Peroxisome Proliferator-Activated Receptors-α and -γ, and cAMP-Mediated Pathways, Control Retinol-Binding Protein-4 Gene Expression in Brown Adipose Tissue

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Retinol binding protein-4 (RBP4) is a serum protein involved in the transport of vitamin A. It is known to be produced by the liver and white adipose tissue. RBP4 release by white fat has been proposed to induce insulin resistance. We analyzed the regulation and production of RBP4 in brown adipose tissue. RBP4 gene expression is induced in brown fat from mice exposed to cold or treated with peroxisome proliferator-activated receptor (PPAR) agonists. In brown adipocytes in culture, norepinephrine, cAMP, and activators of PPARγ and PPARα induced RBP4 gene expression and RBP4 protein release. The induction of RBP4 gene expression by norepinephrine required intact PPAR-dependent pathways, as evidenced by impaired response of the RBP4 gene expression to norepinephrine in PPARα-null brown adipocytes or in the presence of inhibitors of PPARγ and PPARα. PPARγ and norepinephrine can also induce the RBP4 gene in white adipocytes, and overexpression of PPARα confers regulation by this PPAR subtype to white adipocytes. The RBP4 gene promoter transcription is activated by cAMP, PPARα, and PPARγ. This is mediated by a PPAR-responsive element capable of binding PPARα and PPARγ and required also for activation by cAMP. The induction of the RBP4 gene expression by norepinephrine in brown adipocytes is protein synthesis dependent and requires PPARγ-coactivator-1α, which acts as a norepinephrine-induced coactivator of PPAR on the RBP4 gene. We conclude that PPARγ and PPARα-mediated signaling controls RBP4 gene expression and releases in brown adipose tissue, and thermogenic activation induces RBP4 gene expression in brown fat through mechanisms involving PPARγ-coactivator-1α coactivation of PPAR signaling. (Endocrinology 153: 1162–1173, 2012)
as an adipokine, i.e. a protein released by adipose tissue with regulatory functions distinct from its role in vitamin A homeostasis (3). A negative correlation between RBP4 levels (and, specifically, synthesis in WAT) and overall insulin sensitivity has been reported in several rodent models of obesity, and it has been proposed that RBP4 released by WAT acts on liver as a systemic signal promoting insulin resistance. In humans, obesity and the associated insulin resistance have been described as being associated with abnormally high RBP4 protein levels in serum, whereas insulin sensitization achieved via weight loss or exercise appears to reduce serum RBP4 levels (4). However, not all studies in humans have confirmed the relationship of obesity and insulin resistance with RBP4 levels (5–9), and the role of RBP4 as a putative adipokine inducing insulin resistance remains controversial. Moreover, it has been recently reported that the RBP4-vitamin A complex inhibits cellular insulin responses through the interaction with the membrane protein stimulated by retinoic acid 6 present in the cell surface (10).

The two main types of adipose tissue in mammals, WAT and brown adipose tissue (BAT) play opposing roles in energy balance. WAT is an energy-storing tissue, and BAT constitutes a site of energy expenditure due to its thermogenic capacity. Both types of adipose tissue are present in substantial amounts in adult rodents. In adult humans, WAT is predominant, but BAT has been recognized recently to have also a relevant presence (11, 12). Both types of adipose tissue store vitamin A derivatives and have functions in metabolism of retinoids, including release of retinol (13). All-trans retinoic acid acts usually as an antiadipogenic agent in brown and white adipocyte differentiation (14, 15). However, retinoids participate in the differential development of both types of adipose tissues. Thus, all-trans retinoic acid, acting through retinoic acid receptors (RAR), is a powerful inducer uncoupling protein-1 (UCP1) expression, the specific marker of brown-fat-specific thermogenic responses of the brown adipocyte, i.e. enhanced lipolysis and induction of UCP1 gene expression.

The molecular signals controlling RBP4 gene expression and synthesis in adipose tissues are poorly understood. Treatment of rodents with rosiglitazone, an activator of PPARγ, has been reported to reduce RBP4 gene expression in WAT, an effect contributing to the insulin sensitizing effects of this drug (3). However, other reports in humans do not support such an effect, and treatment of human patients with the thiazolidinedione (TZD) pioglitazone, another activator of PPARγ, has been reported to increase RBP4 gene expression in WAT (8, 21).

