Rat Peroxisome Proliferator-activated Receptors and Brown Adipose Tissue Function during Cold Acclimatization*

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Brown adipose tissue (BAT) hyperplasia is a fundamental physiological response to cold; it involves an acute phase of mitotic cell growth followed by a prolonged differentiation phase. Peroxisome proliferator-activated receptors (PPARs) are key regulators of fatty acid metabolism and adipocyte differentiation and may therefore mediate important metabolic changes during non-shivering thermogenesis. In the present study we have investigated PPAR mRNA expression in relation to peroxisome proliferation in rat BAT during cold acclimatization. By immunoelectron microscopy we show that the number of peroxisomes per cytoplasmic volume and acyl-CoA oxidase immunolabeling density remained constant (thus increasing in parallel with tissue mass and cell number) during the initial proliferative phase and the acute thermogenic response but increased after 14 days of cold exposure, correlating with terminal differentiation of BAT. A pronounced decrease in BAT PPARα and PPARγ mRNA levels was found within hours of exposure to cold, which was reversed after 14 days, suggesting a role for either or both of these subtypes in the proliferation and induction of peroxisomes and peroxisomal β-oxidation enzymes. In contrast, PPARβ mRNA levels increased progressively during cold exposure. Transactivation assays in HIB 1B and HEK-293 cells demonstrated an adrenergic stimulation of peroxisome proliferator response element reporter activity via PPAR, establishing a role for these nuclear receptors in hormonal regulation of gene transcription in BAT.

Peroxisome proliferator-activated receptors (PPARs)

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The abbreviations used are: PPAR(s), peroxisome proliferator-activated receptor(s); AOX, acyl-CoA oxidase; BAT, brown adipose tissue; LPL, lipoprotein lipase; tk, thymidine kinase; Luc, luciferase; HEK, human embryonic kidney; PCR, polymerase chain reaction; bp, base pair; CMV, cytomegalovirus; RACE, rapid amplification of cDNA ends; UTR, untranslated region; EMSA, electrophoretic mobility shift assay; PPRE, peroxisome proliferator response element; RXR, retinoid X receptor; DOTAP, N-1-[2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium methyl sulfate, C/EBP, CCAAT/enhances binding protein.
activity (31).

Because PPAR is a known mediator of p eroxisome proliferation and PPAR subtypes have been implicated in adipogenesis, physiological stimulation of BAT offers an excellent in vivo model for studying PPARs in relation to adipogenesis and peroxisome proliferation under physiological conditions. In an attempt to unravel the biological functions of PPARα, PPARγ, and PPARδ in a physiological context, we have investigated the expression of these receptors in rat BAT during cold acclimatization and correlated the expression of the different receptor subtypes with the particular metabolic and developmental state of the tissue. Quantitative analysis of peroxisome proliferation in BAT was performed by inmunoelectron microscopy. The data indicate that peroxisome proliferation correlates with the expression of PPARα and γ. In a transactivation assay utilizing the brown preadipose cell line HIB 1B, it was found that norepinephrine, 8-bromo-cAMP, and forskolin induced AOX-tk-Luc reporter gene activity, suggesting that the β-adrenergic signaling pathway can activate endogenous PPARs. The PPAR dependence of this activation was confirmed in a cell line (HEK-293) that is devoid of detectable endogenous PPARs, suggesting that PPARs are involved in the thermogenic activation of BAT and proliferation of peroxisomes in this tissue. BAT may thus serve as a promising in vivo system for identification of the physiological ligands for PPARs and elucidation of the biological functions of these receptors.

EXPERIMENTAL PROCEDURES

Complementary DNA Cloning of Rat PPARγ and PPARδ—PCR template cDNA was synthesized from 10 μg of BAT RNA utilizing a cDNA synthesis system (Life Technologies, Inc.). Two sets of degenerate oligonucleotide primers were designed to amino acid regions conserved in mouse and Xenopus PPARy. The 5′-primer corresponds to amino acids 1-143 of the amino acid sequence of the Xenopus PPAR (5'-GGCGATCCAGAT/CTGA/ TGAT/CTTTGTTCTTCAACATGAC-3'), and the 3′-primer corresponds to amino acid sequences 161-169 at the carboxyl terminus (5'-GCGGTACCACTAGCCTGATGAGT/CTGA/G/ACT/CTGATGAGT-3'). BamHI and KpnI recognition sites were included at the 5′-end of the olagonucleotide primers for cloning purposes. PCR products were as described previously (39) yielding a 1,951-bp cDNA fragment. This fragment was digested with BamHI and KpnI resulting in a 980-bp fragment that was cloned into the pBluescript II KS cloning vector (Stratagene) and partially sequenced. The isolated clones corresponded to the full-length rat PPARγ (2). The 980-bp fragment was cloned into the pCR2.1 cloning vector (Invitrogen) and the gene-specific primer (5'CGTTCGACAGGGAGGACCGTGAAC-3') was amplified using the PCR (rapid amplification of cDNA ends) was employed to obtain the 5′-UTR and amino-terminal sequences of the PPARγ subtype utilizing the Marathon-Ready cDNA system (CLONTECH) and the gene-specific primer (5'-CGCTTCCAGAAGTGCCTGGAACCTGCTGACG-3'). A 654-bp cDNA fragment was cloned into the pcCR2.1 cloning vector (Invitrogen) and sequenced.

