Brown adipose tissue (BAT) functions in non-shivering and diet-induced thermogenesis via its capacity for uncoupled mitochondrial respiration. BAT dysfunction in rodents is associated with severe defects in energy homeostasis, resulting in obesity and hyperglycemia. Here, we report that the nuclear receptor peroxisome proliferator-activated receptor γ (PPARC), a prostaglandin-activated transcription factor recently implicated as a central regulator of white adipose tissue differentiation, also regulates brown adipocyte function. PPARγ is abundantly expressed in both embryonic and adult BAT. Treatment of CD-1 rats with the PPARγ-selective ligand BRL49653, an anti-diabetic drug of the thiazolidinedione class, results in marked increases in the mass of interscapular BAT.

In vitro, BRL49653 induces the terminal differentiation of the brown preadipocyte cell line HIB-1B as judged by both changes in cell morphology and expression of uncoupling protein and other adipocyte-specific mRNAs. These data demonstrate that PPARγ is a key regulatory factor in brown adipocytes and suggest that PPARγ functions not only in the storage of excess energy in white adipose tissue but also in its dissipation in BAT.

Two types of adipose tissue have been described. White adipose tissue (WAT) is specialized to store triglycerides and to release free fatty acids in response to changing energy requirements. A second type of adipose tissue, termed brown adipose tissue (BAT), is involved in the dissipation of energy via the generation of heat (see below). This unique thermogenic activity of BAT is tightly regulated and can be induced in response to either cold exposure or hyperphagia (1, 2). In rodents, several lines of evidence suggest that BAT plays a central role in maintaining energy balance. First, by increasing energy expenditure in response to increased food intake, BAT thermogenesis acts to prevent (or deter) the development of obesity (3).

Second, transgenic ablation of BAT in mice is sufficient to induce obesity as well as insulin resistance and other metabolic disorders that, as a whole, closely resemble human non-insulin-dependent diabetes mellitus (5–7). Third, defects in BAT function are thought to play a significant role in the development of obesity and diabetes in several animal models (8, 9). These data suggest a tight link between BAT function and the regulation of glucose and lipid metabolism.

The unique thermogenic activity of BAT results from the action of a BAT-specific protein termed uncoupling protein (UCP). UCP is a mitochondrial proton translocator that uncouples fatty acid oxidation from ATP synthesis, releasing the energy as heat (10, 11). Analysis of the UCP gene has resulted in the identification of a 220-bp enhancer located from 2.3 kilobase pairs upstream of the UCP gene that is responsible for brown adipocyte-specific gene expression in cell culture models (12, 13). This enhancer region contains a cAMP response element as well as thyroid hormone receptor and retinoid receptor response elements (12–15). Thus, multiple signaling pathways converge to regulate UCP gene expression.

Recently, we identified a response element in the UCP enhancer for peroxisome proliferator-activated receptor γ (PPARγ), a member of the steroid/retinoid/thyroid hormone receptor superfamily of ligand-activated transcription factors (16). The PPARγ subtype is selectively expressed in white adipose tissue (17, 18) where it has been shown to play a pivotal role in the differentiation of white adipocytes (17, 19, 20). PPARs modulate expression of target genes by binding to short DNA sequences, termed PPAR response elements, at heterodimers with the retinoid X receptors (21–24). PPAR response elements have been identified in the regulatory regions of several genes encoding proteins involved in energy balance (25), including UCP (16). These data indicate a primary role for the PPARs in modulating energy homeostasis.

We and others recently demonstrated that naturally occurring prostanooids, including the PGJ2 metabolite 15-deoxy-
\[\Delta^4_{12,14}\]-PGJ2, bind and activate PPARγ (26–28). In addition to being activated by these prostanooids, PPARγ is also activated by a class of synthetic compounds termed thiazolidinediones (TZDs) (28–30). These agents are promising anti-diabetic drugs that increase peripheral insulin responsiveness and glucose tolerance in both animal models of non-insulin-dependent diabetes mellitus and in man (31–33). In addition to their anti-hyperglycemic effects, these compounds also dramatically reduce circulating levels of triglycerides and non-esterified free fatty acids. The unexpected finding that TZDs are high affinity PPARγ ligands suggested a role for this nuclear receptor in
glucose as well as lipid homeostasis.

