this late step, and the question could be raised as to whether this was caused by early convergence: could rosiglitazone, e.g., by itself increase cAMP levels and through this cause UCP1 gene expression through classical pathways? We therefore examined cAMP levels in these cell cultures. We found low levels of cAMP in nontreated cells (~3 pmol/well), and the level was increased >10-fold after of 10 min NE stimulation (to 35 pmol/well). However, neither acute nor chronic treatment with rosiglitazone affected the cAMP levels in the cell cultures (not shown). Thus rosiglitazone did not induce UCP1 gene expression through early convergence with the classical cAMP pathway.

As seen, the acquisition of both general adipogenic and brown-fat specific characteristics of cultured brown preadipocytes was markedly accelerated by the continuous treatment with rosiglitazone (cf. Ref. 46). In terms of morphological (lipid accumulation) and gene expression features (the expression of aP2 and UCP1), the majority of rosiglitazone-treated cells already after 4 days in culture represented mature brown adipocytes. Most importantly, brown adipocytes grown in the presence of rosiglitazone expressed UCP1 even in the absence of NE stimulation. We also examined whether another PPARγ agonist, ciglitazone (30 μM), could similarly induce high UCP1 gene expression in the absence of adrenergic stimulation. This was the case (not shown), but quantitatively the level reached was somewhat lower than that observed with rosiglitazone. These experiments indicate that the effects on UCP1