RNA Isolation and Northern Blots—Male Harlan Sprague-Dawley rats (B&K Universal AB, Stockholm) weighing about 150 g were preacclimated to standard animal house conditions for 1 week before handling. For thermoregulatory studies, rats were housed individually at 28 °C (controls) or in the cold (4 °C) for the periods of time indicated. Two separate time course experiments were carried out, one with three rats in each time point (up to 21 days in the cold) and another experiment with two animals in each time point (up to 28 days in the cold). Interscapular BAT was dissected out and processed individually for RNA purification using the Ultraspec system (Biotec). This method was also utilized to isolate RNA from HIB 1B and HEK-293 cells. The RNA samples were resolved in 1.5% agarose gels containing 20 μg/mL ethidium bromide. The gels were exposed to Kodak X-Omat film containing 10 μg of total RNA/well, were blotted onto Hybond-N nylon membranes (Amersham Pharmacia Biotec) by capillary transfer. Membranes were hybridized with 32P-labeled cDNA probes in a hybridization buffer containing 5% SDS, 400 μg/mL DNA, 1 mm EDTA, 1 mg/mL bovine serum albumin, and 50% formamide. PPAR Northern analyses were carried out with full-length probe to PPARα (accession number M86952 (33), PPARγ, and δ described here. Full-length AOx (34), δ2,δ3-enoyl-CoA isomerase (35), LPL (36), and CEBPα and β (37, 38) were those earlier described. After 16-h hybridizations at 42 °C, blots were washed at 53 °C in 0.1 × SSC, 0.1% SDS, and 1 mm EDTA and exposed to a PhosphorImaging plate (Fuji).

Immunocytochemical Studies—Female Harlan Sprague-Dawley rats weighing approximately 120 g were housed individually at standard animal house conditions (23 °C). All rats had free access to food (rat pellets, Oriental M, Tokyo) and water. Tissue processing and immunohistochemical staining of Lowicryl-K4M embedded specimens, using the protein A-gold technique, were performed as described previously (39). The infiltration of the resin into BAT isolated from control rats was highly inefficient, probably because of high fat content. Therefore, tissues isolated from rats housed in the cold for 4 days represent the shortest time point available for analysis. After the immunostaining, the sections were stained with 1% uranyl acetate solution and were examined in a Hitachi H700 electron microscope at an accelerated voltage of 150 kV. The morphometric analysis of changes in the peroxisome volume and labeling densities was performed as described previously (39), using 20 electron micrographs of each period of cold acclimatization selected from 200 pictures. The immunostaining was performed with all samples at the same time to equalize the staining conditions. The analysis was performed with a computer-assisted image analyzer, Digimizer G (Muto Co., Tokyo, Japan). The peroxisome volume density was calculated as the ratio of the area of peroxisomes to the cytoplasmic area excluding the lipid droplets. The labeling density of peroxisomes was calculated as the number of gold particles/μm2 of peroxisome area, and the immunolabel concentration was expressed as the number of gold particles/unit of cytoplasmic volume by multiplying the peroxisomal volume density with the labeling density. The statistical analyses were done using analysis of variance and Student's t tests.

Electrophoretic Mobility Shift Assay (EMSA)—The PPRE probe in pGEO1 was a double-stranded oligonucleotide, 5'-CGACCCAG- GAGCTCTTCTTCTGTGGCT-3', extended from the PPARδ reporter. The probe was labeled by a Klenow fill-in reaction in the presence of [α-32P]dCTP. Nuclear protein extracts were obtained from cells harvested in TEN buffer (40 mM Tris, pH 7.9, 10 mM EDTA, 150 mM NaCl) by extraction in the presence of complete TM (EDTA-free) protease inhibitor mixture from Roche Molecular Biochemicals. PPARγ (6 × His-tagged full-length) and RXXR (6 × His-tagged full-length) were translated utilizing the Promega TNT coupled Reticulocyte Lysate system. 25-μl incubation mixtures contained 2–5 μg of protein (nuclear extract), 20,000 cpm of probe, 20 mM KCl in 10 mM Tris, pH 7.8, 10% glycerol, 500 ng of poly(dI-dC)poly(dI-dC), and 500 ng of sonicated salmon sperm DNA. Samples were resolved on a 5% polyacrylamide gel. Antibodies used were RXXR mouse monoclonal antibodies directed against the ligand binding domain, which were a kind gift from Dr. Pierre Chambon, and PPARγ mouse monoclonal antibody (sc 7273x) from Santa Cruz Bio- Tech Inc. Specific competitors corresponding to DR2 (5'-CGACCACCGGGTCCTACAGGCCTGCTG-3') and DR5 (5'-TCGACTGGGTTCACCGAATTTCCAGGAGTTCTCTCTGTTG-3') were used as indicated. The dried gels were exposed to film overnight.

