Peroxisome Proliferator-activated Receptor α Activates Transcription of the Brown Fat Uncoupling Protein-1 Gene

A LINK BETWEEN REGULATION OF THE THERMOGENIC AND LIPID OXIDATION PATHWAYS IN THE BROWN FAT CELL*

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High expression of the peroxisome proliferator-activated receptor α (PPARα) differentiates brown fat from white, and is related to its high capacity of lipid oxidation. We analyzed the effects of PPARα activation on expression of the brown fat-specific uncoupling protein-1 (ucp-1) gene. Activators of PPARα increased UCP-1 mRNA levels severalfold both in primary brown adipocytes and in brown fat in vivo. Transient transfection assays indicated that the (−4551)/UCP1-CAT construct, containing the 5′-regulatory region of the rat ucp-1 gene, was activated by PPARα co-transfection in a dose-dependent manner and this activation was potentiated by Wy 14,643 and retinoid X receptor α. The coactivators CBP and PPARγ-coactivator-1 (PGC-1), which is highly expressed in brown fat, also enhanced the PPARα-dependent regulation of the ucp-1 gene. Deletion and point-mutation mapping analysis indicated that the PPARα-responsive element was located in the upstream enhancer region of the ucp-1 gene. This −2485/−2458 element bound PPARα and PPARγ from brown fat nuclei. Moreover, this element behaved as a promiscuous responsive site to either PPARα or PPARγ activation, and we propose that it mediates ucp-1 gene up-regulation associated with adipogenic differentiation (via PPARγ) or in coordination with gene expression for the fatty acid oxidation machinery required for active thermogenesis (via PPARα).

The peroxisome proliferator-activated receptor α (PPARα) is a fatty acid-activated transcription factor that plays a key role in the transcriptional regulation of genes involved in cellular lipid metabolism (1). PPARα together with PPARγ and PPARβ/δ belong to a subgroup of the nuclear hormone receptor superfamily that heterodimerizes with the 9-cis-retinoic acid receptors (RXRs) (2–5). The PPAR-RXR heterodimer binds to specific response elements (PPREs), which consist of a direct repeat of the consensus half-site motif spaced by one nucleotide (DR-1) (6). Fatty acids, peroxisome proliferators, and fibrate hypolipemic drugs can activate PPARα (1, 4), and natural (leukotriene B4) or synthetic (fibrate Wy 14,643) specific ligands for PPARα have been identified (7). In contrast, 15-deoxy-D12,14-prostaglandin J2 and thiazolidinedione anti diabetic agents are selective ligands for PPARγ (8–10). In addition to ligand selectivity, PPAR subtypes have been involved in different biological functions. PPARα is mostly expressed in tissues with high rates of fatty acid oxidation and peroxisomal metabolism, such as brown fat, liver, or heart (1, 11). Recent studies of PPARα-null mice have confirmed that PPARα is necessary in vivo for hepatic fatty acid oxidation and ketone body synthesis during starvation (12). PPARδ, which is ubiquitously expressed, seems to be involved in basic lipid metabolism (11). High expression of PPARγ is mainly restricted to white (WAT) and brown (BAT) adipose tissue (13). Hence, in contrast to the role of PPARα in cellular lipid catabolism, PPARγ regulates adipogenesis (i.e. lipid deposition) (13, 14).

BAT is a major site for nonshivering thermogenesis in mammals. Its thermogenic capacity relies on the presence of an inner mitochondrial protein uniquely expressed in brown adipocytes, the uncoupling protein (UCP) (15), now referred to as UCP-1 since the discovery of the more widely expressed UCP-2 and UCP-3 (for review, see Ref. 16). Brown fat thermogenesis is mainly controlled by norepinephrine released from sympathetic terminals innervating the tissue, although nuclear receptor-mediated pathways have also been described. Thus, activation of PPARγ promotes HIB-1B brown adipocyte differentiation (17), and up-regulates ucp-1 gene expression (18). Furthermore, we demonstrated that retinoic acid is a powerful inducer of ucp-1 gene transcription, acting through retinoic acid receptors and RXRs (19, 20). The 5′-flanking region of the rat ucp-1 gene contains the proximal regulatory promoter, including C/EBP-regulated sites (21) and the main cAMP-regulatory element (22), and an upstream enhancer involved in complex regulation by retinoic acid receptors, RXR, and thyroid hormone nuclear receptors (19, 20, 23). A site responsive to PPARγ activators has also been located in the upstream enhancer of the murine ucp-1 gene (18).

