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Up-Regulation of UCP-2 Gene Expression by PPAR Agonists in Preadipose and Adipose Cells

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UCP-2 is a member of the emerging family of UCP homologues. Upon high-fat feeding, UCP-2 mRNA levels are increased in epididymal fat pads of A/J mice, suggesting that the flux of fatty acids entering adipose tissue may regulate UCP-2 gene expression. Since fatty acids act as positive transcriptional regulators of lipid-related genes by means of peroxisome proliferator-activated receptors (PPARs), the regulation of UCP-2 gene expression by PPAR agonists (carbacyclin, α-bromopalmitate, BRL49653) has been examined in mouse preadipose and adipose cells in primary cultures or from clonal lines (Ob1771, 3T3-F442A, 1B8). In preadipose cells, carbacyclin and α-bromopalmitate are active and BRL49653 shows no effect, whereas all these ligands are active in adipose cells. The stimulatory effect of PPAR agonists is potentiated by RXR agonists in adipose cells. In contrast to the UCP-1 gene, norepinephrine as a cAMP-elevating agent does not enhance the expression of UCP-2 gene. Altogether, the data favor a predominant role of PPARδ in preadipose cells and the involvement of PPARγ2 in adipose cells in up-regulating UCP-2 gene expression. Thus, a potential link between fatty acid metabolism and thermogenesis may exist in PPAR-expressing tissues.

A mitochondrial protein called UCP-2 has been recently cloned and sequenced. This uncoupling protein is widely expressed in human and mouse tissues and is postulated to play an important role in energy balance, body weight regulation and thermoregulation (1). More recently, a new UCP homologue called UCP-3 has been described as being highly specific of skeletal muscle (2). Of particular interest is the fact that UCP-2 is up-regulated in white adipose tissue (**WAT**) of A/J mice which are resistant to high-fat feeding, but not in WAT of B6 mice which gain weight under these conditions. This observation suggests that an increased bulk of fatty acids entering adipose tissue might alter energy expenditure in A/J mice by means of the regulation of UCP-2 gene expression. Fatty acids have been reported to act as transcriptional regulators of the expression of lipid-related genes in adipose cells (3). The effect is likely mediated by peroxisome proliferator-activated receptors (**PPARs**) which are not only activated but also able to bind thiazolidinediones (BRL49653), fatty acids, various eicosanoids and fibrates (4-8). In this study we have examined in preadipose and adipose cells from clonal lines as well as in primary culture of adipose precursor cells the regulation of UCP-2 gene expression by PPAR agonists. The validity of using such cells has been previously shown since they behave as authentic precursors of adipocytes both *in vitro* and *in vivo* (9). Our results show that the expression of UCP-2 gene increases as a function of differentiation. PPAR agonists are able to up-regulate UCP-2 gene expression in both preadipose and adipose cells and this effect is potentiated by an RXR agonist.

MATERIALS AND METHODS

Cell culture. Cells of Ob1771 and 3T3-F442A clonal lines were plated at a density of 2×10^3 cells per cm^2 and grown in Dulbecco's modified Eagle's medium (**DMEM**) supplemented with 8% fetal bovine serum, 200 units/ml of penicillin, 50μg/ml streptomycin, 33mM biotin and 17mM pantothenate (referred as standard medium). Confluence (day 0) was reached within 5 days. Unless otherwise stated

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Abbreviations used: **Act D,** actinomycin D; **a-FABP,** adipocyte fatty acid-binding protein; **cPGI$_2$**, carba(prosta)cyclin or carbacyclin; **Cyclo,** cycloheximide; **DMEM,** Dulbecco's modified Eagle's medium; **Dex,** dexamethasone; **IBMX,** 3-isobutyl-1-methylxanthine; **PGs,** prostaglandins; **PPARs,** peroxisome proliferator-activated receptors; **T3,** tri-iodothyronine; **WAT,** white adipose tissue.

