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Fatty Acid Activation of Peroxisome Proliferator-activated Receptor (PPAR)

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Peroxisome proliferators such as clofibric acid, nafenopin, and WY-14,643 have been shown to activate peroxisome proliferator-activated receptor (PPAR), a member of the steroid nuclear receptor superfamily. We have cloned the cDNA from rat that is homologous to that from mouse, which encodes a 97% similar protein. To search for physiologically occurring activators, we established a transcriptional transactivation assay by stably expressing in CHO cells a chimera of rat PPAR and the human glucocorticoid receptor that activates expression of the placental alkaline phosphatase reporter gene under the control of the mouse mammary tumor virus promoter. 150 μM concentrations of arachidonic or linoleic acid but not of dehydroepiandrosterone, cholesterol, or 25-hydroxy-cholesterol, activated the receptor chimera. In addition, saturated fatty acids induced the reporter gene. Shortening the chain length to $n = 6$ or introduction of an ω-terminal carboxylic group abolished the activation potential of the fatty acid. To test whether a common PPAR binding metabolite might be formed from free fatty acids we tested the effects of differentially β-oxidizable fatty acids and inhibitors of fatty acid metabolism. The peroxisomal proliferation-inducing, non-β-oxidizable, tetradecylthioacetic acid activated PPAR to the same extent as the strong peroxisomal proliferator WY-14,643, whereas the homologous β-oxidizable tetradecylthiopropionic acid was only as potent as a non-substituted fatty acid. Cyclooxygenase inhibitors, radical scavengers or cytochrome *P*450 inhibitors did not affect activation of PPAR. In conclusion, β-oxidation is apparently not required for the formation of the PPAR-activating molecule and this moiety might be a fatty acid, its ester with CoA, or a further derivative of the activated fatty acid prior to β-oxidation of the acyl-CoA ester.

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Can a nutrient regulate gene transcription? Can a nutrient control its own metabolism, and therefore its own intracellular concentration, in a similar way as glucose controls gene expression in prokaryotes (i.e. the lactose operon)? Since Issemann and Green described the first PPAR (peroxisome proliferator-activated receptor) in 1990 [1], and we demonstrated that this transcription factor is activated by free fatty acids [2], the answer to these questions might be affirmative.

Peroxisomes are subcellular organelles that perform metabolic reactions mainly related to lipid metabolism, one of the most important being the β-oxidation of fatty acids. Administration of a number of man-made unrelated compounds, including hypolipidemic drugs, such as fibric acid derivatives, nafenopin, WY-14,643, or sulfur-substituted fatty acids, industrial plasticizers and others, provoke peroxisomal proliferation and activate peroxisomal β-oxidation enzymes [3, 4]. There are two hypotheses which might explain the generation of peroxisomal proliferation by such a wide variety of inducers. One of these theories proposes that the intracellular overload of fatty acids, which is produced by these chemicals as well, is the real stimulus for promoting peroxisomal proliferation. The other theory postulates the existence of a receptor and an as yet unknown messenger [3]. This latter proposal is now well supported by the discovery of mouse peroxisome proliferator-activated receptor (mPPAR), a member of the steroid nuclear receptor superfamily, which is a

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large class of ligand-activated transcription factors regulating gene expression [1]. Similarly to other nuclear receptors of this superfamily, the amino acid sequence of the PPAR can be divided into distinct regions or domains (A–F), based on homology with the other members [5]. Taking advantage of this property, chimeric nuclear hormone receptors containing the putative ligand-binding domain (LBD) of PPAR and the N-terminal and DNA-binding domains (DBD) of either the estrogen (ER) or the glucocorticoid (GR) receptor were constructed. It was demonstrated that compounds promoting proliferation of peroxisomes also induce PPAR transcriptional activity [1]. However, none of these substances shows direct binding to the PPAR.