Cell Culture, DNA Plasmids, and Transfection—All cell culture experiments, all reagents were purchased from Life Technologies unless specified otherwise. HEK-293 cells were purchased from American Type Culture Collection and were maintained in a 50:50 mix of Dulbecco's modified Eagle's medium and Ham's F-12 medium supplemented with 10% heat-inactivated fetal calf serum and gentamycin (25 μg/ml). HIB 1B cells, kindly provided by Dr. Bruce Spiegelman, were maintained in preadipocyte medium, a 50:50 mix of Dulbecco's modified Eagle's medium and Ham's F-12 medium supplemented with 10% fetal bovine serum.

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Eagle’s medium and Ham’s F-12 medium supplemented with 10% heat-inactivated fetal calf serum and gentamicin (25 μg/ml).

The luciferase reporter plasmids utilized in our experiments contain the herpesvirus thymidine kinase (tk) basal promoter linked upstream of the luciferase gene (tk-Luc). PPAR activity was monitored by the measurement of luciferase activity using the luciferase assay system (Promega). Duplicate plates were used in all experiments for both control and treated conditions. All experiments were repeated several times with consistent results. Unless specified otherwise, data shown are the mean ± S.E. of the mean for three or four independent experiments.

RESULTS

CDNA clones encoding the PPARγ and δ subtypes were isolated from a rat interscapular BAT cDNA library. Two rat PPARγ isoforms, γ1 and γ2, were obtained, which has been reported for the mouse PPARγ isoforms, probably result from differential splicing of PPARγ RNA. The nucleotide and deduced amino acid sequences of the rat PPARγ and γ2 subtypes are shown in Fig. 1. Of the four positive clones isolated, only one encoded PPARγ1. The rat PPARγ1 cDNA clone contains an open reading frame that encodes a protein of 475 amino acids with a calculated molecular mass of 54.5 kDa (Fig. 1A). The PPARγ2 cDNA clone encodes a 57.6-kDa protein containing 30 additional amino acids at the amino terminus (Fig. 1B) compared with PPARγ1. A partial rat PPARδ cDNA clone was also isolated. This clone contained a sequence encoding amino acids 85–440 and approximately 2 kilobases of 3′-UTR. The 5′-end of the rat PPARδ cDNA was isolated by RACE, resulting in amplification and cloning of a 654-bp cDNA fragment that contains part of the 5′-UTR and the nucleotides encoding the amino-terminal 137 amino acids of the protein. The combined sequence information obtained from the RACE, PCR, and library screen products results in a PPARδ cDNA encoding a predicted protein of 440 amino acids with a calculated molecular mass of 49.7 kDa. The published PPARδ sequence (40) differs slightly from the sequence obtained here. In the sequence published previously, the nucleotide differences in the coding region which would encode different amino acids are: C<sub>305</sub> (Thr instead of Met<sup>25</sup>), T<sub>436</sub> (Ser instead of Pro<sup>65</sup>), and T<sub>1271</sub> (Val instead of Asp<sup>343</sup>). The predicted rat PPARδ sequence information obtained from the RACE, PCR, and library screen results in a PPARδ cDNA encoding the predicted protein sequence and is therefore not included in this figure.

BATT PPAR and C/EBP Subtype mRNA Expression during Cold Adapation—The PPAR subtype mRNAs are expressed in a tissue-specific manner. As reported recently, the PPARγ subtype is expressed predominantly in adipose tissue where it is thought to play a crucial role in adipogenesis (14). Because this process is pivotal to BAT function and the mammalian adaptation to cold, we hypothesized that the PPARα, γ, and δ subtypes may be expressed in BAT and that the mRNA levels could be affected during adipogenesis triggered by cold exposure. We have tested this hypothesis by analyzing expression of these
PPAR subtypes in rat BAT as a function of time in the cold (4 °C). Two independent time course experiments were performed. In the first experiment rats were exposed to cold for up to 21 days, and in the second time course experiment rats were exposed to the cold for up to 28 days. RNA samples were analyzed from each animal by Northern analysis. Our results demonstrate that the PPAR subtype mRNAs are regulated differentially during cold exposure (Fig. 2, A and B). PPARα mRNA was decreased markedly after 5 h of cold exposure and was almost undetectable after 1 day in the cold. After about 10 days of cold exposure, PPARα mRNA increased gradually but remained lower than in the controls. PPARγ mRNA levels were also repressed profoundly within hours of cold exposure and remained decreased for 5–10 days in the cold (30–35% of control levels). After 10 days in the cold PPARγ levels increased to control levels after 28 days in the cold. In contrast, the PPARδ mRNA level increased progressively during acclimatization to cold. Fig. 2B shows PhosphorImager quantitation of Northern blots on RNA samples obtained at various time points of cold exposure, analyzed from three different rats at each time point. In another cold exposure experiment, RNA samples were analyzed from two rats at each time point with essentially the same results (as shown in Fig. 2A).