BAT highly coexpresses not only PPARγ and PPARδ subtypes but also PPARα (24). BAT stores triglycerides but, in contrast to WAT, it uses lipids as oxidative substrates to generate heat. Since PPARα induces the expression of fatty acid oxidation enzymes in tissues other than BAT (6), it may do so in BAT in association with thermogenic requirements. Here we
report that PPARα activators induce ucp-1 gene expression in brown adipocytes and in BAT in vivo, acting through a PPRE located in the upstream enhancer of the ucp-1 gene that is also responsible for PPAR-dependent regulation. PPARα is proposed to coordinate the activation of lipid oxidation and thermogenic activity in brown fat.

**EXPERIMENTAL PROCEDURES**

**Materials**—Wy 14,643 (pirimic acid) and 15-deoxy-Delta(12,14)-prostaglandin J2 were obtained from Cayman Chemicals. Troglitazone and BRL 49653 were kind gifts from Dr. T. LeF (Parke-Davis Research) and Dr. L. Castella (Ciba-Geigy, Basel, Switzerland) respectively. Clofibrate, fenofibrate, thyronine, and methoprene were obtained from Sigma. Methoprene was from Dr. L. Casteilla (Toulouse, France). RNeasy Mini Kit was extracted using the RNeasy Mini Kit, kindly provided by Dr. B. Spiegelman, was cultured in each respective binding reaction. When indicated, 1 μg of PPAR agonists resulted in a 2-fold (15-deoxy-Delta(12,14)-prostaglandin J2) to 8-fold (10 μM BRL 49653) increase in UCP-1 mRNA levels. When expressed in each culture, 30 μM Wy 14,643, 10 μM BRL 49653, or 30 μM Wy 14,643 was used to maintain a constant concentration of 0.5 μg/ml of ruxigor in vitro. For the gel retardation assays, the UCP1-PPRE oligonucleotide was used to assess the transcriptional activity of PPARα activators in each culture.

**Cell Culture**—Primary culture of differentiated brown adipocytes was performed as described previously (19), and grown in 5 ml of Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum, 2 mM glutamine, 10% heat-inactivated fetal calf serum, and 100 units of penicillin. Cells were also exposed to various PPAR agonists for 24 h, except for 15-deoxy-Delta(12,14)-prostaglandin J2 which was added at a final concentration of 10 μM for 6 h.

**RESULTS**

**Activators of PPARα Induce the Expression of the ucp-1 Gene in Differentiated Brown Adipocytes**—To analyze whether PPARα agonists modulate the expression of the ucp-1 gene, primary cultures of murine brown adipocytes were used since they express all three PPAR subtypes (24). As shown in Fig. 1, exposure of brown adipocytes differentiated in culture (day 9) to PPARα activators resulted in a 2-fold (15-deoxy-Delta(12,14)-prostaglandin J2) to 8-fold (10 μM BRL 49653) increase in UCP-1 mRNA levels. When PPARα activators, such as several fibrates and the PPARα-specific ligand Wy 14,643, were tested an even higher (3–12-fold) increase in UCP-1 mRNA expression was detected. In contrast, COII mRNA expression did not respond in any of the three plasmids. PPAR activation resulted in a 2-fold (15-deoxy-Delta(12,14)-prostaglandin J2) to 8-fold (10 μM BRL 49653) increase in UCP-1 mRNA levels. When PPARα activators, such as several fibrates and the PPARα-specific ligand Wy 14,643, were tested an even higher (3–12-fold) increase in UCP-1 mRNA expression was detected. In contrast, COII mRNA expression did not respond to PPAR activators, thus indicating that the effect of PPAR activators is specific for UCP-1 mRNA.