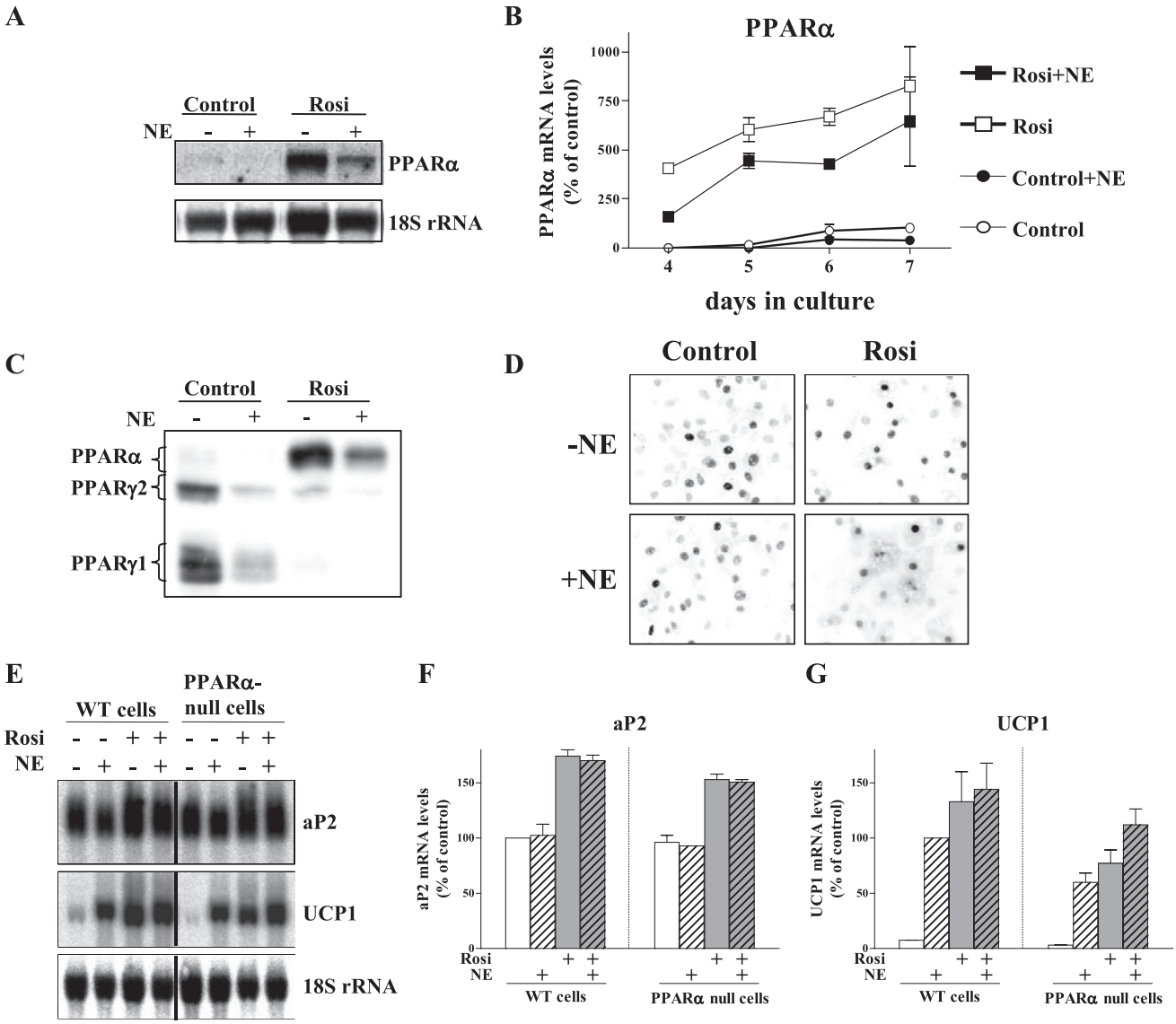

Fig. 3. Rosiglitazone effects on brown adipocytes are not mediated by PPARα. Cells as in Fig. 1 were analyzed in *A* and *B*. *A*: representative Northern blot, *day 6* of culture. Total RNA (10 μg) was used for each lane, and the blot was hybridized with the PPARα and 18S rRNA probes. *B*: PPARα mRNA levels during spontaneous and rosiglitazone-stimulated differentiation. The PPARα mRNA levels were normalized to the 18S rRNA levels in each sample. The points are means ± SE of 2 independent experiments, each performed in duplicate. The control at *day 7* was set in each experiment to 100%, and the PPARα mRNA levels on the other days were expressed relative to this value in each individual experiment. *C*: primary cultures of brown adipocytes were grown for 6 days in the absence or presence of 1 μM rosiglitazone. Where indicated, 1 μM NE was added 4 h before harvest. Whole cell lysates were analyzed by high-resolution SDS-PAGE and probed with an antibody that recognizes all PPAR protein isoforms (Santa Cruz Laboratories, sc-7273). PPARα protein was identified by analysis of total lysates of BAT, liver, and heart originating from PPARα-null mice (see below) and corresponding wild-type (WT) mice. The band corresponding to the markedly induced band in rosiglitazone-treated brown adipocytes could not be detected in BAT, liver and heart lysates of PPARα-null mice (not shown). Also, the same band was not detectable with a PPARγ-specific antibody (Cell Signaling, no. 2443; not shown). Thus the *top* band (resolvable from PPARγ only by high-resolution SDS-PAGE) represents PPARα protein. *D*: primary cultures of brown adipocytes were grown for 7 days in the absence or presence of 1 μM rosiglitazone. Where indicated, 1 μM NE had been present for 4 h. Brown adipocytes were analyzed by immuno-fluorescence cell staining using an antibody against PPAR (as in *C*). Cells were examined with a fluorescence microscope and representative negative images were processed (by applying identical parameters to all images) in Adobe Photoshop software. *E*, *F*, and *G*: expression levels of aP2 and UCP1 in control and rosiglitazone-treated brown adipocytes originating from wild-type and PPARα-null mice. Primary cultures of brown adipocytes originating from wild-type and PPARα-null mice were grown for 7 days in the absence (control) or presence of 1 μM rosiglitazone. PPARα-null mice were a kind gift from Dr. F. Gonzalez (32), backcrossed on a DBA background. As control mice, age-matched DBA mice were used. Where indicated, 1 μM NE had been added 2 h before harvest. *E*: representative Northern blot. Total RNA (10 μg) was used for each lane and the blot was hybridized with the aP2, UCP1, and 18S rRNA probes. aP2 (*F*) and UCP1 (*G*) mRNA levels in control and rosiglitazone-treated brown adipocytes originating from wild-type and PPARα-null mice. aP2 and UCP1 mRNA levels were normalized to the 18S rRNA levels in each sample. The values represent means and range of 1 experiment performed in duplicate (a second experiment yielded qualitatively identical results). The control level of aP2 and the NE-induced level of UCP1 in cultures originating from wild-type mice were set to 100%, and the aP2 and UCP1 levels in the other samples were expressed relative to this value.