In the present study, we show that RBP4 gene expression is under the control of PPARα and PPARγ-mediated regulation as well as by noradrenergic, cAMP-mediated, stimulus involving PPARγ-coactivator-1-α (PGC-1α) in brown adipocytes These pathways of control may contribute to the differential regulation of RBP4 gene expression and protein release by BAT and WAT.

Materials and Methods

Wy14,643, bezafibrate, GW7647, GW501516, GW6471, GW9662, all-trans and 9-cis retinoic acid, norepinephrine, dibutyryl-cAMP, and cycloheximide were from Sigma (St. Louis, MO). Rosiglitazone was from Cayman (Ann Arbor, MI). GW7647 and 4-[(E)-2-(5,6,7,8-Tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-1-propenyl]benzoic acid (TTNPB) were from Tocris (Ellisville, MO).

The care and use of mice were in accordance with the European Community Council Directive 86/609/EEC. For studies in lactating dams, Swiss mice 15 d after parturition were injected with a single dose of Wy14,643 [50 μg/g body weight (bw)] or bezafibrate (100 μg/g bw) in 20% dimethylsulfoxide/saline, or equivalent volumes of vehicle solution, and killed 6 h later. To determine the effects of cold exposure, two-month-old PPARα-null mice and strain-matched wild-type mice were acclimated to a thermoneutral temperature (28°C) for 1 wk and then exposed to 4°C for 24 h. Interscapular BAT, perivisceral WAT, and liver were dissected for further analysis.

RNA was extracted from BAT, WAT, liver, and cultured cells (RNAeasy kit; QIAGEN, Valencia, CA). RT was performed in 20 μl, using random hexamer primers (Applied Biosystems, Foster City, CA) and 0.5 μg of RNA. PCR were conducted in the ABI/Prism-7700 Sequence-Detector System using 25 μl of reaction mixture containing 1 μl of cDNA, 12.5 μl of TaqMan Universal PCR Master Mix, 250 nm probes, and 900 nm primers from the following Assays-on-Demand (Applied Biosystems) kits: Mm00803266, mouse RBP4; Hs00198930, human RBP4; Mm00447183, PGC-1α; and Hs99999901, 18S rRNA. Each sample was run in duplicate, and the mean value of the duplicate was normalized to that of the 18S rRNA gene using the comparative (2−ΔΔCT) method following the manufacturer’s instructions.

Primary brown adipocytes were differentiated in culture as described previously (16). Brown adipocytes were exposed to treatments, and cells and medium were harvested 24 h later,
unless otherwise indicated. The HIB-1B brown adipocyte cell line was cultured as reported elsewhere (22). Primary cultures of mouse white adipocytes were performed using preadipocytes isolated from mouse sc WAT, as described (23). Experiments were performed on d 12 of differentiation, and results are presented as mean ± SE. SGBS human adipocyte cells were cultured and differentiated as already described (24), and experiments were performed on d 12 after induction of differentiation, when more than 70% cells were differentiated.

Mouse embryonic fibroblasts (MEF) were isolated from 13-d-old (embryonic age) wild-type and PGC-1α-null mice (kindly provided by B. Spiegelman) according to standard procedures, and adipogenic differentiation of MEF was achieved as already described, with slight modifications (25, 26). Experiments were performed when 80–90% of the cells were differentiated, on the basis of acquisition of adipocyte morphology.

The plasmids −1192-RBP4-Luc, −535-RBP4-Luc, and −285-RBP4-Luc, in which the corresponding human RBP4 promoter fragments drive Firefly luciferase, have been described previously (27). The plasmid −1192–Mut-RBP4-Luc is a version of −1192-RBP4-Luc, in which point mutations to disrupt the putative PPAR-responsive element at position −765/−745 had been included. The point mutant construct was generated using a QuickChange Site-Directed Mutagenesis kit (Strategene, La Jolla, CA), the forward primer 5′-GAA ACT AAA GAA CAA ATA TTT Aage GAG GaG cCC aCA aCG CTT CgG AAA GAG AG-3′, and 5′-CTC TCT TTT AGC AGG GCT GTG GGT CCC TCG CTT CAA TAT TTGTTCTTTAGTTTC-3′ (lower case indicates the mutated base pairs), the complementary reverse primer. The plasmids were transfected into HIB-1B cells using FuGENE 6 (Roche, Indianapolis, IN). Each point was assayed in triplicate and contained 0.3 μg of RBP4-Luc reporter plasmid, 0.5 ng of the expression vector pRL-CMV (Promega, Madison, WI), and, when indicated, 0.06 μg of pSG5-PPARα, pSG5-PPARγ, or pSV-PGC-1α. Cells were incubated for 48 h after transfection and, when indicated, treated for 24 h with 1 μM GW7647, 10 μM rosiglitazone, or 1 mM dibutyryl-cAMP. Luciferase activities were measured using the Dual-LuciferaseReporter assay (Promega). Firefly luciferase activity was normalized for transfection efficiency using Renilla luciferase as an internal control.