C/EBPα, C/EBPβ, and PPARs are expressed sequentially and seem to determine the adipocyte phenotype in concert (14, 41–43), therefore we also analyzed the expression of these mRNAs in BAT during cold exposure. RNA from two animals at each time point were analyzed, and the results demonstrated that the expression of C/EBPα and C/EBPβ mRNAs is rapidly and differentially regulated during the cold exposure of rats (Fig. 2A). PhosphorImager quantitation showed that the C/EBPα mRNA level was decreased transiently within 5 h of cold exposure, to less than 50% of the control levels, then returned to control level after 5–10 days and remained at about this level throughout the experiment (not shown). In contrast, the C/EBPβ mRNA level increased rapidly, almost 3-fold within 1 h of cold exposure, after which the expression returned to near control level at 24 h and remained slightly elevated for the remaining experimental period.

AOX, LPL, and Enoyl-CoA Isomerase mRNA Expression in BAT during Cold Adaptation—Because the AOX gene is transcriptionally regulated by PPARα in the liver (2, 3), and peroxisomal β-oxidation is induced about 10-fold in BAT (31) during acclimatization to cold, BAT AOX mRNA levels were analyzed. AOX mRNA steady-state levels increased immediately upon cold exposure, peaking at 5 h. The mRNA amount
days of cold exposure were unchanged during the acute cold phase (Fig. 3). After 5 days of cold exposure and peaked after 24 h of cold exposure. The lower panel shows EtBr staining of the membrane with the positions of the 28 S and 18 S bands.

was near the control at 1 and 5 days and increased thereafter at 10–14 days, ultimately resulting in a 4.2-fold (±0.4) increase in AOX mRNA in BAT after 4 weeks in the cold (Fig. 3).

It has also been shown that the gene encoding LPL is transcriptionally regulated in adipose tissue by PPARγ (44) and that cis-D3, D3-enoxy-CoA isomerase is strongly induced by peroxisome proliferators (45). To correlate the expression of the LPL and D3, D3-enoxy-CoA isomerase genes with the expression of the PPAR genes we analyzed the expression of these genes in BAT during cold acclimatization. LPL mRNA levels increased after only 1 h of cold exposure and peaked after 24 h of cold exposure (Fig. 3). This rapid increase was followed by a sharp decrease in the expression of the LPL gene within 5 days in the cold. LPL mRNA levels were reduced further during the prolonged cold treatment, almost reaching control levels after 28 days of cold exposure. D3, D3-Enoyl-CoA isomerase mRNA levels were unchanged during the acute cold phase (Fig. 3). After 5 days of cold exposure D3, D3-enoxy-CoA isomerase mRNA levels increased and remained elevated during later time points (Fig. 3).

Peroxisomal Proliferation and Induction of Immunoreactive AOXs during Cold Acclimatization—Experiments involving disruption of the gene encoding PPARα have demonstrated the important role of this nuclear receptor in activation of target genes and in hepatic peroxisome proliferation (9). Because PPARs appear to be key regulators of adipogenesis, a process intimately related to thermogenesis in BAT, the effects of cold acclimatization on the number of peroxisomes in BAT were investigated by electron microscopy. In preliminary experiments the immunoreactivity of BAT embedded in two kinds of resins, general epoxy resin and Lowicryl-K4M, was compared. Using ultrathin sections of BAT embedded in Lowicryl-K4M, strong immunoreactivity with antibodies to catalase, AOX, and peroxisomal thiolase was obtained, but when using epoxy-resin sections, only anti-catalase showed good immunoreactivity. Therefore Lowicryl-K4M was employed in all of the experiments described. However, it was not possible to embed and section BAT samples obtained from control rats probably because of interference by the high content of triglycerides in the tissue. Therefore, samples prepared from rats housed at 4 °C for 4 days represent the earliest time point in our studies.

BAT was isolated from rats acclimatized to cold for 4 days and analyzed by immuno-electron microscopy. Small spherical organelles with a diameter of about 0.1 μm were immunoreactive for catalase (Fig. 4, A and C) and peroxisomal β-oxidation enzymes (Fig. 4, B and D) in BAT, indicative of the presence of microperoxisomes containing β-oxidation enzymes in BAT. The

structure of the tissue and the immunoreactivity with peroxisomal enzymes were studied throughout the cold acclimatization process of 4 weeks. During this process, the shape of the immunolabeled peroxisomes changed from being simple ovoid organelles at 4 days to becoming much more elongated and lobulated in rats cold acclimatized for 4 weeks. There was no obvious increase in the density of immunolabel for catalase (compare Fig. 4, A and C) or AOX (compare Fig. 4, B and D) between 4 and 28 days of cold exposure. Morphometric quantitation (Fig. 5) showed that the peroxisome volume in the cytoplasm (o/oo) increased significantly after a delay of 2 weeks in the cold, from 6.62 ± 0.964 (at 4 days) to 10.9 ± 1.39 at 4 weeks (Fig. 5A). The AOX labeling density (gold particles/μm² of peroxisome area) did not change during the first 2 weeks of cold acclimatization but increased significantly from 5.50 ± 0.41 particles/μm² peroxisomal area (at 4 days) to 6.59 ± 0.56 (at 3 weeks) and to 7.77 ± 0.82 particles/μm² of peroxisome area (at 4 weeks) (Fig. 5). Thus, the immunolabel concentration (labeling density/cytoplasm volume) of AOX increased from 36/μm² unit of cytoplasmic area (μm²) (at 4 days) to 84/unit of cytoplasmic area (μm²) (at 4 weeks) (Fig. 5B). During acclimatization to cold, BAT mass increases about 3-fold (31), therefore the total increase in peroxisome volume and in the content of peroxisomal AOX can be calculated to be about 7-fold. This is close to the earlier reported 10-fold increase in peroxisomal β-oxidation, suggesting a strong correlation of peroxisome proliferation to the previously reported increases in β-oxidation activity during cold acclimatization (31).