In our laboratory it was possible to clone a PPAR cDNA from rat that is homologous to the PPARα cDNA from mouse [2]. To find physiological activators, or putative ligands, a transcriptional transactivation assay was established by stably expressing in CHO cells a chimera of the N-terminal and DNA-binding domains of the GR and the ligand binding domain of rat PPARα together with placental alkaline phosphatase as a reporter gene. The reporter gene was under the control of the mouse mammary tumor virus promoter which is normally inducible by glucocorticoids via the GR. It is well known that a high-fat diet is also capable of inducing peroxisomal proliferation [6]. Therefore, a broad range of free fatty acids as well as other compounds related to lipid metabolism were tested for activation of this receptor chimera. On the one hand, as was to be expected, the reporter gene was activated in these CHO cells by the known activators of mPPAR, WY-14,643 and clofibric acid. On the other hand, fatty acids like linoleic (all-*cis*-Δ9,12-C18:2) and arachidonic (all-*cis*-Δ5,8,11,14-C20:4) acid, but not dehydroepiandrosterone (DHEA, a steroid hormone that also stimulates peroxisomal proliferation), DHEA sulfate, cholesterol, or 25-hydroxycholesterol, activated the reporter gene in cells expressing the receptor chimera.

To study the specific structural requirements of fatty acids, if any, in activation of PPAR, unsaturated as well as saturated fatty acids were tested. Neither the degree of unsaturation, the position of the double bonds, nor the steric conformation influenced the potency of the different, unsaturated fatty acids to activate the GR-PPAR receptor chimera. This is interesting since most of the fatty acids found in nature bear double bonds in the *cis*-configuration, however, elaidic acid (*trans*-Δ9-C18:1) activated the PPAR chimera with the same potency as its *cis*-homologue, oleic acid (*cis*-Δ9-C18:1). With regard to the saturated fatty acids, lauric acid (C12:0) was able to induce reporter gene activity, whereas caproic acid (C6:0) and 1,12-dodecanedioic acid did not substantially activate the receptor chimera. Therefore, the structural requirements for fatty acids to activate PPAR did not seem to be very strict since only reduction of the chain length to six carbons or introduction of a second carboxylic group at the ω-position abolished the inducing activity. So, the only structural property that seems to be common to all the PPAR-activating compounds is the presence or easy formation of a poorly metabolized acidic function and a minimal lipophilic backbone [2]. The activation of PPAR by fatty acids is in agreement with the fact that fatty acids provoke similar physiological actions as peroxisomal proliferators, i.e. they lower serum triglycerides, increase lipid deposition in the liver and produce proliferation of liver peroxisomes [7].

Since, at least in the case of linoleic acid, the PPAR activating-dose of fatty acids was found within the range of serum levels of the nonesterified acid, a potential physiological role of fatty acids as activators of PPAR might be proposed. Furthermore, using the GR–PPAR chimeric receptor stably expressed in CHO cells, it was possible to isolate activators from human serum. Basically, the analytical biochemistry approach to fractionate serum according to polarity and charge, described by Banner *et al.* [8] for the isolation of steroids from plasma, was complemented by the screening for PPAR-activation in the reporter cell line. The only PPAR-activators isolated from human serum by this approach were fatty acids (i.e. palmitic acid, oleic acid, linoleic acid, and arachidonic acid), which further emphasizes the role of these compounds as potential endogenous activators of PPAR [9]. However, identification of activators with this approach did not include high affinity/specificity binding and therefore the identified activators did not necessarily represent specific ligands. These results might constitute the link between the receptor and fatty acid hypotheses (cf. above) respectively, as proposed recently [10]; peroxisome proliferators might promote intracellular accumulation of lipids (e.g. fatty acids), which would induce the transcriptional activity of PPAR, affecting several genes related to lipid metabolism and ultimately resulting in peroxisomal proliferation.