Differentiated primary brown adipocytes were transduced with an adenoviral vector expressing RNA interference against PGC-1α (28) or a scrambled RNA sequence as a control. Multiplicity of infection was 200, and cells were exposed 24 h after transduction to 0.5 μM norepinephrine for 6 h. Differentiated SGBS adipocytes were transduced with adenoviral vectors driving human PPARα (AdCMV-hPPARα) (29), murine PGC-1α (AdCMV-PGC-1α, provided by B. Spiegelman), or green fluorescent protein (GFP) (AdCMV-GFP, control) at a multiplicity of infection of 400, for 4 h. Experiments were performed after further incubation in differentiation medium for 48 h. Efficiency of transduction was approximately 80%, on the basis of GFP fluorescence. Cells were treated for 24 h with 1 μM GW7647, 10 μM rosiglitazone, or vehicle (dimethylsulfoxide).

Immunoblot analysis of RBP4 protein in cell culture medium (35 μl) was performed using 13% SDS-PAGE, RBP4 antiserum reacting against human or mouse RBP4 (1:2000; Alexis, San Diego, CA), and the enhanced chemiluminescence detection kit (Amersham, Piscataway, NJ). Quantitative analysis was performed by densitometry (Phoretics ID Software, Newcastle, UK). Quantification of mouse RBP4 plasma levels was performed using a specific ELISA kit (AdipoGen, Incheon, Korea).

Chromatin immunoprecipitation (ChIP) experiments were performed as described elsewhere (30). HIB-1B cells were transfected with the RBP4 promoter constructs, pSG5-PPARα or pSG5-PPARγ, and exposed to 1 μM GW7647 or 10 μM rosiglitazone, respectively, as described above. Immunoprecipitation was carried out using anti-PPARα (sc-9000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or anti-PPARγ (sc-7196; Santa Cruz Biotechnology, Inc.) antibodies or an equal amount of an unrelated immunoglobulin (Sigma). Input DNA and immunoprecipitated DNA were analyzed by quantitative PCR using SYBR green fluorescent dye and the following primers encompassing the putative PPAR-responsive region in the RBP4 gene: forward, 5′-ACC AGG GTG CCG TTT CTG GAG AAT-3′; reverse, 5′-TCT GAG GTC CAC TTG TTC GAG AAT-3′. Primers amplifying a 237-bp fragment of the cyclophilin-A gene, used as control, were: forward, 5′-CCA TGC CAT TCA GTC TT-3′ and reverse, 5′-TTA CAG GAC ATG GCC AGG AG-3′. The protein-bound DNA was calculated as a ratio to input DNA. The specificity of amplification using the primers above was assessed by PCR amplification (30 cycles of 30 sec at 95°C, 30 sec at 60°C, and 30 sec at 72°C), electrophoreses on 1.5% agarose gel, and staining with ethidium bromide and visualization of the expected bands. For ChIP analysis of the endogenous RBP4 gene promoter, we followed essentially the same procedures, but HIB-1 cells were not transfected with the RBP4 promoter plasmids, and a DNA fragment, encompassing the corresponding PPAR-responsive region in the mouse RBP4 gene promoter, was amplified using the specific primers: forward, 5′-GCT TCT ACT GAG GAC ATG GCC AGG AG-3′; and reverse, 5′-GGA CGA TCA ACC ACT CGG CCA TTG-3′.

Where appropriate, statistical analysis was performed using the Student’s t test; significance is indicated in the text.