Transfection Experiments Using the BAT-derived Cell Line HIB 1B and HEK-293 Cells—Cold exposure triggers immediate and chronic adrenergic stimulation of BAT. To investigate if adrenergic stimulation of BAT can modulate PPAR activity it was necessary to develop a system where endogenous PPAR activity can be measured in an adipocyte environment. As demonstrated in Fig. 6A, HIB 1B cells express PPARγ and δ mRNA, but PPARα mRNA was not detected. In contrast, it was not possible to detect any PPAR subtype mRNA in HEK-293 cells, which makes these cell lines suitable for ligand activation studies of PPARs. To validate further the use of HIB 1B cells...
for the study of endogenous PPARs, nuclear proteins were isolated from both cell lines for EMSAs using an AOx PPRE probe. As demonstrated in Fig. 6B, both in vitro translated PPARγ/RXRα and HIB 1B nuclear extracts formed a complex with the same mobility which was, however, not obtained with HEK-293 nuclear extracts. The complex formed with the HEK-293 nuclear extract had a different mobility and could not be competed with unlabeled PPRE probe (lane 15) or supershifted with antibodies directed against PPARγ (lane 18). However, a weak supershift was observed when using antibodies to RXR (lane 17), indicating that this band may at least in part be due to binding of RXR to the probe. In contrast, the retarded complex seen in the presence of HIB 1B nuclear extracts was competed efficiently by the unlabeled PPRE probe (lane 6) but not by a DR2 probe (lane 7) and only weakly by a DR5 probe (lane 8). The mobility of the formed complex was supershifted efficiently by antibodies directed against RXR (lane 11) and, although to a lesser extent, also by antibodies directed against PPARγ (lane 13). These results indicate that the observed complex in HIB 1B extracts is formed in part by a PPARγ-RXRα heterodimer that specifically binds to the AOx PPRE sequence, and therefore endogenous PPAR activation can be measured in HIB 1B cells by transient transfection of the AOx-tk-Luc reporter plasmid. In vivo, cold exposure induces norepinephrine-mediated activation of BAT. To model this process, AOx-tk-Luc-transfected HIB 1B cells were treated with norepinephrine. As shown in Fig. 7, this treatment resulted in a 6.5-fold increase in luciferase activity, in sharp contrast to tk-Luc-transfected cells where norepinephrine treatment did not increase luciferase activity. To determine the type of adrenergic receptor that mediates the activation of the AOx-tk-Luc reporter, cells were treated with various agents that can trigger the cAMP signal transduction cascade. Forskolin (an activator of adenylate cyclase) and 8-bromo-cAMP treatments of AOx-tk-Luc-transfected HIB 1B cells mimic the norepinephrine effects and resulted in 12- and 5.7-fold increases of luciferase activity, respectively. This induction was not observed in tk-Luc-transfected cells, indicating that the AOx-PPRE sequence mediates the changes in luciferase activity. These results suggest that the β-adrenergic receptor signaling is involved in regulating AOx-reporter activation. Further evidence for this was provided by the lack of activation of the AOx reporter by 12-O-tetradecanoylphorbol-13-acetate (protein kinase C activation) and Ca2+, which are known to mimic α1-adrenergic receptor stimulation. We also found that propranolol could block the activation of the AOx-tk-Luc reporter by norepinephrine and that albuterol, a β-agonist, activated the AOx-tk-Luc reporter, whereas 6-fluoronorepinephrine, an α-agonist, was without effect. Furthermore, several PPAR activators (like Wy-14643, LY-171883, and linoleic acid) activated the AOx-tk-Luc reporter to an extent similar to that of norepinephrine (6–9-fold) (Fig. 7C).