**Exposure to Wy 14,643 led to a time-dependent increase in UCP-1 mRNA expression (Fig. 2A) and maximum induction was observed at 10 μM Wy 14,643 (Fig. 2B).**
The Stimulation of ucp-1 Gene Expression by the PPARα Ligand Wy 14,463 Is Independent of Protein Synthesis and Synergizes with the Effects of an RXR-specific Agonist—Brown adipocytes were exposed for 12 h to 10 μM Wy 14,463 in the absence or presence of 5 μg/ml cycloheximide, an inhibitor of protein synthesis (Fig. 3A). Cycloheximide treatment led to lower basal expression of UCP-1 mRNA, as already described (19), but it did not affect the ability of Wy 14,463 to increase UCP-1 mRNA.

When the effects of the RXR-specific agonist methoprene were analyzed (Fig. 3B), results showed that besides its reported direct action upon UCP-1 mRNA expression (20), there was a synergistic effect when both the PPARα and the RXR ligands were added, suggesting a PPARα-RXR heterodimer-mediated effect on ucp-1 gene expression.

PPARα Induces the Rat ucp-1 Gene Promoter Activity—Primary brown adipocytes were transiently transfected with a plasmid containing the upstream 4.5 kb of the rat ucp-1 gene fused to a CAT reporter gene. As shown in Fig. 4, PPARα activators increased the (−4551)UCP1-CAT activity at least 2-fold, in the same range of the effect caused by BRL 49653. Responsiveness of (−4551)UCP1-CAT to PPAR activators was enhanced 6-fold by co-transfection of the expression vector for PPARα. Thus, expression of both endogenous ucp-1 gene and transfected ucp-1 gene promoter are up-regulated by PPARα activators in primary brown adipocytes.

RXRα Enhances the PPARα-dependent Induction of the ucp-1 Gene Promoter—To further investigate the transcriptional regulation by PPARα of the ucp-1 gene promoter, we used the brown adipocyte-derived HIB-1B cell line. These cells express PPARγ and PPARβ but not PPARα (24). Thus, HIB-1B cells provide a useful model of brown fat-derived cell in which PPARα-dependent regulation rely on transfected receptor. In agreement, Wy 14,643 did not modify (−4551)UCP1-CAT activity (Fig. 5A). However, co-transfection of pSG5-PPARα induced (−4551)UCP1-CAT activity 3-fold in the absence and nearly 7-fold in the presence of 10 μM Wy 14,463. Co-transfection of pRSV-RXRα caused a synergistic increase in the PPARα-dependent effect upon (−4551)UCP1-CAT activity. We next performed co-transfection experiments using HepG2 cells to avoid any interference of PPARγ in the observed effect. The HepG2 cell line was chosen because, in contrast to HIB-1B cells, does not express PPARγ nor PPARα (31), and has been widely used to analyze PPARα regulation of gene transcription (31–33). As shown in Fig. 5B, co-transfection of pSG5-PPARα enhanced (−4551)UCP1-CAT activity and its responsiveness to Wy 14,463 in a dose-dependent manner, and maximal effects were observed at 0.3 μg of pSG5-PPARα. This amount of vector was the same at which the maximum synergistic enhancement by
When co-transfected together with pSG5-PPAR2 (4551)UCP1-CAT activity 5- and 7-fold, respectively (Fig. 6). transfection of pCMX-CBP or pSV-PGC1 alone enhanced basal a—
er—9 Element in the Upstream Region of the ucp-1 Gene Enhanc-
ing vector pSG5-PPARm were exposed or not to 10μM Wy 14,643, 10μM BRL 49653, or 30μM Ly171883. Results are expressed as CAT activity relative to control, but no effect was observed due to PPARd agonists on (4551)UCP1-CAT. When indicated, 3μM of the expres-
sion in Brown Adipose Tissue in Vivo in Different Physiological Situations—to assess the in vivo significance of PPAR activat-
ors on the expression of the ucp-1 gene, mice at different physiological situations were injected with single doses of the PPAR-specific ligand Wy 14,643 or, for comparative purposes, of the PPARγ activator troglitazone. We have previously re-
ported that sensitivity of gene expression to PPARα activators in acute treatments in vivo depends on the status of lipid metabolism able to provide endogenous PPARα ligands (36). In adult mice (Fig. 9), Wy 14,643 caused a moderate 1.5-fold increase in UCP-1 mRNA abundance in BAT. When lactating mice were analyzed, Wy 14,643 significantly increased (5-fold) UCP-1 mRNA levels. During lactation, functional atrophy of BAT, including diminished lipolytic and lipoprotein lipase ac-
viability, and reduced expression of the ucp-1 gene contribute to energy sparing (37, 38). In contrast, troglitazone only had a moderate effect on brown fat UCP-1 mRNA abundance. When newborn mice at thermoneutrality were analyzed, in-
jecktion of pups with Wy 14,643 caused a significant sustained increase in UCP-1 mRNA levels whereas injection of the PPARγ-ligand BRL 49653 did not significantly change UCP-1 mRNA expres-
sion. The action of PPAR agonists was specific for the ucp-1 gene since COII mRNA levels were essentially unaffected by PPAR activators in BAT (see Fig. 9, bottom). Present results demonstrate an acute regulation of the ucp-1 gene in vivo by the PPARα-ligand Wy 14,643 that is more potent than that observed for PPARγ ligands.
PPARα activates UCP-1 gene transcription