**Results**

RBP4 gene expression and induction by thermogenic activation in mouse BAT

Basal expression of RBP4 mRNA was not significantly different between BAT and WAT in mice reared at thermoneutral temperature. Exposure of mice to cold (4°C) caused a significant increase in RBP4 mRNA levels in BAT, whereas no effects were observed in WAT (Fig. 1A). In fact, mice placed at the usual room temperature (21°C), in which a mild thermogenic stimulus occurs, showed significantly higher levels of RBP4 mRNA in BAT than in WAT (3 ± 0.25-fold, P < 0.05), thus confirming the sensitivity of the RBP4 gene to the thermogenic stimulus in BAT. However, exposure of mice to a 4°C environment caused a significant reduction in RBP4 gene expression in the liver from cold-exposed mice (65 ± 13% RBP4 mRNA levels respect to thermoneutral liver, P < 0.05). Plasma levels of RBP4 were lowered in cold-exposed mice (54.5 ± 4.6 μg/ml) respect to thermoneutral controls (78.1 ± 2.4 μg/ml, P < 0.05).
The effects of acute treatment of mice with drugs distinct from a noradrenergic stimulus but known to influence brown fat activity, i.e. PPAR activators, were studied. Previous studies have indicated that the action on BAT of acute treatment of mice with PPAR activators was optimally visualized when controls were mice in a physiological state involving low levels of circulating free fatty acids, i.e. lactating dams (19). Thus, when lactating mice were treated with a single dose of bezafibrate, a PPAR activator with poor selectivity for PPAR subtypes, a significant rise in RBP4 mRNA was observed (Fig. 1B). The PPARα-specific activator Wy14,643 also significantly induced RBP4 mRNA levels. To further establish the role of PPARα in the control of RBP4 gene expression in BAT, PPARα-null mice were studied. No differences in RBP4 mRNA levels were observed in BAT from mice in basal, thermoneutral conditions (Fig. 1C). However, cold exposure caused a minor induction of RBP4 mRNA levels in PPARα-null mice relative to controls (Fig. 1C), thus suggesting an interaction between noradrenergic and PPAR-dependent pathways of control of the RBP4 gene in BAT. In liver, levels of RBP4 mRNA expression were lower in PPARα-null respect to wild-type mice in thermoneutral conditions and were similarly lowered by cold exposure (Table 1). Plasma levels of RBP4 in PPARα-null mice were reduced respect to wild-type mice in thermoneutral conditions and after cold exposure (Table 1).

**Effects of noradrenergic activation, PPAR agonists, and retinoids on RBP4 gene expression in brown adipocytes**

Norepinephrine as well as cAMP caused a dramatic induction of RBP4 mRNA levels in brown adipocytes in primary culture. Bezafibrate, PPARα (GW7647), and PPARγ (rosiglitazone)-specific activators also significantly induced RBP4 mRNA levels, whereas a PPARβ/δ activator (GW501516) did not (Fig. 2A). Both 9-cis retinoic acid and a synthetic activator of RXR, AGN194204 (a gift from R. Chandraratna), also increased RBP4 mRNA abundance. In contrast neither all-trans retinoic acid nor TTNPB, a specific activator of RAR, induced RBP4 mRNA expression. To establish the consequences of the increase in RBP4 mRNA levels, RBP4 protein levels

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**TABLE 1.** Plasma RBP4 levels and RBP4 mRNA expression in liver from PPARα-null mice and wild-type controls

<table>
<thead>
<tr>
<th></th>
<th>Plasma RBP4 (µg/ml)</th>
<th>Liver RBP4 mRNA (% vs. thermoneutral wild type)</th>
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</thead>
<tbody>
<tr>
<td>Wild type</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thermoneutral</td>
<td>80 ± 3</td>
<td>100 ± 14</td>
</tr>
<tr>
<td>4 C, 24 h</td>
<td>55 ± 4a</td>
<td>61 ± 8a</td>
</tr>
<tr>
<td>PPARα-null</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thermoneutral</td>
<td>57 ± 5b</td>
<td>62 ± 5b</td>
</tr>
<tr>
<td>4 C, 24 h</td>
<td>43 ± 2a,b</td>
<td>40 ± 3a,b</td>
</tr>
</tbody>
</table>

Effects of cold exposure. Data are means ± SEM of four to five mice.

a Statistically significance of differences due to cold exposure (4 C, 24 h); \( P < 0.05 \).
b Statistically significance of differences between PPARα-null and wild-type controls at each thermal environment condition; \( P < 0.05 \).