gene expression are not limited to rosiglitazone but that probably any potent PPARγ agonist is able to induce high UCP1 gene expression in the absence of adrenergic stimulation. Thus rosiglitazone treatment not only promoted differentiation and enhanced NE-stimulated UCP1 gene expression in young cultures but could also, in itself, in the absence of NE, induce UCP1 gene expression to its full extent (Fig. 2). These results should be contrasted with earlier observations in which chronic

treatment of the brown adipocyte-like HIB-1B adipocytes with rosiglitazone (46) or darglitazone (39) was shown only to promote responsiveness of the UCP1 gene to later NE stimulation, and that acute treatment of primary brown adipocytes with pioglitazone (16), ciglitazone, or rosiglitazone (our unpublished results) induced UCP1 expression but not to its full extent; UCP1 gene expression was still highly responsive to NE stimulation.

The results obtained here can be seen as support for the hypothesis that physiologically the expression of UCP1 in nonsympathetically stimulated BAT may be explainable by the presence of ligand-activated PPARγ, i.e., by PPARγ that is maintained in an active state because of the presence of endogenous (still unknown) PPARγ ligands. Published in vivo studies have to date only investigated the effects of treatment in animals already sympathetically stimulated by exposure to a normal ambient temperature (rodent BAT is constantly sympathetically activated even at normal ambient temperatures). Positive responses were clearly seen on tissue hyperplasia and hypertrophy (4), but the effect on specific UCP1 expression reported in vivo has been absent or small. Thus the specific levels of UCP1 mRNA and protein (i.e., expressed per mRNA or protein units, respectively) were unchanged or only slightly increased (~60% as a mean of the articles quoted below) in BAT of rodents treated with PPARγ agonists (2, 7, 9, 15–17, 28–31, 36, 44, 46, 47) (however, total UCP1 content in BAT of treated animals was somewhat more increased, ~2-fold, due to tissue enlargement). In two recent investigations, robust 3-fold increases in specific UCP1 gene expression were reported (15, 29), demonstrating that also in vivo PPARγ agonists have the ability to affect UCP1 gene expression. However, even threefold increases are marginal compared with the ~1,000-fold increase in UCP1 gene expression seen here in the cell culture system (estimated from quantitative PCR measurements of RNA from cultures grown as those examined in Fig. 2*C*, *day 7*). Thus, in vivo, the true potency of PPARγ agonists to increase UCP1 gene expression seems to be masked, perhaps due to continuous exposure of the receptor to endogenous ligands or simply to the effect of the concomitant sympathetic activation. Indeed, we have observed that in cell cultures continuously stimulated with NE (as is the case for brown fat in vivo at normal ambient temperatures) for 7 days, additional chronic stimulation with rosiglitazone was unable to further increase UCP1 gene expression, implying that experimental conditions in vivo preclude any observation of the true potential of PPARγ agonists.

Rosiglitazone effects on brown adipocytes are not mediated by PPARα. Brown adipose tissue is a unique tissue in that it coexpresses high levels of all three PPAR receptors (α, β/δ, and γ; Ref. 6). PPARα is only found in brown and not in white adipose tissue (14, 20), and it has been reported to be expressed only after brown adipocyte differentiation has commenced (49). The distal enhancer of the UCP1 gene contains a PPAR responsive element that can bind either PPARα or PPARγ (27), and both PPARγ and PPARα agonists can induce UCP1 expression (2).

Surprisingly, we observed a very low expression of PPARα in cultured brown adipocytes both at the mRNA (Fig. 3, *A* and *B*) and the protein level (Fig. 3*C*). However, upon chronic exposure to rosiglitazone, gene expression was dramatically augmented (Fig. 3, *A* and *B*) so that the PPARα protein now represented the dominant PPAR isoform (Fig. 3*C*). Importantly, these changes in PPAR transcriptional machinery were not followed by alteration of their cellular localization, i.e., these PPAR proteins remained located in nuclei (Fig. 3*D*; both the dominant PPARα isoform and the repressed PPARγ isoform are recognized by the antibody). The absence of translocation of PPAR nuclear receptors into the cytoplasm under any of the circumstances studied indicates their active transcriptional role in brown adipocytes (43), as nuclear receptor translocation to the cytoplasm would evidently abolish the ability to control gene expression (52).