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one day post-confluent cells, i.e. lipid-free preadipose cells known to express early markers of differentiation only, were used. Differentiated triacylglycerol-containing cells, which express late markers of differentiation, were also used in some experiments. In this case confluent Ob1771 preadipocytes were maintained for 14 to 18 days in standard medium containing 17nM bovine insulin and 2nM triiodothyronine (**T_3**) (termed as standard differentiation medium) and supplemented with 10nM dexamethasone (**Dex**) plus 100μM 3-isobutyl-1-methylxanthine (**IBMX**) during the first three days in order to trigger differentiation. Media were changed every other day. Before adding the various effectors, both undifferentiated preadipose cells and differentiated adipose cells were transferred to a serum-free medium composed of DMEM supplemented with 850 nM bovine insulin, 10μg/ml human transferrin and 0.04% bovine fatty acid-free serum albumin (referred to as IT medium). Differentiation of cells of 1B8 clonal line into brown adipocytes (10) was performed as described previously (11,12). Primary cultures of adipose precursor cells from epididymal fat pads of C57BL/6J mice were carried out of as described previously (13).

RNA analysis. RNAs were prepared by the guanidium thiocyanate technique (14); a minimum of three dishes were pooled for each condition. Northern-blot analysis using full length mouse UCP-2 cDNA (1) were performed as described previously (12,15). Autoradiographs were quantitated using Fujix PhosphorImager. All results were normalized to β-actin signals.

Materials. Culture media were obtained from Gibco (Cergy-Pontoise, France) and Gibco BRL (Gaiterburg, U.S.A.). Fetal bovine serum was a product of Seromed (Berlin, Germany). [α-^{32}P]dCTP, random priming kit and Hybon membranes from Amersham (Les Ulis, France). Enzymes for nucleic acid manipulations were from Eurogentec (Seraing, Belgium) and New England Biolabs (Beverly, U.S.A.). Other chemical products were purchased from Amersham (Les Ulis, France). All prostanoids were products from Cayman Chemicals (SpiBio, Massy, France). BRL49653 was obtained from SmithKline Beecham Pharmaceuticals (Welwyn, U.K.). CD2809 (patent n° E.P. 0679630) was obtained from CIRD-Galderma (Sophia-Antipolis, France) and LGD1069 (16) was a kind gift of Glaxo-Wellcome (Research Triangle Park, USA).

RESULTS

UCP-2 gene expression during adipose cell differentiation. UCP-2 mRNA was detectable at confluence (day 0), began to accumulate 8 days later and continued to increase until day 16. When compared with the expression of the mitochondrial PIL-7 gene, which encodes for a subunit of cytochrome C oxidase (17), the expression of UCP-2 and that of PIL-7 genes were similar. These observations are in agreement with the characterization of UCP-2 as a mitochondrial carrier (1) and suggest that some mitochondriogenesis is taking place during adipocyte formation. A similar increase of UCP-2 gene expression ($\sim$ 4-fold) was observed during differentiation of 3T3-F442A preadipose cells and that of 1B8 (10) preadipose cells (not shown). These results indicate that the differentiation-dependent expression of UCP-2 gene was not restricted to a particular preadipocyte cell line and that both white and brown adipose cells express UCP-2 gene.

Regulation of UCP-2 gene expression in committed lipid-free preadipose cells. In order to study the effects of PPAR agonists on the expression of UCP-2 gene,