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**FIG. 1.** RBP4 mRNA levels in brown and WAT. Effects of thermogenic stimulus and PPARα-dependent stimulation. A, RBP4 mRNA levels in BAT and WAT from Swiss mice maintained at thermoneutral temperature (28 C) (TN) or exposed to 4 C ambient temperature during 24 h. B, RBP4 mRNA levels in BAT from lactating mice 6 h after treatment with a single injection of Wy14,643 (50 µg/g bw) or bezafibrate (100 µg/g bw) (BZ). C, RBP4 mRNA levels in BAT from PPARα-null and wild-type mice exposed to 4 C ambient temperature over 24 h. Bars indicate the mean ± SEM of six to seven mice from at least three different litters. Data are shown as fold-induction relative to control values for each panel. Significant differences between controls and cold exposed (A and C) or with respect to drug-injected mice (B) are shown by *, \( P < 0.05 \); and the comparison between wild-type and PPARα-null mice by #, \( P < 0.05 \). DMSO, Dimethylsulfoxide.
were determined in brown adipocyte culture medium. Results indicated that norepinephrine, cAMP, bezafibrate, and the PPARγ-specific (rosiglitazone) and PPARα-specific (GW7647) activators caused a significant rise in the abundance of RBP4 protein in the medium (Fig. 2B).

To further analyze the role of PPARα in the control of the RBP4 gene and its interaction with the cAMP-dependent, noradrenergic pathway of regulation, primary cultures of brown adipocytes were established from PPARα-null mice (Fig. 3). Morphological differentiation of adipocytes from PPARα-null mice was unaltered respect to cultures from wild-type mice, as described elsewhere (31). Basal levels of RBP4 mRNA expression were also unaltered in PPARα-null brown adipocytes, whereas the increase in RBP4 mRNA levels due to the PPARα agonist GW7647 was completely suppressed (Fig. 3). The effects of the RXR activator AGN194204 were essentially unchanged, and the action of 9-cis retinoic acid and of rosiglitazone was slightly reduced. The effects of norepinephrine, although not completely impaired, were significantly reduced in adipocytes lacking PPARα, thus further indicating a cross talk between PPARα-dependent and noradrenergic pathways of regulation of RBP4 gene expression (Fig. 3).

In a second approach, the PPARα antagonist GW6471 was used. This drug not only suppressed the action of the PPARα activator GW7647, but it also significantly reduced the action of norepinephrine (Fig. 4). The PPARγ antagonist GW9662 also significantly decreased the effects of norepinephrine on RBP4 mRNA expression, whereas treatment with both PPAR subtype-specific drugs completely suppressed noradrenergic induction. This indicated that an active PPAR-dependent pathway of regulation, either through PPARα or PPARγ, is required for effective noradrenergic induction of the RBP4 gene in brown adipocytes.

Transcriptional control of the RBP4 gene by PPAR and cAMP in brown adipocytes

To determine the action of cAMP and PPARα-dependent pathways in transcriptional control of the RBP4 gene in brown adipocytes, transfection assays on HIB-1B
brown fat-derived cells were performed using a luciferase construct driven by the 1192-bp promoter region of the human RBP4 gene. Exposure of the transfected construct to PPAR activators indicated significant induction by PPARγ activation (rosiglitazone), a weaker but significant induction by PPARα activation (GW7647), and a significant induction due to cAMP (Fig. 5A). Cotransfection with PPARα, and particularly further addition of GW7647, caused a dramatic induction of the RBP4 promoter activity. Similar observations were obtained when PPARγ was cotransfected and cells were exposed to rosiglitazone. No effects were observed with PPARβ/δ cotransfection, further indicating the lack of sensitivity of the RBP4 gene to this PPAR subtype (data not shown).

Through a deletion mutant analysis, it was determined that the RBP4 promoter construct lacking the fragment from −1192 to −535 retained most of the basal activity but became completely nonsensitive to PPAR-dependent regulation (Fig. 5A). Most of cAMP-mediated responsiveness was lost in this short −535-RBP4-Luc construct. Further deletion of the reporter construct between −353 and −285 dramatically reduced basal promoter activity (11% of the full-length construct levels), and −285-RBP4-Luc was completely insensitive to PPAR- or cAMP-dependent regulation (data not shown). The −1192 to −535 region of the RBP4 gene was previously reported to contain a putative PPAR-responsive sequence at −765 to −745 (27). When we analyzed a construct of the RBP4 promoter in which point mutations had been introduced to disrupt PPAR binding at this site (−1192-Mut-RBP4-Luc) (Fig. 5C), the responsiveness to PPAR and to cAMP was essentially lost (Fig. 5A). ChIP in conditions of PPAR-dependent induction of the RBP4 promoter in HIB-1B cells using antibodies against PPARα and PPARγ indicated a specific enrichment in the PCR amplification of both the endogenous and the transfected RBP4 promoter region (Fig. 5B). When −1192-Mut-RBP4-Luc mutated version of the RBP4 promoter construct was analyzed in comparison with the wild-type promoter construct, a significant impairment in the enrichment of amplified DNA after ChIP using PPARα and PPARγ antibodies was observed (Fig. 5D). Collectively, this data indicate that PPARα and PPARγ regulate the RBP4 gene promoter mainly through the −765 to −745 region of the RBP4 gene.