Based on these results, and the general concept that PPARα is a transcription factor regulating lipid catabolism (11), it could be suggested that the rosiglitazone effect on the differentiation of brown adipocytes was mediated through this induction of PPARα. To address this question, we utilized primary cultures of brown adipocytes originating from PPARα-null mice. As shown in Fig. 3, *E* and *F*, expression of the general adipogenic marker aP2 did not differ between mature brown adipocytes originating from wild-type and PPARα-null mice. Importantly, the UCP1 gene was responsive to rosiglitazone and NE treatment in both control and PPARα-null brown adipocytes (Fig. 3, *E* and *G*). Therefore, since the PPARα-null brown adipocytes were able to differentiate in the same way as were the wild-type cells, the rosiglitazone-induced increase in PPARα mRNA was not essential for the mechanism by which rosiglitazone increased UCP1 gene expression.

Chronically rosiglitazone-treated brown adipocytes are competent in demonstrating NE-induced UCP1-mediated respiration (thermogenesis). Thermogenesis (heat production) takes place in the mitochondria. Thermogenic responses in brown-fat mitochondria (34) and brown-fat cells (35) are fully UCP1 dependent. Physiologically, UCP1 is the only protein capable of mediating adaptive nonshivering thermogenesis in the cold (19), and it is essential for the recruitment of adaptive adrenergic nonshivering thermogenesis (18). However, mitochondrial biogenesis is also an essential part of BAT recruitment (3). Importantly, the UCP1 protein is rapidly degraded unless incorporated into mitochondria (38). If PPARγ activation is a competent alternative pathway for BAT recruitment, it is essential that PPARγ activation also increases the mitochondrial complement. Rosiglitazone has been shown to induce mitochondrial biogenesis and mitochondrial remodeling in white fat of *ob/ob* mice (51) and in fully differentiated 3T3-L1 adipocytes (50). Therefore, we examined whether the continuous rosiglitazone treatment was capable of increasing mitochondrial biogenesis in the cultured brown adipocytes and whether it enhanced mitochondrial brown fat-specific characteristics.

To estimate the amount of mitochondria, we used MitoTracker green, a membrane potential-independent mitochondrial-specific fluorescent dye. We compared mitochondrial staining in untreated and cultures continuously treated with rosiglitazone. In untreated cells, there was only a very faint staining with MitoTracker Green (Fig. 4*A*, *left*). However, cultures treated with rosiglitazone showed cells with intense staining, indicating a dramatic enhancement of mitochondrial biogenesis in these cells (Fig. 4*A*, *right*; it may be noted that in these cells, mitochondrial staining is often distinctly localized). Also, an increased content of subunit 4 of cytochrome oxidase