The specific peroxisome proliferator responsive element (PPRE) consists of a direct repeat of an AGGTCA element spaced by a single nucleotide. It has been shown that induction of cytochrome *P*450 IVA expression by peroxisome proliferators, as well as of acyl-CoA oxidase and peroxisomal β-oxidation bifunctional enzyme (enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase), is mediated through a PPRE in the 5′-regulatory region of these genes [11–13]. More recently, an additional PPRE has also been found in the regulatory sequence of mitochondrial HMG-CoA synthase gene [14]. The existence of this PPAR responsive element has enabled other groups to corroborate that fatty acids actually activate PPAR transcriptional function, without using the chimera approach. Thus, PPAR expression vector and reporter plasmid, carrying the gene for chloramphenicol acetyltransferase under the

control of the PPRE of acyl-CoA oxidase and thymidine kinase or β-globin gene promoter, were cotransfected into a suitable cell line. Testing a number of free fatty acids, both Keller *et al.* [15] and Issemann *et al.* [16] confirmed that PPAR is activated by fatty acids. However, Issemann *et al.* found that saturated fatty acids induced PPAR activity better than unsaturated fatty acids. This might be due to differences in PPAR responsiveness to these compounds among different species; recently it has, e.g., been proposed that methylpalmitate is an endogenous activator of the human PPAR (NUCI) [17] whereas we found that the methyl esters of fatty acids were unable to induce PPAR from rat [9]. To date, five PPARs have been identified in mouse (mPPAR α, δ and mNUCI, and the isoforms mPPAR γ1 and γ2), three in *Xenopus* (xPPAR α, β and γ), three in the human (hPPAR α and γ, and hPPAR/NUCI, a β-homologue) and finally only one in rat (rPPARα). Thus, as shown by Kliewer *et al.* [18], distinct types of PPAR within the same species can present different responsiveness to activators (i.e. fatty acids and peroxisome proliferators) and their stimulation can even depend on the nature of the PPRE [19].

To further characterize the common fatty acid metabolite which activates, and possibly binds, PPAR, the roles of the cyclooxygenase and lipoxygenase pathways (arachidonic acid dependent signals), the cytochrome *P*450 dependent metabolism of fatty acids and the formation of potential activators of PPAR via the β-oxidation pathway were evaluated. Besides, since hydrogen peroxide and hydroxy radicals appear to play a role in the activation of some transcription factors (e.g. the NF-κB) [20], and since peroxisomal β-oxidation is known to produce peroxides and reactive oxygen intermediates, the role of reactive oxygen species for the activation of the GR–PPAR chimera was also tested [21]. First of all, since PPAR is supposed to play a key role in the induction of peroxisome proliferation, the requirement of fatty acid β-oxidation for the activation of PPAR was tested. Fatty acid derivatives which have either the β-carbon (the 3-thia-fatty acid, tetradecylthioacetic acid) or the γ-carbon (the 4-thia-fatty acid, tetradecylthiopropionic acid) replaced by sulphur were used. The 3-thia-fatty acid, which is blocked for β-oxidation, induced peroxisomal proliferation as palmitoyl-CoA oxidase activity *in vivo*, whereas the 4-thia-fatty acid, which undergoes at least one round of β-oxidation, was only a weak proliferator. A quite similar result was obtained using the GR–PPAR chimera stably expressed in CHO cells, the 3-thia fatty acid being equally potent as a strong peroxisome proliferator such as WY-14,643, in agreement with the finding reported by Issemann *et al.* [16], whereas the 4-thia-fatty acid was only as potent as a non-substituted fatty acid. All these results indicate that PPAR is stimulated by high concentrations of an as yet unknown fatty acid derivative. Furthermore, β-oxidation of the inducing fatty acid is not required and blocking of this pathway increases the potency of a fatty acid derivative for activating PPAR [21]. These results would be in agreement with the suggestion made by Issemann *et al.* [16] that rapid metabolism of fatty acids within the cell might explain why high concentrations of β-oxidizable fatty acids are required to activate PPAR. In fact, Kaikaus *et al.* [22] have shown that oleic acid alone was unable to stimulate peroxisomal β-oxidation or FABP (fatty acid-binding protein), but in the presence of 2-tetradecylglycidic acid (TDGA), an inhibitor of CPT I (carnitine palmitoyltransferase I), induced peroxisomal β-oxidation and FABP pretranslationally.