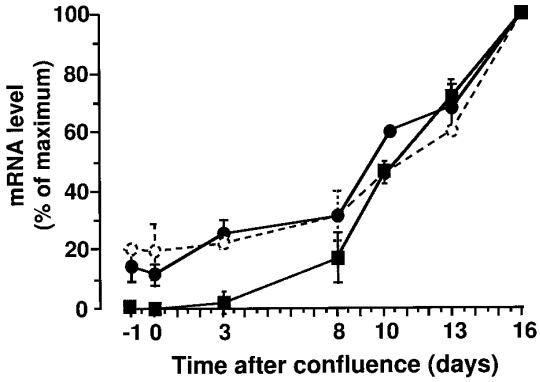

FIG. 1. Kinetics of UCP-2 gene expression in Ob1771 cells. Cells were grown in standard medium and transferred at confluence in standard differentiation medium as described in Materials and Methods. At the indicated times, RNAs were isolated and Northern-blot analysis was performed (20μg/lane). The results are given as means of two independent experiments. UCP-2 (●), a-FABP (■), and PIL-7 (○) mRNA signals were normalized to those of β-actin and expressed as % of the maximal response obtained for each probe.

Ob1771 preadipose cells were treated for 24h in serum-free conditions and exposed or not to various effectors. At that stage PPARδ and PPARγ2 were present at high and low levels, respectively, whereas PPARα was not detected (18). As shown in Figure 2, Ob1771 preadipose cells, maintained in IT medium, were able to respond rapidly (within 24h) and dose-dependently (0.1-1μM) to carbacyclin with a $\sim$ 4-fold increase in the levels of both UCP-2 mRNA and the adipocyte fatty acid-binding protein (**a-FABP**) mRNA. It should be stressed that this effect does not require serum components and appears independent from the differentiation process *per se* as it occurs in the absence of adipogenic hormones which are required to terminate differentiation (9). These results are in agreement with reports showing that carbacyclin (**$cPGI_2$**) is an activator of all three PPAR isoforms in transactivation assays (19) and is a ligand of PPARδ and γ (6). Other prostaglandins (**PGs**) such as PGE_1, PGE_2, PGD_2, 6-keto-PGE_1 and 6-keto-$PGF_{1\alpha}$, when present at 1μM, were unable to exert any significant effect on the level of UCP-2 mRNA. Similar results were obtained for the regulation of a-FABP gene, with the exception of PGD_2 which was slightly active as previously reported (15). It is striking that PGE_1 and 6-keto-PGE_1 were inactive despite their binding to the same cell surface receptor as prostacyclin, their ability to alter intracellular cAMP and free calcium levels (20) and to induce terminal differentiation of Ob1771 preadipose cells although to a lower extent than carbacyclin (21). α-bromopalmitate, reported to be the most potent activator of the expression of a-FABP gene in PPARδ-expressing fibroblasts (22), was also active ($\sim$ 5-fold increase) in the 1-10μM range of concentrations at a fixed concentration of 6μM serum albumin (molar ratio from 0.17 to 1.7). In contrast to

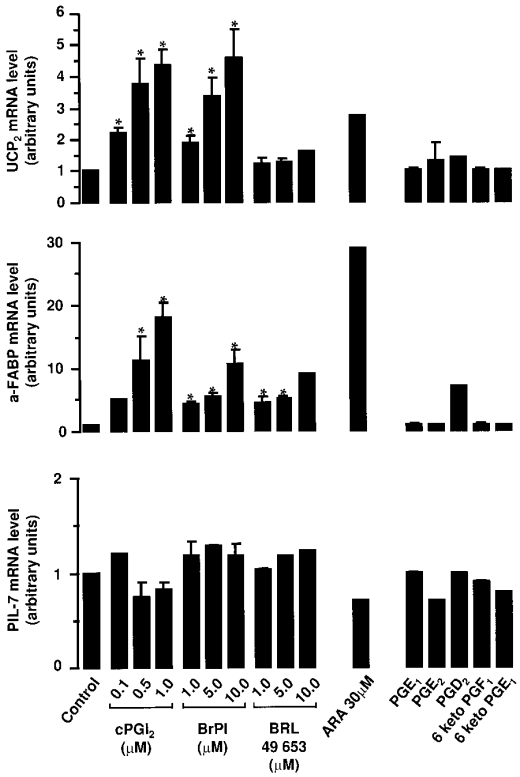

FIG. 2. Accumulation of UCP-2, a-FABP and PIL-7 mRNAs in Ob1771 preadipose cells exposed to PPAR agonists. One-day post-confluent preadipose cells were maintained in serum-free medium alone or supplemented with various PPAR agonists. 24 h later, RNAs were extracted and analyzed by Northern-blot (20μg/lane). The intensities of the signal were normalized to those of β-actin and expressed by taking as 1 the signal obtained with untreated cells. The data are the means ± S.E.M. of values obtained in three separate experiments and a single experiment in the case of arachidonic acid, PGD_2, and 10μM BRL49653. *, significantly different from control cells, $p < 0.001$.