The identification of a PPARγ response element in the UCP enhancer raised the possibility for a role for PPARγ in BAT function. In this report, we show that PPARγ is expressed at high levels in BAT and that TZD activation of PPARγ promotes brown adipocyte growth in vitro and in vivo. These data indicate a central role for PPARγ in BAT formation and, furthermore, suggest that BAT may be a target tissue for the lipid- and glucose-lowering effects of the TZDs.

EXPERIMENTAL PROCEDURES

In Situ Hybridization Analyses—Digoxigenin-labeled riboprobes were generated using Riboprobe Plus™ 4 RNA labeling kit from Boehringer Mannheim. PPARγ sense and antisense probes were generated from a plasmid containing full-length mouse PPARγ1 inserted into pBS-SK. UCP probes were generated from a plasmid containing the 1.2-kilobase pair EcoRI fragment of the rat UCP cDNA inserted into pBS-RS. Probes of average size (200 base pairs) were prepared by partial alkaline denaturation, and washed according to the manufacturer’s instruction. UCP, GPD, PPARγ, and 36B4 cDNA probes were labeled with [α-32P]dATP (3000 Ci/mmol) by the random priming method (35).

Cell Culture—Heat-inactivated sera were used for culture of HIB-1B cells. HIB-1B preadipocytes were maintained in Dulbecco’s modified Eagle’s medium (DMEM, Life Technologies, Inc.) plus 10% calf serum (Life Technologies, Inc.) by capillary action. The membrane was cross-linked, and washed according to the manufacturer’s instruction. UCP, GPD, PPARγ, and 36B4 cDNA probes were labeled with [α-32P]dATP (3000 Ci/mmol) by the random priming method (35).

RESULTS

PPARγ Is Expressed in Embryonic and Adult Marine Rat—

PPARγ has been shown to be specifically expressed at high levels in epididymal WAT, where it functions as a key regulator of adipocyte determination and differentiation (17, 20). Previous studies have shown that PPARγ expression begins at 13.5 days postconception; however, the site of this expression was not investigated (37). We investigated the site for fetal PPARγ expression by in situ hybridization to day 18 (E18) mouse embryos. As shown in Fig. 1, PPARγ mRNA was detected in the interscapular and cervical regions of the embryo (Fig. 1, A and B), areas known to contain BAT. In situ analysis performed with a BAT-specific UCP probe revealed the co-localization of the UCP and PPARγ transcripts in the cervical region (Fig. 1, C–E) and I-BAT (data not shown). Thus, PPARγ expression is localized to BAT in day 18 mouse embryos.

The selective expression of PPARγ in embryonic BAT led us to investigate whether PPARγ is expressed in adult BAT. Northern blot analysis of RNA isolated from several tissues of adult mice revealed that PPARγ mRNA levels are comparable in BAT and WAT (Fig. 2). As controls, we observed high level expression of the adipocyte-specific lipid binding protein αP2 in BAT and WAT while UCP expression was observed only in BAT. The blot was also probed with 36B4 (a ribosomal protein probe) cDNA to demonstrate equivalency of loading and hybridization (38). The identification of BAT as a site for high level expression of PPARγ suggests that activation of this nuclear hormone receptor may play an important regulatory role in BAT development and function.

Treatment with BRL49653 Promotes I-BAT Hyperplasia in CD-1 Rats—Since PPARγ is a ligand-activated nuclear receptor, we investigated the effects on BAT of chronic PPARγ activation by treating CD-1 rats for 4 weeks with the PPARγ-selective ligand, BRL49653. Interestingly, marked increases in the I-BAT mass were observed in the BRL49653-treated versus vehicle-treated animals upon necropsy. Measurement of tissue wet weights following dissection revealed a greater then 3-fold increase in the amount of I-BAT in BRL49653-treated animals relative to control animals (Table 1). I-BAT comprised 0.44% of the total body weight in BRL49653-treated rats and 0.14% of the total body weight in vehicle-treated rats (Table 1). We confirmed that the tissue was BAT via Northern analysis using UCP as a probe; as shown, the I-BAT from BRL49653- and vehicle-treated rats expressed comparable amounts of UCP mRNA (Fig. 3). β-Actin mRNA levels were also determined as a control. Analysis of I-BAT sections via light microscopy revealed no increases in cell size in BRL49653- versus vehicle-treated animals (data not shown), suggesting that the increases in I-BAT wet weight were due to tissue hyperplasia and increased brown adipocyte differentiation.