To investigate the PPAR dependence of the AOx-tk-Luc reporter activation seen in HIB 1B cells, a gene transfer system was established in HEK-293 cells. This cell line does not express detectable PPAR mRNA levels as determined by Northern blot and EMSAs (see Fig. 6), making it possible to study the behavior of the PPAR subtypes without interference from endogenously expressed PPARs. To study the transcriptional activity of the full-length rat PPARs the AOx-tk-Luc construct was cotransfected into HEK-293 cells with the empty expression plasmid (CMV) or with an expression plasmid containing the full-length PPARα, γ2, or δ. Activators of several cellular signaling pathways were tested for their ability to activate the AOx-tk-Luc reporter in the HEK-293 transfection system. As shown in Fig. 8, forskolin/isobutylmethylxanthine and 8-bromo-cAMP treatment of HEK-293 cells resulted in increased luciferase activity only in cells expressing PPARα or PPARγ but not in cells expressing PPARδ or empty expression plasmid. In contrast, treatment with calcium (in combination with BayK-8644, an 1-type Ca2+ channel activator) or 12-O-tetradecanoylphorbol-13-acetate did not result in significant changes in luciferase activity. Known PPAR activators (Wy-14643, LY-171883, and linoleic acid) activated the AOx-tk-Luc reporter to an extent similar to that of cAMP-elevating compounds only when cotransfected with full-length PPARα, γ2, or δ expression vectors (Fig. 8B). Taken together, these transfection experiments support the conclusion that activation of the AOx-tk-Luc reporter plasmid in HIB 1B cells requires activation of the β-adrenergic pathway and that the activation was dependent on PPAR. The adrenergic stimulation of the HIB 1B cells did not lead to increased PPAR mRNA expression, as analyzed by Northern blotting (data not shown), strongly suggesting that the increased AOx-tk-Luc reporter gene activity is indeed due to a ligand-dependent activation via PPAR.

**DISCUSSION**

The first PPAR subtype described was PPARα, expressed predominantly in the liver and kidneys, the main target tissues for chemical compounds known as peroxisome proliferators (46). It was also found that these compounds can activate PPARα in transfection experiments. Disruption of the PPARα gene by homologous recombination demonstrated conclusively that PPARα directly mediates the effects of peroxisome proliferators on hepatic peroxisomes and expression of β-oxidation

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**Fig. 5. Changes in peroxisome volume density and AOx immunolabel concentration during cold acclimatization. Panel A,** the volume density of peroxisomes was expressed as the ratio of the area of peroxisomes to the cytoplasmic area, excluding lipid droplets (open circles). Panel B, the immunolabel concentration was expressed per unit of cytoplasmic area by multiplying the peroxisome volume density with the labeling density (open circles). The values shown are means ± S.E. (in panel A). The asterisk (*) indicates significance from the 4-day acclimatized rats (Student's t test).

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enzymes (9). The identification of two additional PPAR subtypes with selective tissue expression suggested that these nuclear receptors are involved in multiple physiological processes unrelated to hepatic peroxisome proliferators. PPAR\(_{g}\) was cloned as a highly adipose-specific transcription factor important for adipogenic gene expression (13). Ectopic expression of PPAR\(_{g}\) promotes lipid accumulation and expression of adipose genes in fibroblasts. The anti-diabetic thiazolidinediones can bind to and activate this PPAR subtype, providing further evidence for a possible role for this receptor in lipid metabolism and energy balance. The role of the PPAR\(_{d}\) subtype is not yet understood, but it has been suggested that it can act as a negative modulator of nuclear receptor function (47), and it has been identified as a fatty acid-activated factor mediating transcriptional activity in adipocytes (15). We cloned the rat PPAR\(_{g}\) subtype from a BAT cDNA library and demonstrated that all three PPAR subtypes are expressed in BAT, the thermogenic center in small mammals. It is therefore of special interest to elucidate the functions of the different PPARs in BAT under various physiological conditions.

In rats, cold acclimatization results in only weak proliferation of hepatic peroxisomes (48) but a pronounced proliferation of BAT peroxisomes (30) and induction of peroxisomal \(\beta\)-oxidation enzymes (31). However, feeding rats peroxisome proliferators results in a much smaller induction of peroxisomal enzymes in BAT than in liver, which is also dependent on the thyroid status and acclimatization temperature (49–51). It is clear that the cold acclimatization process leads to a series of distinct events that ultimately increase the activity, mass, and oxidative capacity of BAT. However, our results indicate that during the initial proliferative phase of adaptation to cold (the first 2 weeks) there is no significant increase in peroxisome proliferation or induction of peroxisomal enzymes. This is in accordance with our earlier findings that the specific activity of peroxisomal \(\beta\)-oxidation activity or catalase is not increased during the first 2 weeks of cold acclimatization (31). This phase has previously also been shown to be characterized by a dramatic proliferation of preadipocytes (measured as DNA synthesis) (26). In the present investigation we find that during this phase a near depletion of PPAR mRNA from the tissue occurs. The physiological relevance for the down-regulation of PPAR mRNA expression could be that sustained expression of PPAR\(_{a}\) and \(g\) could interfere with the proliferative phase. The reason for the lack of down-regulation of peroxisomal enzymes under this period is in accordance with the findings in the PPAR\(_{a}\)-null mice that contain normal levels of peroxisomes and peroxisomal enzymes (9).