carbacyclin and α-bromopalmitate, BRL49653, a thiazolidinedione which promotes adipose differentiation of Ob1771 cells (23), and which binds specifically PPARγ2 and promotes adipose differentiation of PPARγ2-expressing fibroblasts (24-26), proved to be ineffective between 1 and 10μM. Similar results were obtained with 3T3-F442A preadipose cells as 1μM carbacyclin, 10μM α-bromopalmitate and 1μM BRL49653 were able to increase within 24h in IT medium the UCP-2 mRNA content by 5.8-fold, 4-fold and only 1.35-fold, respectively. Thus the regulation of UCP-2 gene expression is clearly distinct from that of a-FABP gene as both carbacyclin, α-bromopalmitate ***and*** BRL49653 were able to stimulate in Ob1771 preadipose cells the expression of a-FABP gene up to 17-fold, 10-fold and 8-fold, respectively (Fig. 2) and in 3T3-F442A cells up to 7-fold, 7-fold and 3-fold, respectively (not shown).

Similar to the effect of BRL49653 (23), arachidonic acid was clearly active in increasing the expression of a-FABP gene but showed a weak effect on that of UCP-2 gene. Under all conditions, in contrast to UCP-2 and a-FABP genes, the expression of PIL-7 gene remained unchanged, strongly suggesting a specific role of PPAR agonists in the regulation of UCP-2 gene expression.

As shown in Figure 3, the accumulation of UCP-2 and a-FABP mRNA in preadipose cells, when exposed to 1μM carbacyclin for 15h, was abolished in the presence of actinomycin D or cycloheximide. Since the regulation of a-FABP gene expression was shown to be primarily transcriptional and to require protein synthesis (3), this suggests that the regulation of UCP-2 gene expression might also be regulated at the transcriptional level under these conditions.

Regulation of UCP-2 gene expression in adipose cells. As the UCP-2 mRNA content is present at high levels in differentiated cells (Fig. 1) and as adipocytes represent the major cell type present in adipose tissues (9), studies of the regulation of UCP-2 gene expression have been carried out in Ob1771 adipose cells exposed to PPAR agonists in the absence or the presence of a spe-