**Regulation of RBP4 gene expression in white adipocytes**

We determined whether the observed effects were specific to brown adipocytes or were shared by white adipose cells. Differentiated 3T3-L1 and 3T3-F442A adipocytes express extremely low levels of RBP4 mRNA, hardly detectable even using highly sensitive quantitative real-time PCR. In contrast, mouse white adipocytes differentiated in primary culture expressed substantial levels of RBP4 mRNA. Norepinephrine and the PPARγ agonist rosiglitazone significantly induced RBP4 mRNA levels in white adipocytes, whereas the PPARα activator GW7647 had no effect (Fig. 6A). Parallel observations were made for RBP4 protein levels in the adipocyte culture medium in response to these agents (Fig. 6B). The study of SGBS human white adipocytes led essentially to similar results (Fig. 6C). This indicated that the main features of regulation of RBP4 gene expression in white adipocytes were shared by human and murine cells.

The lack of effect observed for the PPARα agonist on RBP4 gene expression in white adipocytes was likely to be due to the much lower expression of PPARα in white, in comparison with brown, adipocytes (18). We therefore investigated whether increasing PPARα expression in white adipocytes could confer to these cells sensitivity to activation of RBP4 gene expression. Adenoviral transduction of human SGBS white adipocytes led to highly PPARα expression, with levels around 30-fold those in basal conditions. PPARα expression led to a significant induction of RBP4 mRNA levels in SGBS white adipocytes after exposure to GW7647 (Fig. 6D). This confirmed the positive action of PPARα on the RBP4 gene in the context of adipose cells, either brown or white.

**Effects of PGC-1α on RBP4 gene regulation**

The requirement of intact PPAR signaling pathway for cAMP responsiveness of RBP4 gene expression led us to study whether the effects of noradrenergic, cAMP-depen-
dent, stimulus of the RBP4 gene in brown adipocytes could occur through indirect processes. Treatment of brown adipocytes with cycloheximide, a suppressor of protein synthesis, blunted RBP4 mRNA induction in response to noradrenaline (Fig. 7A), indicating that, effectively, RBP4 mRNA induction was protein-synthesis dependent. We examined the role of PGC-1α in RBP4 gene regulation in brown adipocytes, considering that PGC-1α expression is induced by norepinephrine in brown adipocytes via cAMP-dependent signaling and it is a powerful coactivator of PPARα and PPARγ (32). The time course of norepinephrine effects on gene expression in brown adipocytes was analyzed with transfections of promoter constructs (analysis of the endogenous murine RBP4 gene promoter) or after transfection with the −1192-RBP4-Luc plasmid. PPARα and PPARγ expression vectors were cotransfected, and cells were treated with 1 μM GW7647 and 10 μM rosiglitazone (Rosi), respectively. PPARα or PPARγ antibodies (Ab) or an unrelated IgG were used to immunoprecipitate the protein-DNA complexes. Bars are mean ± SEM of relative enrichment of the PCR amplification signal due to PPAR antibodies respect to IgG (top). Significant differences respect to IgG are shown by *, P < 0.05. An example of PCR amplification in the ChIP assay is shown (bottom). RBP4 indicates the amplified DNA corresponding to the RBP4 promoter region, and Cyp, that corresponding to the cyclophilin-A gene used as control (see Materials and Methods).