During the second phase, corresponding to weeks 3 and 4 in the cold, PPAR\(_{a}\) and \(g\) mRNA levels increased to near normal levels, and a significant (about 2-fold) increase in the peroxisomal contribution to the total cytoplasmic volume was measured. The increase in immunoreactive AOx correlated to in-
increased AOx mRNA, and with respect to the 3-fold increase in tissue mass, these relatively modest increases in peroxisome volume and enzyme would therefore represent an approximately 7-fold increase in the total peroxisomal capacity in cold-adapted rats. These changes therefore correlate well to the earlier described increase in peroxisomal \( \beta \)-oxidation enzyme activity (31). AOx gene expression is regulated by PPAR, thus the data presented here suggest an active function of these nuclear receptors during BAT thermogenesis. Notably, expression of AOx also closely correlates to C/EBP\( \beta \) expression, indicating that also C/EBP\( \beta \) may regulate expression of the AOx gene. The final stage of BAT differentiation also coincides with increased PPAR\( \alpha \) and PPAR\( \gamma \) mRNA expression, which substantiates previously described adipogenic properties for these subtypes. In contrast, the PPAR\( \delta \) mRNA levels undergo a progressive increase during cold exposure, reflecting an additional difference between the PPAR\( \delta \) subtype and the other PPARs. It therefore seems clear that proliferating preadipocytes do not express high levels of PPAR\( \alpha \) or \( \gamma \) and that these two receptor subtypes become abundant only during the final differentiation phase. To our knowledge, only glucocorticoids and diurnal rhythm have been shown to affect PPAR\( \alpha \) expression \textit{in vivo} (53), and therefore the regulated expression of PPARs described here is the second example of physiological regulation of PPARs \textit{in vivo}. Fig. 9 aims at summarizing the short and long term effects of cold acclimatization on PPAR mRNA expression in relation to tissue hyperplasia, peroxisome proliferation, induction of peroxisomal enzymes, and adipose differentiation.

The expression of LPL mRNA did not appear to correlate to PPAR\( \gamma \) (or PPAR\( \alpha \)) mRNA expression. LPL mRNA was increased severalfold during the first 24 h in the cold and subsequently decreased to near control levels during the 4-week period in the cold. This pattern of LPL mRNA expression is in accordance to the changes in LPL activity during acclimatization to cold (54). The promoter of LPL apparently contains a functional PPRE that can be activated by PPAR\( \alpha \) and \( \gamma \) in transactivation assays (44); however, activation through PPARs does not seem to regulate LPL expression in BAT during cold acclimatization. BAT LPL activity has previously been

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**Fig. 7. Adrenergic activation of the AOx-tk-Luc reporter in HIB 1B cells.** HIB 1B cells were transiently transfected with the tk-Luc (open bars) or AOx-tk-Luc (filled bars) plasmids. Histograms demonstrate luciferase activity levels relative to untreated transfected cells (CON). Panel A, cells were treated with norepinephrine (NE, 0.1 \( \mu \)M), norepinephrine + propranolol (NE/P, 0.1 \( \mu \)M and 5 \( \mu \)M, respectively), albuterol (ALB, 0.1 \( \mu \)M), or 6-fluoronorepinephrine (6-F-NE, 0.1 \( \mu \)M). Panel B, cells were treated with forskolin + isobutylmethylxanthine (F/I, 12 \( \mu \)M and 1 \( \mu \)M, respectively), 8-bromo-cAMP (8Br, 1 \( \mu \)M), 12-O-tetradecanoylphorbol-13-acetate (TPA, 1 \( \mu \)M), and BayK (Ca\( ^{2+} \), 1 \( \mu \)M). Panel C, cells were treated with Wy-14643 (Wy, 100 \( \mu \)M), LY-171883 (LY, 1 \( \mu \)M), linoleic acid (LA, 30 \( \mu \)M), and norepinephrine (NE, 0.1 \( \mu \)M). The results are the means of duplicate experiments.

**Fig. 8. PPAR-dependent activation of the AOx-tk-Luc reporter in HEK-293 cells.** HEK-293 cells were cotransfected with the PPRE-tk-Luc reporter and either the pBKCMV plasmid (CMV) or expression constructs for PPAR\( \alpha \) (CMV-\( \alpha \)), PPAR\( \gamma \) (CMV-\( \gamma \)), or PPAR\( \delta \) (CMV-\( \delta \)). Panel A, luciferase activity in untreated transfected cells (Con) or in cells treated with forskolin and isobutylmethylxanthine (F/I, 12 \( \mu \)M and 1 \( \mu \)M, respectively), 8-bromo-cAMP (8-Br, 1 \( \mu \)M), or BayK (1 \( \mu \)M) or 12-O-tetradecanoylphorbol-13-acetate (TPA, 1 \( \mu \)M). Data shown represent the mean ± S.E. of the mean for three or four experiments. Panel B, cells were treated with Wy-14643 (Wy, 100 \( \mu \)M), LY-171883 (LY, 1 \( \mu \)M), and linoleic acid (LA, 30 \( \mu \)M).
shown to be increased through a β-adrenergic pathway and insulin (54, 55). The regulation of expression of Δ⁵,Δ⁷-enoyl-CoA isomerase does not appear to be PPAR-dependent in BAT during cold acclimation, but the very strong induction in rat liver (activity 25-fold and mRNA 29-fold (45)) by clofibrate treatment suggests that the Δ⁵,Δ⁷-enoyl-CoA isomerase gene may contain a PPRE. The Δ⁵,Δ⁷-enoyl-CoA isomerase mRNA was not increased during the 1st day of cold exposure. However, after 5 days in the cold, the expression was induced strongly. The pattern of induction does not correlate to PPAR expression and may suggest another mechanism of regulation.