Activation of HIB-1B Cell Differentiation by the PPARγ-selective Ligand BRL49653—To further examine the effects of BRL49653 activation of PPARγ on brown adipocyte function, we used the HIB-1B brown preadipocyte cell line. HIB-1B cells can be maintained as preadipocytes that express PPARγ at low levels (16) and can be induced to differentiate by treatment with a mixture of adipogenic compounds (see “Discussion”) (39, 40). Differentiation of the HIB-1B cells is reflected by the induction of adipocyte-specific gene expression as well as morphologic changes that include the cells becoming spherical. HIB-1B adipoцитesthat are not preadipocytes express high levels of UCP mRNA following adrenergic receptor stimulation (39, 40). Thus, HIB-1B cells represent a useful model system to study brown adipocyte differentiation.

To determine whether PPARγ regulates brown adipocyte differentiation, HIB-1B preadipocytes were cultured in CT-
FCS. The charcoal and dextran treatment removes small, lipophilic molecules that might activate PPARγ. After the cells reached confluence, they were treated with either 1 mM high affinity PPARγ ligand BRL49653 or vehicle (Me2SO). BRL49653 does not activate the PPARα or PPARδ subtypes at this concentration (28, 30, 41). The cells were examined by phase contrast microscopy daily, and morphological changes were first noted 2 days following BRL49653 treatment (data not shown). Six days postconfluence, the BRL49653-treated cells were approximately 80% differentiated as assessed by their spherical shape, while the vehicle-treated cells remained fibroblastic in appearance (Fig. 4A).

Differentiation of the HIB-1B cells was also examined by Northern analysis. RNA samples from Me2SO- or BRL49653-treated cells were isolated daily from confluence until 5 days postconfluence (Fig. 4B). Four hours prior to harvest, the cells were treated with 100 nM norepinephrine to elevate cAMP levels and induce UCP mRNA expression. We examined the levels of mRNA for the adipocyte-specific fatty acid binding protein (aP2), C/EBPα, and GPD; all three genes are known to be activated in a differentiation-dependent manner in 3T3-F442A adipocytes (42–44). Increased expression of aP2, C/EBPα, and GPD mRNAs was observed after 1 day of drug treatment and increased dramatically by the 5th day (Fig. 4B). Expression of UCP was readily detected by the 3rd day of drug treatment and was increased dramatically by day 5 of BRL49653 treatment (Fig. 4B). Expression of the control, 36B4 mRNA, was unaffected by the treatment. We conclude from these data that activation of PPARγ by BRL49653 results in the differentiation of HIB-1B cells to mature brown adipocytes as judged by both morphologic and molecular criteria.

We next investigated whether continuous treatment of HIB-1B cells with BRL49653 is required for induction and maintenance of the adipocyte phenotype. Confluent HIB-1B cells were incubated for 36 h in CT-FCS medium containing either 1 mM BRL49653 or 0.1% Me2SO as vehicle control. The cells were subsequently cultured for 2.5 days in CT-FCS medium and then for 3 days in untreated FCS. In the BRL49653-treated cultures 80% of the cells appeared differentiated, i.e. spherical shape, while less than 10% of the cells in the Me2SO-treated cultures appeared to be differentiated (data not shown). As shown in Fig. 4C, the mRNA levels of the four differentiation-dependent genes were substantially increased in the BRL49653-treated cells while 36B4 mRNA levels were not affected by the BRL49653 treatment. These data demonstrate that transient activation of PPARγ is sufficient to commit the
PPARγ Promotes Brown Adipocyte Differentiation

TABLE I
BRL49653 treatment increases I-BAT mass in CD-1 rats

|               | BRL49653 | Control |
|---------------|----------|---------|
| Day 0         | Day 28   | Day 0   | Day 28   |
| Body weight (g) | 324 ± 7.57 | 343 ± 8.80 | 326 ± 5.09 | 329 ± 6.25 |
| I-BAT (g)      | 1.55 ± 0.181 | 0.44 ± 0.043 | 0.44 ± 0.422 | 0.14 ± 0.011 |
| Body weight (%) | 0.00005   | 0.00005  | 0.0005   | 0.0422 |

* p < 0.0005.