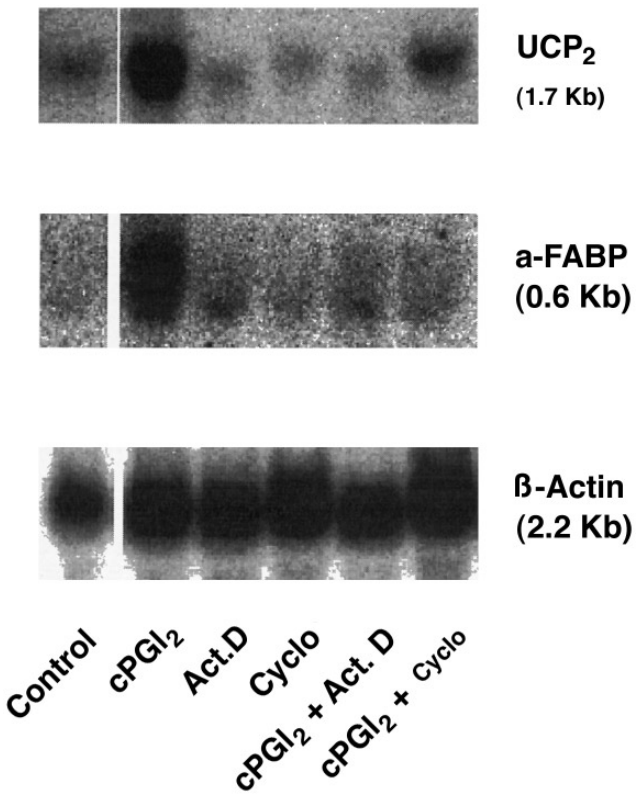

FIG. 3. Effect of actinomycin D and cycloheximide on UCP-2 and a-FABP mRNA levels in Ob1771 preadipose cells exposed to carbacyclin. One-day post-confluent preadipose cells were maintained for 15 h in serum-free medium supplemented or not with 1μM $cPGI_2$, 2μg/ml actinomycin D (**Act D**), 1μg/ml cycloheximide (**Cyclo**), or a combination of $cPGI_2$ and these later compounds ($cPGI_2$ + Act D and $cPGI_2$ + Cyclo, respectively). RNAs were then extracted and analyzed by Northern-blot (20μg/lane). The results are representative of three separate experiments.

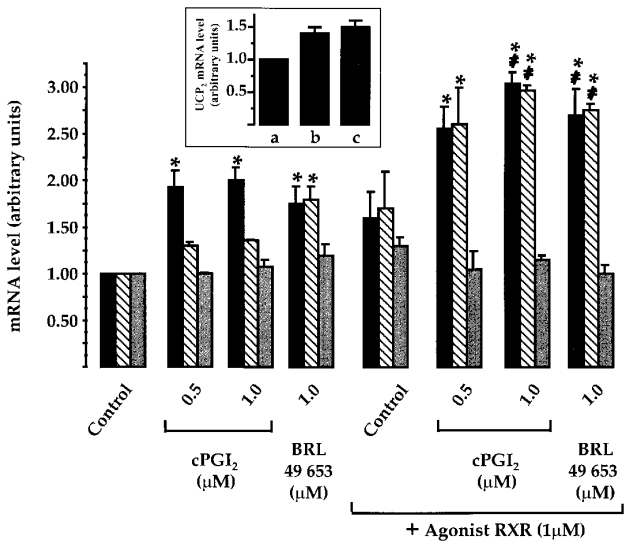

FIG. 4. Expression of UCP-2, a-FABP, and PIL-7 genes in Ob1771 adipose cells exposed to PPAR agonists and to PPAR/RXR agonists. Ob1771 cells, grown and differentiated as described in Materials and Methods, were transferred to serum-free medium for 24h. Fresh serum-free medium was then supplemented with PPAR agonists in the absence or the presence of 1μM CD2809 as RXR agonist. Twenty-four hours later, RNA were extracted and analyzed by Northern-blot. The intensity of the signals obtained for UCP-2 (black bars), a-FABP (hatched bars), and PIL-7 (grey bars) was normalized to those of β-actin and expressed by taking as 1 the signal obtained for each probe with untreated cells. The data are the means ± S.E.M. of values obtained in four and two separate experiments in the absence or the presence of RXR agonist, respectively. Inset: UCP-2 mRNA level in 8-day post-confluent differentiated adipose cells in primary culture either untreated (**a**) or treated for 4h with 10μM α-bromopalmitate (**b**) or 1μM BRL49653 (**c**). The data are representative of two separate experiments which gave similar results. *, significantly different from control cells, $p < 0.005$; #, significantly different from RXR agonist-treated control cells, $p < 0.05$.