**FIG. 5.** PPARα-dependent induction of \((-4551)\)UCP1-CAT expression in transiently transfected HIB-1B and HepG2 cells: influence of RXR co-transfection. A, HIB-1B cells were transfected with \(1 \mu g\) of \((-4551)\)UCP1-CAT vector, and included or not 0.5 \(\mu g\) of pSG5-PPARα, and/or 0.1 \(\mu g\) of pRSV-RXRα. After transfection, cells were exposed (dark bars) or not exposed (open bars) to \(10 \mu M\) Wy 14,643 for 24 h. Results are shown as relative to the basal expression of \((-4551)\)UCP1-CAT, which is set to 1. Bars are means of at least two independent experiments, each one done in duplicate. B, HepG2 cells were transfected with \(1 \mu g\) of \((-4551)\)UCP1-CAT vector together with increasing amounts of the expression vector pSG5-PPARα. After transfection, cells were exposed ( ) or not exposed (○) to \(10 \mu M\) Wy 14,643 for 24 h. C, as in B, but 0.1 \(\mu g\) of pRSV-RXRα was also co-transfected. Points are means of at least two independent experiments, each one done in duplicate.

**DISCUSSION**

Here we have established that PPARα activators regulate the expression of the ucp-1 gene both in primary brown adipocytes and in BAT in vivo. Brown adipocytes differentiated in primary culture were used since they highly coexpress all PPAR subtypes, equally to BAT (24). In contrast, the HIB-1B brown adipocyte cell line lacks PPARα expression (24), and therefore, the results of previous studies using HIB-1B cells to determine the effects of PPAR activators on the expression of the ucp-1 gene must be viewed with caution. Present results also demonstrate that PPARα induces the rat ucp-1 gene promoter activity upon treatment with its specific ligand Wy 14,643, but it can also activate transcription in the absence of exogenously added ligand. This has been widely described for other PPARα-responsive gene promoters (32, 39), and could be explained by either the presence of endogenous activators, such as fatty acids or their metabolites, or by ligand-independent activity of these nuclear receptors (40). The responsiveness of the ucp-1 gene promoter to PPARα-ligand is increased by co-transfection with expression vectors for either coactivator CBP or PGC-1. Furthermore, the synergistic effect observed when adding both coactivators points to the involvement at the same time of CBP and PGC-1 in coactivating PPARα. In this way, PPARα can interact directly with CBP (41) and also with PGC-1 (42). In addition, CBP can form a complex with PGC-1 (43), thus providing multiple contact points to stabilize the complex assembly. Furthermore, CBP can also interact with other transcription factors, such as CREB and C/EBP, known to regulate transcription of the rat ucp-1 gene through its proximal regulatory region (22, 21).