Fig. 3. BRL49653 treatment causes I-BAT hyperplasia in CD-1 rats. For BRL49653-treated (B) rats, body weight increased 1.55-fold from Day 0 to Day 28. This response required the PPARγ agonist BRL49653 (B) in the presence of the UCP enhancer (URE1) that responds to the differentiation state of the cells (16). This enhancer contains a consensus cAMP response element (CRE2) that responds to β-adrenergic receptor activation (13) and contains a PPARγ response element (URE1) that responds to the differentiation state of the cells (16).

The presence of a PPARγ response element in the UCP enhancer suggested that anti-diabetic TZDs, which function as PPARγ ligands, might modulate UCP gene expression through the enhancer elements. To explore this possibility, HIB-1B preadipocytes were transfected with a CAR reporter construct driven by the UCP enhancer and a minimal UCP promoter (220/−73CAT) or a similar construct containing a mutated URE1 site (220mURE1/−73CAT) (16). Transfected cells were treated with either BRL49653 or Btc2cAMP, or both, or MeSO. The resulting CAT activity is presented in Fig. 5. Treatment with either BRL49653 or Btc2cAMP increased reporter expression roughly 5-fold. Notably, treatment with both compounds together resulted in a synergistic increase in reporter expression (>17-fold). This response required the PPARγ response element URE1, as deletion of the entire enhancer (−73CAT) or specific mutation of URE1 (220mURE1/−73CAT) abolished the effects of these compounds on reporter gene expression (Fig. 5). These data demonstrate that the PPARγ and cAMP signaling pathways cooperate in the activation of UCP gene expression in HIB-1B cells.

**DISCUSSION**

It is well established that PPARγ plays a central role in the differentiation of white adipocytes. However, whether PPARγ functions in an analogous role in brown adipocytes had not been examined. In this report, we present three lines of evidence that indicate that PPARγ also regulates the differentiation and function of brown adipocytes. First, PPARγ is highly expressed in both embryonic and adult BAT in rodents. Second, the PPARγ-selective ligand BRL49653 induces dramatic increases in the mass of the interscapular BAT depot in rats. Third, BRL49653 promotes the efficient differentiation of the HIB-1B brown preadipocyte cell line. The finding that PPARγ is involved in the differentiation of both brown and white adipocytes, which are involved in the dissipation and storage of excess energy, respectively, suggests an important role for this nuclear receptor in regulating systemic energy balance.

In addition to its effects on HIB-1B cell differentiation, we have shown that BRL49653 can activate the UCP enhancer in transfected HIB-1B cells. This suggests that PPARγ may directly modulate the thermogenic capacity of BAT. Moreover, we show that BRL49653 synergizes with Btc2cAMP in the activation of the UCP enhancer. These data suggest that PPARγ ligands, such as TZDs and prostanoids, might cooperate with agents that elevate cAMP levels, such as catecholamines and β-agonists, to regulate thermogenesis in vivo. This cooperativity might be relevant in vivo since the increased lipolysis that occurs in response to β-adrenergic receptor activation would provide increased levels of fatty acids that may be endogenous activators of PPARγ (see below). Indeed, two previous reports provided evidence for interactions between the PPARγ and the β-adrenergic signaling pathways in the induction of thermogenesis. The TZD ciglitazone, a known PPARγ activator, was found to potentiate the thermogenic effects of the β-adrenergic receptor agonist BRL26830 in lean rats (45). Furthermore, the β-adrenergic receptor antagonist propranolol was found to block ciglitazone-induced increases in basal metabolic rates in rats (46). Interestingly, both TZDs and β-adrenergic receptor agonists are known to have significant anti-diabetic effects and exert marked effects on BAT morphology in rodents. Our data showing that TZDs and Btc2cAMP synergize in the induction of UCP gene expression suggest a molecular basis for the reported coupling of the TZD and β-adrenergic signaling pathways in vivo.

Our finding that PPARγ is a key regulator in brown adipocytes has two possible physiologic implications. First, in addition to its classical role in cold-induced thermogenesis, BAT also modulates changes in metabolic rate associated with variations in food intake (3). Chronic hyperphagia, or even a single meal, can increase BAT thermogenesis and consequently raise the basal metabolic rate (47). The importance of BAT in overall energy homeostasis is underscored by the finding that ablation of BAT in mice results in severe obesity accompanied by insulin resistance, hyperglycemia, hyperlipidemia, and hypercholesterolemia (5–7). Our finding that PPARγ functions as a regulator of BAT differentiation and UCP gene expression suggests a novel means for coupling dietary signals to BAT thermogenic activity. As PPARγ is activated by a variety of fatty acids and prostanoids (28–29), an increase in serum lipid concentrations due to feeding might lead directly to the activation of PPARγ in BAT. Alternatively, dietary stimulation of the sympathetic nervous system could result in increased lipolysis in BAT and consequent increases in PPARγ ligand concentrations. Ligand-activated PPARγ could in turn stimulate UCP gene expression in pre-existing BAT and promote the differentiation of nascent brown adipocytes. In this manner, PPARγ might serve as a
molecular link between nutritional state and energy expenditure via BAT.