cific RXR agonist. The expression of UCP-2 gene was compared to that of a-FABP and PIL-7 genes. At that stage, Ob1771 adipose cells still express PPARδ but now express also high levels of PPARγ2 (18). As shown in Figure 4, Ob1771 adipose cells remained able to respond to carbacyclin (×1.9-fold) but then became able to respond to BRL49653 (×1.7-fold), a specific ligand of PPARγ2 (24). A similar response to BRL49653 (×1.4-fold) and a response to α-bromopalmitate (×1.5-fold) were also observed in differentiated mouse adipose precursor cells in primary culture (inset of Fig. 4). CD2809, a specific RXR agonist, showed a weak effect when present alone but potentiated significantly the effect of carbacyclin and BRL49653 with a 2.5- to 3.0-fold increase of UCP-2 mRNA level above control values. In the case of a-FABP gene expression, carbacyclin alone was less potent than BRL49653 but, in combination with the RXR agonist, similar levels of a-FABP mRNA were obtained with carbacyclin or BRL49653. PPAR agonists, present alone or in combination, did not have any significant effect on the expression of PIL-7 gene, suggesting again a specific role of PPAR and RXR agonists on the expression of UCP-2 and a-FABP genes.

Similar results were also observed in the brown 1B8 adipose cells. Figure 5 shows that, in the presence of PPARγ2 and RXR agonists, UCP-2 mRNA levels were increased 2.7-fold above the level observed in standard medium alone, and 1.8-fold over the increase observed with differentiation alone. In addition, these data also show that the cAMP pathway does not regulate UCP-2 in the same manner as UCP-1. The addition of norepinephrine to the cells for 4 hours (a treatment known to potently induce UCP-1 expression in this and other brown adipose tissue cell lines; ref.12) did not affect the expression of UCP-2 under any of the growth or differentiation conditions.

DISCUSSION

In the present study, using a mouse full-length cDNA which recognizes a single transcript of 1.6 kb, UCP-2 mRNA can be easily detected by Northern-blot analysis strongly suggesting a significant level of expression of UCP-2 protein which still remains to be shown. Since UCP-2 mRNA has been reported to be present in white and brown adipose depots of rodents (1), it is not surprising that UCP-2 gene is expressed in adipose cells from clonal lines known to express UCP-1 protein

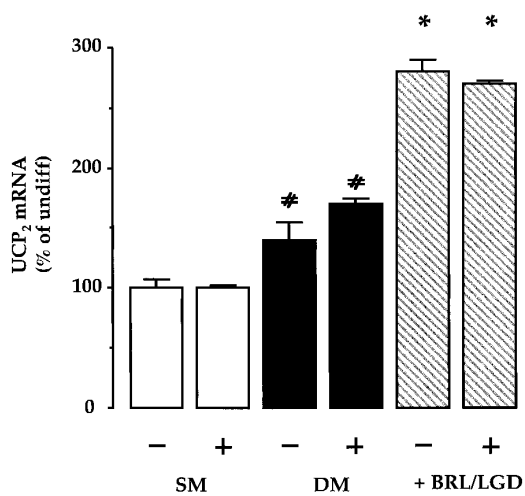

FIG. 5. Increased expression of UCP-2 in 1B8 cells by PPAR/RXR agonists. 1B8 cells were cultured as previously described (10). Some cells were maintained for 5 days in standard medium supplemented (**DM**) or not (**SM**) with insulin and T_3, whereas other cells were also treated (BRL/LGD) with 1μM BRL49653 and 0.1μM LGD1069, a specific RXR agonist. During the final 4 h prior to harvest, cells were treated with 1μM norepinephrine (+) or aqueous vehicle (−). Total cellular RNA was isolated for Northern-blot analyses. Cyclophilin mRNA levels were used as internal control (12). The results shown are from two independent experiments of four samples each. #, significantly different from SM samples, $p<0.05$; *, significantly different from SM and DM samples, $p<0.001$.

(clonal line 1B8) or not to express UCP-1 protein (clonal lines Ob1771 and 3T3-F442A). Moreover, since mouse stromal-vascular cells of epididymal fat pads are able to express UCP-2 after differentiation in primary cultures, it can be ruled out that some irrelevant expression of UCP-2 gene takes place preferentially in preadipocyte clonal lines. Quite to the contrary, the differentiation-dependent expression of UCP-2 gene, parallel to that of the PIL-7 mitochondrial gene, suggests that UCP-2 is implicated in mitochondriogenesis within adipose cells and thus could play a role in energy expenditure of mature cells.