FIG. 5. Effects of PPARα and PPARγ activation and of cAMP on RBP4 promoter. A, HIB-1B cells were transfected with the −1192-RBP4-Luc plasmid, the deleted plasmid construct −535-RBP4-Luc, or the −1192 hRBP4-Mut-Luc mutated version (see C) and, when indicated, were cotransfected with the plasmid expression vectors for PPARα or PPARγ and exposed to the PPARα activator GW7647, the PPARγ activator rosiglitazone, and 1 mM dibutyryl-cAMP (cAMP). Bars indicate the mean ± SEM of normalized luciferase activity (see Materials and Methods). Significant differences (P < 0.05) due to PPAR agonists or cAMP relative to controls for each construct and cotransfection setting are shown by *, those due to PPARα or PPARγ for each condition are shown by #, and those between wild-type and mutated forms of the promoter constructs at the same condition are shown by Δ. B, ChIP of PPARα and PPARγ binding to RBP4 gene promoter. HIB-1B cells were analyzed without transfection of promoter constructs (analysis of the endogenous murine RBP4 gene promoter) or after transfection with the −1192-RBP4-Luc plasmid. PPARα and PPARγ expression vectors were cotransfected, and cells were treated with 1 μM GW7647 and 10 μM rosiglitazone (Rosi), respectively. PPARα or PPARγ antibodies (Ab) or an unrelated IgG were used to immunoprecipitate the protein-DNA complexes. Bars are mean ± SEM of relative enrichment of the PCR amplification signal due to PPAR antibodies respect to IgG (top). Significant differences respect to IgG are shown by *, P < 0.05. An example of PCR amplification in the ChIP assay is shown (bottom). RBP4 indicates the amplified DNA corresponding to the RBP4 promoter region, and Cyp, that corresponding to the cyclophilin-A gene used as control (see Materials and Methods). C, Sequence of the PPAR responsive element in the RBP4 promoter; site-directed mutations to generate 1192-RBP4-Mut-Luc are shown in lower case. D, ChIP of PPARα and PPARγ binding to the wild-type and mutant forms of the RBP4 gene promoter. HIB-1B cells were transfected and treated as in B but including either the −1192-RBP4-Luc or −1192-RBP4-Mut-Luc plasmids. Bars are mean ± SEM of relative enrichment of the PCR amplification signal respect to input. Significant differences between the wild-type (wt) and the mutated (mut) promoter constructs are shown by *, P < 0.05.
pocytes showed a much earlier peak of induction of PGC-1α mRNA than RBP4 mRNA (Fig. 7B). Cycloheximide did not suppress PGC-1α mRNA induction (17-fold induction of PGC-1α mRNA in the presence of cycloheximide vs. 15-fold induction in the absence, 2-h exposure to norepinephrine). These results were compatible with the hypothesis that PGC-1α may play a role in stimulus of the RBP4 gene in response to noradrenergic cAMP-mediated signaling, via coactivation of PPAR. This possibility was assessed by determining the effects of impairing PGC-1α expression in the responsiveness of the RBP4 gene to norepinephrine. Adenoviral-mediated expression of PGC-1α small interfering RNA (siRNA) reduced PGC-1α mRNA levels to 31 ± 5% respect to those in controls transduced with scrambled siRNA. Under these conditions, the capacity of norepinephrine to induce RBP4 mRNA expression was significantly impaired (Fig. 7C). Basal expression of RBP4 mRNA was slightly impaired in PGC-1α-null adipocytes (71 ± 28% respect to wild-type adipocytes). Adipocytes derived from PGC-1α-null embryos showed a significant impairment in cAMP-mediated induction of RBP4 mRNA expression (Fig. 7D), thus indicating that PGC-1α is required for cAMP-mediated regulation of the RBP4 gene. A further analysis using transfection of HIB-1B cells was undertaken. PGC-1α cotransfection induced RBP4 gene promoter activity as well as enhanced the activation elicited by rosiglitazone and PPARγ cotransfection (Fig. 7E). Similar results were observed for PPARα and GW7647 effects (data not shown). The effects of PGC-1α cotransfection were lost for the −1192 hRBP4-Mut-Luc construct, in which the PPAR responsive region is mutated. Finally, the

FIG. 6. Effects of hormonal agents on RBP4 mRNA and protein levels in murine white adipocytes differentiated in primary culture and in differentiated human SGBS white adipocytes. A, RBP4 mRNA levels in mouse white adipocytes differentiated in primary culture and exposed to the indicated agents (see Materials and Methods). B, RBP4 protein in culture medium from white adipocytes differentiated in culture. C, RBP4 mRNA levels in human SGBS white adipocytes differentiated in culture and transduced with adenoviral vectors driving PPARα or GFP as a control and treated or not with the PPARα activator GW7647 (1 μM). Bars indicate the mean ± SEM of four to six independent cell cultures for each condition. Data are shown as fold-induction relative to control (C, untreated cells) values for each panel. Significant differences in comparison with controls are shown by *, P < 0.05. NE, Norepinephrine; Rosi, rosiglitazone.
effects of PGC-1α on RBP4 gene expression were analyzed in SGBS white adipocytes, which lack a substantial expression of PGC-1α in contrast with brown adipocytes but express PPARγ. Adenoviral-mediated overexpression of PGC-1α was enough to cause a significant induction of RBP4 mRNA expression (Fig. 7F).