A second possible implication of this study relates to the mechanism of action of the anti-diabetic TZDs. While their therapeutic properties are well documented, their mechanism of action has remained obscure. In this report, we show that treatment of wild-type rats with the potent PPARγ ligand BRL49653 results in a marked increase in the mass of the interscapular BAT. BAT is known to be relatively atrophied and thermogenically quiescent in several rodent models of diabetes and obesity. It has been proposed that this BAT dysfunction contributes to the metabolic efficiency and resulting obesity and diabetes in these animals (48, 49). Our findings raise the intriguing possibility that BAT may be a target tissue for the actions of BRL49653 and other TZDs in rodents. Interestingly, the TZD ciglitazone has also been reported to partially restore GDP binding activity, a measure of BAT thermogenic activity, in brown adipocytes isolated from obese and diabetic (ob/ob) mice (50). Taken together, these data suggest that BAT may represent a target tissue for the palliative effects of TZDs on circulating glucose and lipid levels in rodent models of diabetes.

In summary, we have shown that PPARγ plays a central role in the differentiation of BAT. These data further establish the importance of this nuclear receptor in the initiation of adipogenesis. Moreover, our data suggest that PPARγ may serve as a molecular means for maintaining energy balance, coordinating the storage of excess energy in WAT and its dissipation in BAT. Thus, the modulation of levels of PPARγ and/or its ligand may afford novel therapeutic opportunities for the treatment of metabolic diseases that affect glucose and lipid homeostasis.

Acknowledgments—We thank Peter Tontonoz for helpful discussions; Larry Hamacher, Jim Lenhard, and Linda Moore for communicating unpublished results; Jeri Edwards, Barbara Munch, Michelle

FIG. 4. BRL49653 activation of PPARγ promotes brown pre-adipocyte differentiation. A, HIB-1B preadipocytes were treated with 1 μM BRL49653 or vehicle (0.1% Me₂SO (DMSO)) alone for 6 days and photographed under phase contrast microscopy (×200). B, HIB-1B preadipocytes were treated at confluence with 1 μM BRL49653 or vehicle. 10 μg of total RNA isolated at different days postconfluence was loaded onto a gel, blotted to nylon membrane, and hybridized with

FIG. 5. BRL49653 activates the UCP enhancer in HIB-1B preadipocytes. HIB-1B preadipocytes were transfected with 8 μg of CAT reporter constructs −73CAT, 220/−73CAT, and 220mURE1/−73CAT. Twelve hours after transfection the cells were treated with Bt₂cAMP or BRL49653, or both, or 0.1% Me₂SO as vehicle control. The resulting CAT activity is presented as percent conversion. The results from three separate experiments are reported.

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32P-labeled UCP, aP2, C/EBPα, and GPD cDNA probes. Equal loading of RNA in each lane was determined by hybridization to a 36B4 cDNA probe. BRL, BRL49653; DMSO, Me₂SO as vehicle control. All cells were treated with 100 nM norepinephrine 4 h prior to RNA isolation. C, HIB-1B preadipocytes were treated at confluence with 1 μM BRL49653 or 0.1% Me₂SO for 36 h. The cells were cultured for another 2.5 days in CT-FCS media and then cultured in normal FCS medium for 3 days. Cells were treated with norepinephrine for 4 h before collection. 10 μg of total RNA was analyzed as described above. B, BRL49653; D, Me₂SO as vehicle control.
Kim, and Betty Gaskill for technical assistance; and Thomas Perlmann and Chiayang Wang for critical reading of the manuscript.

**Note Added in Proof**—Recently, it was shown that the thiazolidinedione pioglitazone synergizes with norepinephrine to increase UCP mRNA levels in primary brown adipocytes (Foellmi-Adams, L. A., Wyse, B. M., Herron, D., Nedergaard, J., and Kletzien, R. F. (1996) Biochem. Pharmacol. 52, 693–701)

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