In rodents, high-fat feeding for 2-3 weeks leads to adipocyte hypertrophy followed by adipose tissue hyperplasia (27). In A/J mice but not in B6 mice, UCP-2 mRNA content is increased after high-fat feeding (1), suggesting also that the sustained and increased fatty acid flux entering adipose tissue may lead to increase UCP-2 gene expression. In support of our hypothesis that increased UCP-2 levels in A/J mice contribute to the resistance to obesity, we have found that core body temperature in A/J mice consuming a high-fat diet is significantly increased as compared to B6 mice (S. Collins and R. Surwitt, unpublished observations). In the study presented herein, the importance of a fatty acid flux has been examined directly in preadipose and adipose cells which are able to respond to natural and non-metabolizable fatty acids by enhanced transcription of various lipid-related genes (3,28). This effect appears to be due to the involvement of PPARs which have been very recently shown to be able to bind the thiazolidinedione BRL49653 as well as various amphipatic carboxylates, i.e. fatty acids, eicosanoids and fibrates (5-7,24). Although the differential activation of the PPAR family members by these compounds is of interest, a comparison of transactivation data indicates that the specificity of PPARα, PPARδ and PPARγ2 for a given inducer depends to a large extent on the experimental design, i.e. the nature of the recipient cells and/or the expression vectors and their levels of expression (22,26,29,30). Consequently, the role of PPARs in the regulation of UCP-2 gene expression has been examined in the present study in their natural cellular context, i.e. preadipose and adipose cells, in which they appear to be implicated at various stages of adipocyte differentiation (31). It is likely that PPARδ plays a critical role in the regulation of UCP-2 gene expression in preadipose cells as i) PPARα is undetectable in contrast to high and low levels of PPARδ and PPARγ2, respectively, ii) $cPGI_2$ and α-bromopalmitate are stimulators of UCP-2 gene expression whereas BRL49653 appears inactive (Fig. 2). Since the latter compound is a specific ligand and a potent activator of PPARγ2 whereas it activates weakly and does not bind to PPARδ (6,7,24), and since $cPGI_2$ and α-bromopalmitate can bind and activate only PPARδ and PPARα (6,7), a role of PPARδ appears predominant, although the involvement of uncharacterized members of the PPAR subfamily cannot be excluded.

Once preadipose cells have undergone terminal differentiation, the fact that BRL49653 is now active in adipose cells is likely due to the presence of PPARδ ***and*** that of PPARγ2 then expressed at high levels (18). RXR specific ligands have been reported as potent adipogenic agents in cells expressing PPAR/RXR heterodimers in particular the PPARγ2/RXRα heterodimer. RXRα and RXRβ are present respectively at high and low levels in Ob1771 adipose cells (32) and it is of interest to note that simultaneous treatment of adipose cells with both PPARγ2 (or PPARδ) and RXR specific agonists results in an additive increase of UCP-2 mRNA content, suggesting that activation of PPARγ2 (or PPARδ)/RXRα heterodimer may provide a new approach to increase UCP-2 protein in adipose tissue.

The effect of PPAR agonists on the regulation of UCP-2 gene expression appears quite specific as other hormones such as glucocorticoids, 17β oestradiol, T_3 and growth hormone proved to be ineffective in adipose cells. Interestingly enough, insulin showed no effect in both preadipose and adipose cells maintained in serum-free or serum-supplemented medium despite robust responsiveness of other genes in these cells in response to insulin under these conditions (not shown). Finally, although the expression of PPARγ2 appears most prominent in adipose tissue and that of PPARδ appears also important, the fact that these PPARs as well as RXRs are expressed in other tissues may provide a pharmacological mean to regulate UCP-2 levels in various organs.

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