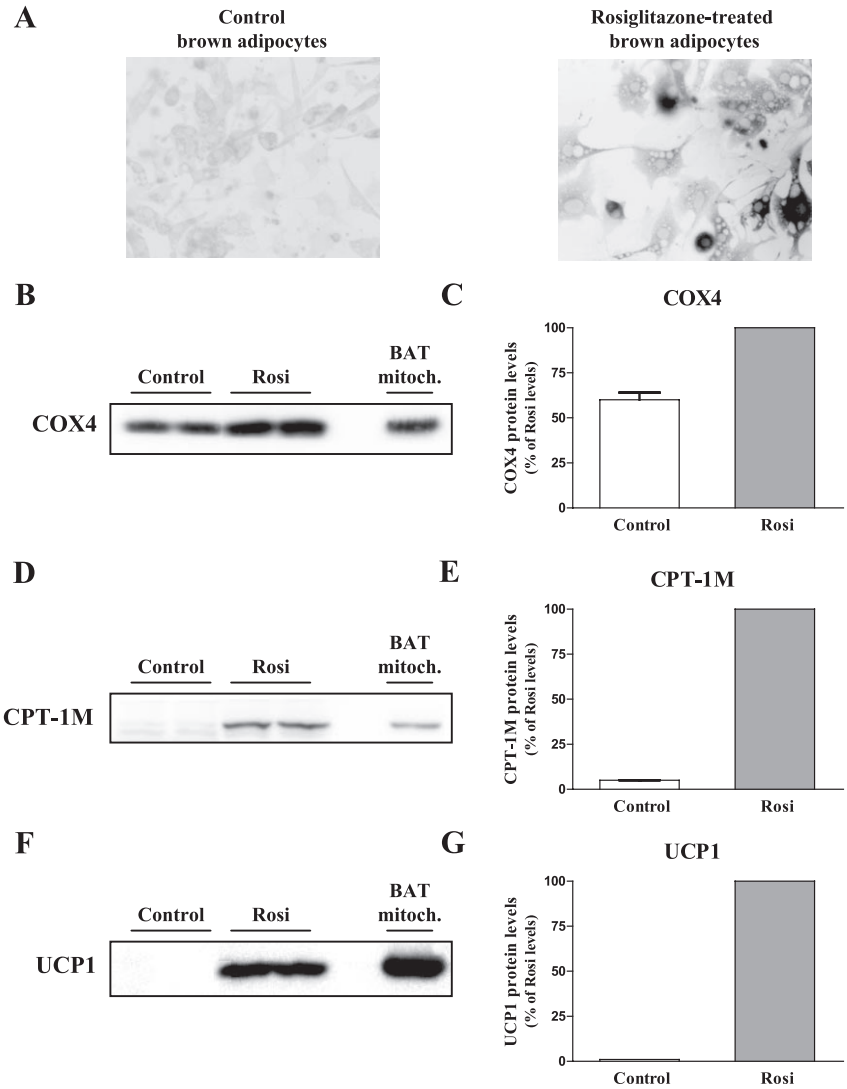

Fig. 4. Rosiglitazone promotes mitochondrial (mitoch.) biogenesis in brown adipocytes. Primary cultures of brown adipocytes were grown for 7 days in the absence (control) or presence of 1 μM rosiglitazone. *A*: control (*left*) and rosiglitazone-treated brown adipocytes (*right*) were analyzed for mitochondrial abundance by MitoTracker Green staining. Cells were examined with a fluorescence microscope and representative negative images were processed (by applying identical parameters to all images) in Adobe Photoshop software (thus MitoTracker Green fluorescence is represented as gray or black). *B–G*: protein levels of COX4, CPT-1M, and UCP1 in control and rosiglitazone-treated brown adipocytes. *B*, *D*, and *F*: representative Western blots. Total brown adipocyte lysate (30 μg of protein/lane) were examined by Western blot using antibodies against COX4 (*B*), CPT-1M (*D*), and UCP1 (*F*). COX4 (*C*), CPT-1M (*E*), and UCP1 protein (*G*) levels in control and rosiglitazone-treated brown adipocytes; isolated BAT mitochondria (5 μg of protein/lane) were included as markers. Values are means ± SE of 3 independent experiments, each performed in duplicate. The rosiglitazone-induced level was in each experiment set to 100%, and control levels were expressed relative to this value in each individual experiment.

(COX4) in the rosiglitazone-treated brown adipocytes (Fig. 4, *B* and *C*) demonstrates that these cells have enhanced respiratory capacity.

An even more pronounced effect of rosiglitazone was seen on the amount of carnitine palmitoyl transferase (muscle isoform, CPT-1M; Fig. 4, *D* and *E*). This enzyme mediates the transport of long chain fatty acids across the mitochondrial membrane, thus enabling lipid substrates to be used for respiration. CPT-1M was almost absent in untreated cells. However, rosiglitazone treatment induced remarkably high levels of CPT-1M (Fig. 4, *D* and *E*): the levels were increased 20 times. Thus the presence of a PPARγ agonist altered brown adipocyte mitochondria in both a quantitative (enhanced number) and, what is perhaps more important, a qualitative (remodelling) way.

The data presented in Fig. 2, *A* and *C*, clearly demonstrate that the presence of a PPARγ agonist enabled brown adipocytes differentiating in culture to fully express mRNA for the brown-fat cell-defining gene UCP1, but this may not necessarily result in high amounts of UCP1 protein (cf. Ref. 38). However, importantly, these very high levels of UCP1 mRNA were indeed reflected in remarkably high levels of UCP1 protein (Fig. 4, *F* and *G*). Thus rosiglitazone-treated brown adipocytes were well endowed with the cellular effectors of thermogenesis: an increased number of mitochondria and a relative increase in the mitochondrial proteins crucial for thermogenesis (UCP1), respiratory activity (COX4), and lipid substrate oxidation (CPT-1M).