In vivo exposure to a cold environment leads to chronic and immediate adrenergic stimulation of BAT. Our data also demonstrate that adrenergic stimulation of a PPRE reporter-transfected adipose cell line can result in PPAR-dependent reporter activation. In these experiments the levels of AOx-tk-Luc activity appear to result from PPAR activation as validated by the EMSA results that indicate that PPAR-RXR heterodimers efficiently bind to the AOx PPRE sequence, thus retarding its electrophoretic migration, and by the observation that nuclear extracts derived from HEK-293 cells with undetectable PPAR levels are unable to display retarded mobility of the probe. As further evidence for the PPAR dependence of the activation of the reporter gene in HIB 1B cells, we demonstrate in the HEK-293 gene transfer experiments that elevated levels of cAMP result in increased AOx-tk-Luc activity only if this reporter is cotransfected with a PPARα or PPARγ expression plasmid. The norepinephrine dependence of activation was characterized further in HIB 1B cells, and the data showed that albuterol, a β-agonist, activated the AOx-tk-Luc reporter, whereas 6-fluoronorepinephrine, an α-agonist, did not. Therefore, it appears that activation of the PPAR pathway in BAT is mediated through the β-adrenergic pathway. Adrenergic stimulation of brown fat cells results in cAMP-dependent activation of hormone-sensitive lipase, which rapidly hydrolyzes the stored triacylglycerol (56) and releases high concentrations of fatty acids that may act as activators of PPARs. The physiological ligands activating PPARs in vivo have not yet been identified conclusively, but several bioorganic compounds such as the less abundant lipid 15-deoxy-Δ¹²,¹⁴-prostaglandin-J₂ (33, 57–61) have been shown to activate PPARs in transactivation assays. In addition, fibrates and fatty acids were recently shown to bind to PPARα, γ, and δ, suggesting that these lipids may regulate gene expression through the direct interaction with these PPARs (58, 59). Our results from the gene transfer experiments using the HIB 1B cell line show that adrenergic stimulation of the cells results in activation of the PPRE reporter gene, although the data do not prove conclusively that the activation of the PPRE reporter gene by adrenergic stimulation is mediated by direct ligand-mediated activation. However, in view of the well characterized adrenergic stimulation of BAT which involves mobilization of endogenous fatty acids by hydrolysis of stored triglycerides, it is likely that the released free fatty acids act as ligands that activate PPARα and PPARγ, resulting in differentiation of adipocytes and metabolic activation via induction of lipid-metabolizing enzymes.

Several recent publications have shown that PPARα and γ are phosphoproteins and that phosphorylation events correlate with PPAR activity (62–65). Phosphorylation of these PPARs was shown to be mediated by mitogen-activated protein kinase. Mitogen-activated protein kinase is activated in BAT by adrenergic stimulation and cold exposure (66, 67), which may result in phosphorylation and inactivation of PPARs α and γ. The molecular basis for the down-regulation of PPARα and γ expression is at present not known. However, the differential regulation of PPAR subtype mRNAs during cold exposure could be related to the state of PPAR activation in a manner reminiscent of receptor down-regulation upon chronic exposure to a ligand released upon chronic adrenergic stimulation. If PPAR activity disfavors adipose proliferation, adrenergic PPAR activation may explain the need to reduce PPAR mRNA levels during early thermogenesis. Taken together, our results suggest an involvement of PPARs in cold-induced differentiation and activation of BAT possibly mediated by adrenergic stimulation of the tissue. Indeed, recent studies have demonstrated that treatment of rodents with thiazolidinediones promotes hyperplasia and differentiation of BAT and together with norepinephrine synergizes to increase expression of uncoupling protein (52, 68, 69). BAT may therefore be a very suitable tissue for elucidation of physiological functions of PPARs.

REFERENCES
1. Muehlfuss, A. S., Griffin, K. J., and Johnson, E. F. (1992) J. Biol. Chem. 267, 19051–19053
2. Tugwood, J. D., Issemann, I., Anderson, R. G., Bundell, K. R., McPheat, W. L., and Green, S. (1992) EMBO J. 11, 433–439
3. Zhang, B., Marcus, S. L., Sajjadi, F. G., Alaves, K., Reddy, J. K., Subramani, S., Rachubinski, R. A., and Capone, J. P. (1998) Proc. Natl. Acad. Sci. U.S.A. 95, 192, 16710–16714
4. Bardot, O., Aldridge, T. C., Latruffe, N., and Green, S. (1993) Biochem. Biophys. Res. Commun. 192, 37–45
5. Motojima, K., Passilly, P., Peters, J. M., Gonzalez, F. J., and Latruffe, N. (1998) J. Biol. Chem. 273, 16710–16714
6. Vu-Duc, N., Schoonjans, K., Laine, B., Fruchart, J. C., Auwerx, J., and Staels, B. (1994) J. Biol. Chem. 269, 31012–31018
7. Gulick, T., Cresci, S., Cairns, T., Moore, D. D., and Kelly, D. P. (1994) Proc. Natl. Acad. Sci. U.S.A. 91, 11012–11016