By deletion and mutation analysis we have identified the PPARα-responsive element in the upstream enhancer region of the rat ucp-1 gene. This \(-2485/-2458\) region contains a potential PPRE consensus formed by two direct repeats separated by one nucleotide (DR-1). Highly comparable elements are also found in the human and mouse ucp-1 genes (see Fig. 5A), indicating that these sequences may have an important regulatory role in response to PPARα. In fact, the murine element has been described to mediate PPARγ responsiveness (18). Our present results further demonstrate that the \(-2485/-2458\) element in the rat ucp-1 gene behaves as a promiscuous responsive site to either PPARα and PPARγ activation, but not PPARβ. From the analysis of various natural PPREs, it has been reported that the binding strength and functional transcription for each PPAR subtype on the same PPRE was similar (33). Only some significant PPARγ specificity was described, and it was related to the 5’-flanking sequence with respect to the DR-1 element, which is essential for PPARα binding (33). However, present results indicate a similar capacity of PPARα and PPARγ to bind and activate ucp-1 transcription through the UCP1-PPRE. The predominant role of any subtype at any one time may thus depend on: 1) the relative amount of each subtype. For instance, PPARα and PPARγ gene expression in brown adipocytes are under opposite regulation by their ligands and retinoic acid: up-regulation of PPARα but down-regulation of PPARγ (24). 2) Cross-talk with other signaling pathways, like regulation of PPAR transcriptional activity by MAP kinase-dependent phosphorylation, which enhances PPARα (44) but decreases PPARγ activity (45). 3) Ligand availability. Several PPAR ligands have been described to be highly subtype-specific (6), although identification
of endogenous ligands and how their synthesis is regulated, is far from being established. 4) Interaction with coregulators. The interaction of PGC-1 with PPAR\(_\alpha\) is ligand-dependent whereas that with PPAR\(_\gamma\) is not (42, 30). These and other possible events may determine which PPAR subtype activates transcription of \(ucp-1\) in response to brown adipocyte physiological condition, mainly PPAR\(_\alpha\) in association with differentiation-dependent events or PPAR\(_\alpha\) in coordination with increased lipid catabolism in active BAT.

Other PPAR target genes have been described to be induced by both PPAR\(_\alpha\) and \(\gamma\) activators through the same PPRE (39, 46). However, since they have been studied in tissues such as liver, which highly expresses PPAR\(_\alpha\) but not PPAR\(_\gamma\), or WAT, which predominantly expresses PPAR\(_\gamma\), tissue-specific regulation has been suggested. In contrast, BAT provides a model to study whether PPAR subtypes specifically regulate a PPRE in a target gene or whether a unique element behaves as a common site, as shown by our present findings in the \(ucp-1\) gene promoter. For instance, the lipoprotein lipase (LPL) gene is up-regulated by PPAR\(_\alpha\) (in liver) and PPAR\(_\gamma\) (in WAT) through the same PPRE (46). During BAT differentiation, induction of LPL allows for increased fatty acids delivery to brown adipocytes, which results in triglyceride accumulation, thus promoting the adipocyte phenotype. However, thermogenic stimulus
also up-regulates LPL to increase fatty acids uptake, which increases the supply of substrate for oxidation. Expression of LPL mRNA is increased by PPARα and γ activators in differentiated brown adipocytes, suggesting that LPL gene transcription in BAT could be activated by both PPARα and -γ. Other genes, such as the fatty acid transport protein and the acyl-CoA synthetase genes, which also regulate cell uptake of fatty acids, might be similarly regulated in BAT since they are induced by PPARα and -γ activators (39, 47).