Discussion

The present study demonstrates that RBP4 gene expression is tightly regulated in BAT by cAMP-mediated pathways, PPARγ and PPARα. Moreover, the present findings support the notion that, in contrast to other adipokines that are preferentially released by white (rather than brown) adipocytes, substantial RBP4 expression and release occurs in BAT, specially after thermogenic activation.

The cAMP-dependent regulation observed in brown adipocytes may be related to the induction of RBP4 gene expression by the thermogenic stimulus in BAT. A likely scenario after thermogenic stimulus would be that enhanced lipolysis results in increased hydrolysis of retinyl-esters inside the fat depots of BAT, with the subsequent release of retinol and the potential need for coordinate synthesis of the RBP4 protein to bind it. The capacity of BAT to become an enhanced source of retinol and/or RBP4 protein in response to thermogenic stress has not been analyzed to date, but it might be expected to occur on the basis of the present findings. However, the physiological significance of RBP4 expression and release by BAT under conditions of enhanced thermogenic activity is unclear at present. We observed that serum RBP4 levels were reduced in mice exposed to cold, in association with reduced RBP4 gene expression in liver. In general, changes in serum
RBP4 levels paralleled changes in liver RBP4 expression in our study, thus supporting a main role of liver RBP4 expression in determining circulating RBP4 levels. It is likely that lowered hepatic expression compensate for enhanced RBP4 production by BAT in conditions of thermogenic stress resulting ultimately in lower systemic levels. The possibility that increased production of RBP4 by BAT causes mainly local, autocrine/paracrine effects should deserve further research. In WAT, adrenergic induction of RBP4 gene expression and release might have a distinct physiological significance, because lipolysis in white fat is enhanced in response to starvation, a state of increased RBP4 levels in serum and reduced insulin action.

Our present findings indicate that PPARγ activation induces RBP4 expression by acting directly on the RBP4 gene promoter. This is in agreement with reports on the induction of RBP4 expression by acting directly on the RBP4 part of the cluster of PPARα-regulated genes, usually encoding lipid catabolism proteins, may be related to the aforementioned need for dealing with the free retinol appearing in BAT as a consequence of a high lipolysis during thermogenic activation. On the other hand, the identification of the RBP4 gene as a target of PPARα in brown adipocytes should lead to exploration of whether this pathway is also active in liver, a main site of PPARα and RBP4 gene expression, and a major target of fibrates, PPARα-activating drugs of current use in some dyslipidemias. Our present data indicating lowered expression of RBP4 mRNA in liver from PPARα-null mice is consistent with this possibility. In a recent study, chronic treatment of obese mice with the PPARα agonist fenofibrate was reported to cause a reduction of RBP4 mRNA expression in WAT (41), although it is unclear whether this was a direct or indirect effect as a consequence of long-term treatment “in vivo.” In that report, the action of fenofibrate causing a slight decline in RBP4 mRNA expression in 3T3-L1 adipocytes should be considered to the light of the present findings and other previous reports (42) indicating an extremely low basal expression of RBP4, close to detection limits, in 3T3-L1 adipocytes.

A close interplay between the cAMP and PPAR-dependent pathways of regulation of the RBP4 gene in BAT is shown in the present study, and most of the cAMP action on RBP4 gene transcription requires intact PPAR-dependent pathways. A role for PPARα in mediating noradrenergic, cAMP-dependent, regulation of gene transcription in BAT has been proposed (43, 44), and it was attributed to the action of cAMP in promoting lipolysis and the intracellular generation of free fatty acid derivatives, natural activators of PPAR. However, modulation of PPARα activity via protein kinase A-dependent phosphorylation cannot be excluded (45). Nonetheless, our present data strongly support that it is PGC-1α induction by cAMP and the corresponding action of PGC-1α in coactivating PPARα and/or PPARγ what determines most of the cAMP-dependent RBP4 gene regulation.

In summary, we report that PPARγ and PPARα, as well as cAMP, control RBP4 gene expression in brown adipocytes. Induction of PGC-1α expression by cAMP and subsequent coactivation of PPAR play a pivotal role in mediating cAMP effects. Drugs activating PPARγ, such as TZD, promote RBP4 gene up-regulation in adipocytes. Fibrates, which act on PPARα, also control RBP4 gene expression in brown adipocytes. Taking into account the recent recognition of active BAT in adult humans (12), further research will be required to establish the role of the induction of RBP4 gene expression and RBP4 protein release by BAT in the context of the overall systemic responsiveness to these drugs when used in the treatment of insulin resistance and dyslipidemias.
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