The most crucial test of whether PPARγ activation truly represents a complete alternative recruitment pathway must, however, be that the brown adipocytes can display NE-induced thermogenesis. The thermogenic capacity of BAT is reflected in the ability of isolated brown adipocytes to respond to an addition of NE with an extremely large increase in oxygen consumption (thermogenesis) (8). To examine the thermogenic capacity of brown adipocytes differentiated in culture, we measured oxygen consumption of cells under basal conditions (before NE addition) and after stimulation with NE, as exemplified in Fig. 5*A*. The basal oxygen consumption rates of untreated and continuously rosiglitazone-treated brown adipocytes were similar (Fig. 5*B*). However, these cells responded very differently to the addition of NE (Fig. 5*B*): control brown adipocytes (that do not contain UCP1 protein; Fig. 4, *F* and *G*), as expected, did not increase oxygen consumption upon stimulation with NE (Fig. 5*A*). In contrast, NE addition led to a

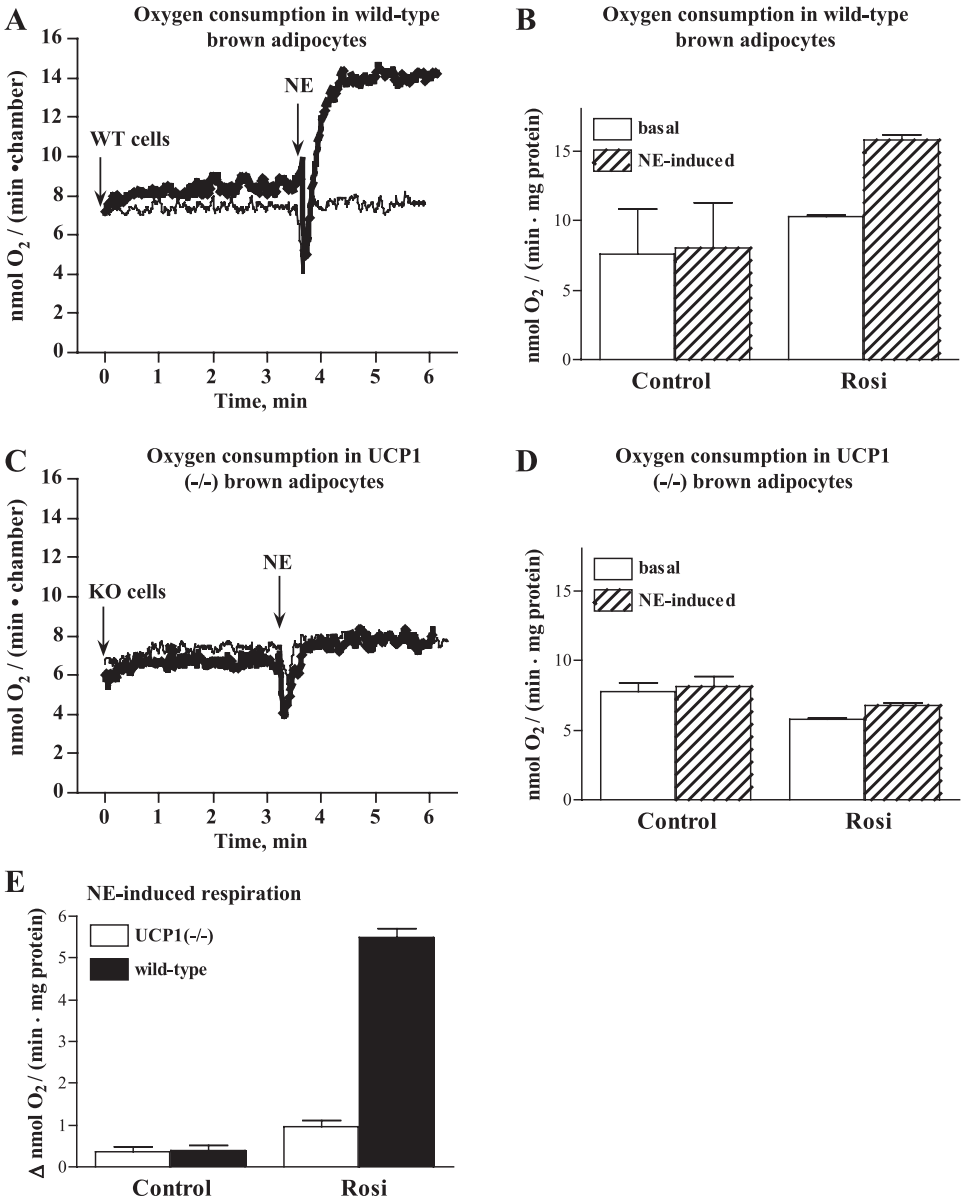

Fig. 5. Rosiglitazone-treated brown adipocytes demonstrate NE-induced UCP1-dependent thermogenesis. Primary cultures of brown adipocytes originating from wild-type and UCP1-ablated mice were grown for 7 days. Cells were continuously treated (from *day 0*) with 1 μM rosiglitazone or were kept untreated (control). The UCP1-ablated mice (13) had been backcrossed on to a C57Bl6 background. As wild-type mice, age-matched C57Bl6 mice were used. *A* and *C*: representative traces showing the effect of NE on oxygen consumption of control (thin line) and rosiglitazone-treated (thick line) brown adipocytes originating from wild-type (*A*) and UCP1-ablated (*C*) mice. At the arrow, 1 μM NE was added. *B* and *D*: basal (white bars) and NE-induced (hatched bars) levels of oxygen consumption in control and rosiglitazone-treated brown adipocytes originating from wild-type (*B*) and UCP1-ablated (*D*) mice. The polarographic output was time-differentiated, sampled, and recalculated per milligrams of total cellular protein. The values are means ± SE of two (wild-type cells) or three [UCP1(−/−) cells] independent experiments. *E*: NE-induced component of respiration (calculated as the difference between the rates of oxygen consumption before and after addition of NE) in control and rosiglitazone-treated brown adipocytes originating from wild-type (filled bars) and UCP1-ablated (open bars) mice.

rapid and marked increase in oxygen consumption in rosiglitazone-treated brown adipocytes (Fig. 5*A*).