Here we also demonstrate that in vivo activation of PPARα by Wy 14,643 up-regulates UCP-1 mRNA expression in BAT. The effects of the acute administration of this synthetic ligand are higher in those physiological situations (lactating dams and newborn pups at thermoneutrality) in which endogenous PPARα-ligands are expected to be low, in agreement with previous findings that PPARα sensitivity in vivo depends on the status of lipid metabolism (36). Furthermore, the higher UCP-1 gene responsiveness to acute treatments with PPARα than PPARγ agonists underlines the in vivo significance of PPARα-dependent regulation of UCP-1 gene expression. In contrast, it has been reported that chronic exposure to PPARα or PPARγ activators led to opposite results: long-term oral treatment of rats with Wy 14,643 did not change UCP-1 mRNA levels and thiazioldinedione administration resulted in a slight up-regulation of UCP-1 mRNA (48). This behavior of UCP-1 is similar to other bona fide PPAR-target genes in BAT, which remain unchanged by chronic exposure to PPARα agonists (49). Positive effects of long-term treatment with thiazioldinediones on UCP-1 gene expression may be a consequence of their reported action promoting overall BAT differentiation (17, 48, 50).

Activation of BAT thermogenesis has been classically recognized to be mediated by norepinephrine. Among other regulatory effects, there is a cAMP-dependent activation of hormone sensitive-lipase, which rapidly hydrolyzes the stored triglycerides and releases high concentrations of fatty acids. These fatty acids, in addition to be the major substrate for thermogenesis and the inducers of UCP-1 uncoupling activity, may also act as PPAR activators. Accordingly, cold exposure and β-adrenergic stimulation of BAT result in activation of the PPAR pathway (51). We have previously reported that norepinephrine directly up-regulates transcription of the ucp-1 gene promoter, mainly through a cAMP responsive region in the proximal promoter region (22). However, the upstream enhancer region of the rat ucp-1 gene is also responsive to norepinephrine, although it lacks a defined cAMP responsive region. Several lines of evidence suggest a role for PPARα in mediating this regulation, although the involvement of PPARγ cannot be ruled out. Mutation of the UCP1-PPRE affects the response of the ucp-1 gene promoter to norepinephrine (17). Furthermore, the mitogen-activated protein kinase pathway is activated in BAT by adrenergic stimulation (52). This may result in activation of PPARα but inactivation of PPARγ, as discussed above. The coactivator PGC-1 is rapidly induced by cold-exposure through β-adrenergic pathways in BAT (30). Present data demonstrate that PGC-1 coactivates PPARα and further increases ucp-1 gene responsiveness to PPARα-ligand dependent activation. Likewise, PGC-1 cooperates with PPARα in the transcriptional control of nuclear genes encoding mitochondrial fatty acid oxidation enzymes (42), and it also induces mitochondrial gene expression by regulating the nuclear respiratory factor system (53). Taken together, these data point to PGC-1/PPARα interaction as playing an important role in mediating changes in gene expression in response to BAT thermogenic requirements. Although basal expression of UCP-1 mRNA in PPARα-null mice has been reported to be unaltered (12), as also reported for other bona fide PPAR-target genes in liver (6), further studies are in course to determine whether ucp-1 gene expression is altered in these mice in response to thermogenic stimulus.

In conclusion, PPARα directly regulates ucp-1 gene transcription and we propose that this transcriptional regulatory mechanism is a component of the coordinate control of thermogenic and lipid oxidation pathways in active BAT. Recently, PPARα has been implicated in obesity (54) and selective PPARα activators have been described to improve insulin sensitivity and reduce WAT mass (55). Part of these effects could be due to an increase in energy expenditure in BAT, and the positive action of PPARα on ucp-1 gene expression opens new perspectives on the molecular targets of PPARα involved in mediating these effects.

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