To examine whether the NE-induced increase in oxygen consumption was UCP1-dependent, we performed the same experiment using brown adipocyte cultures originating from UCP1-ablated mice. NE addition to the UCP1(−/−) brown adipocytes did not lead to any significant increase in oxygen consumption rate (Fig. 5, *C* and *D*), neither in control nor in rosiglitazone-treated brown adipocytes. A comparison of the NE-induced component of respiration (Fig. 5*E*) between wild-type and UCP1(−/−) cells clearly demonstrates its UCP1-dependent nature. Thus we conclude that brown adipocytes grown and differentiated in culture in the presence of the PPARγ agonist rosiglitazone are competent to perform UCP1-dependent respiration (thermogenesis). Thus brown-fat cells differentiated in culture are capable, upon stimulation with NE, of demonstrating UCP1-dependent thermogenesis, and PPARγ activation constitutes a fully competent recruitment pathway.

Conclusion. Sympathetically mediated recruitment of BAT cannot explain all of the physiological conditions that demonstrate enhanced BAT recruitment. There are at least two physiological contexts where this pathway is contraindicated: prenatal and prehibernation recruitment. In these physiological conditions, brown fat needs to be recruited and, what is of great importance, not simultaneously thermogenically activated (which would be expected as a consequence of adrenergic stimulation). Figuratively, BAT recruited in this way represents a nonburning stove that waits for the spark, an adrenergic stimulus, to initiate the heat production. In the present study, using a brown adipocyte culture system as a model, we demonstrate that PPARγ activation represents such a mechanism for BAT recruitment: nonadrenergic, potent, and fully competent.

Thus continuous treatment with the PPARγ agonist rosiglitazone markedly enhanced brown-fat specific characteristics of brown adipocytes in such a way that they became qualitatively different from untreated brown adipocytes: brown-fat specific

genes (UCP1 and PPARα) were highly expressed, the amount of mitochondria was significantly increased, and the competence to demonstrate NE-induced UCP1-dependent thermogenesis was fully established. Thus essential recruitment processes in brown adipocytes were remarkably promoted when they were grown and differentiated in the presence of a PPARγ activator.

Provided that PPARγ activation is a relevant physiological pathway for brown adipocyte recruitment under certain important physiological conditions, questions may be raised as to under which conditions this pathway is activated, what is the nature of the endogenous PPARγ activator (which is in general a still unresolved issue; Ref. 45), and which signaling pathway leads to the production of this activator.

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