A strong inducer of PPAR transcriptional activity is arachidonic acid [2]. Since this compound is the precursor for the synthesis of all eicosanoids, such as prostaglandins, thromboxanes, and leukotrienes, all of which are implicated in several signal-transducing processes within the cell, it was important to demonstrate whether the activation of PPAR was caused by the fatty acid itself or by a metabolite produced during the formation of eicosanoids. The lack of any effect of indomethacin or aspirin on arachidonic acid activation of the PPAR, as we [21] and Keller *et al.* [15] have shown, together with the fact that even saturated fatty acids activate this receptor [2], suggest that the cyclooxygenase pathway is not involved in the formation of the PPAR-activating molecule. When using other specific blockers such as nordihydroguaiaretic acid (NDGA) for the lipoxygenase pathway, and metyrapone for the epoxygenase pathway, basically the same negative results were obtained [15, 21], and even treatment with a number of eicosanoids did not show any activation of PPAR. Nevertheless, Keller *et al.* [15] found that ETYA (5,8,11,14-eicosatetraynoic acid), a blocker of lipoxygenases and cycloxygenases, was 100 times more potent than the previously most efficient activator WY-14,643 or than arachidonic acid to induce xPPARα.

On the other hand, antioxidants and radical scavengers, such as *N*-acetylcysteine and pyrrolidinedithiocarbamate, did not interfere with the activation of the GR–PPAR chimera by arachidonic acid. Not even the direct application of H_2O_2 activated the reporter gene, and therefore it is unlikely that reactive oxygen species mediate the activation of PPAR [21].

The induction of cytochrome *P*450 IVA seems to be a primary event, or at least an intact *P*450 IVA ω-hydroxylase pathway is necessary, in the peroxisome proliferator response [22, 23]. However, neither the non-specific SKF525A nor the specific inhibitor of the cytochrome *P*450 IVA-dependent fatty acid ω-hydroxylation, 1-aminobenzotriazole, were able to antagonize GR–PPAR activation by fatty acids [21]. Moreover, since peroxisome proliferators induce microsomal cytochrome *P*450 IVA activity and non-β-oxidizable derivatives of fatty acids are potent activators of PPAR, it would be expected that the

dicarboxylic fatty acids formed via ω-hydroxylation and subsequent dehydrogenation of the accumulated monocarboxylic fatty acids could serve as primary inducers of PPAR (see Fig. 1). However, dicarboxylic acids such as 1,12-dodecanedioic or 1,16-hexadecanedioic acid did not substantially activate the GR–PPAR chimera in CHO cells, although blocking of both carboxylic groups for β-oxidation by sulphur substitution of the β-carbon atoms (3,14-dithiahexadecanedioic acid) markedly increased the PPAR-activating potential but at concentrations higher than those required for the non-substituted monocarboxylic acids. Nevertheless, it is worth noting that experiments performed by Issemann *et al.* [16] have shown that 1,16-hexadecanedioic acid was a potent inducer of the PPAR transcriptional activity and Kaikaus *et al.* [22] have shown that long-chain dicarboxylic acids can mediate the peroxisome proliferator induction of FABP and peroxisomal β-oxidation. Besides, we have demonstrated [24] that endogenous fatty acids, such as docosahexaenoic acid (all-*cis*-Δ4,7,10,13,16,19-C22:6) and arachidonic acid, act as pretranscriptional regulators of *P*450 IVA when added to primary cultures of rat hepatocytes, and blocking their metabolism (β-oxidation) leads to significant enhancement of their potency. Therefore, the notion that the *P*450 IVA ω-oxidation pathway could be implicated in the generation of a PPAR-activating molecule still remains a possibility.

Another possibility would be that since the sulphur-substituted fatty acid derivatives and peroxisome proliferators of the fibrate and phthalate type are converted to CoA esters [25], but these cannot enter the β-oxidation pathway, their accumulation as CoA-esters, or any other derivative such as acyl-carnitine esters, esters with glycerol or with cholesterol would be expected. Among all these activated compounds that are not subjected to β-oxidation one might still in principle

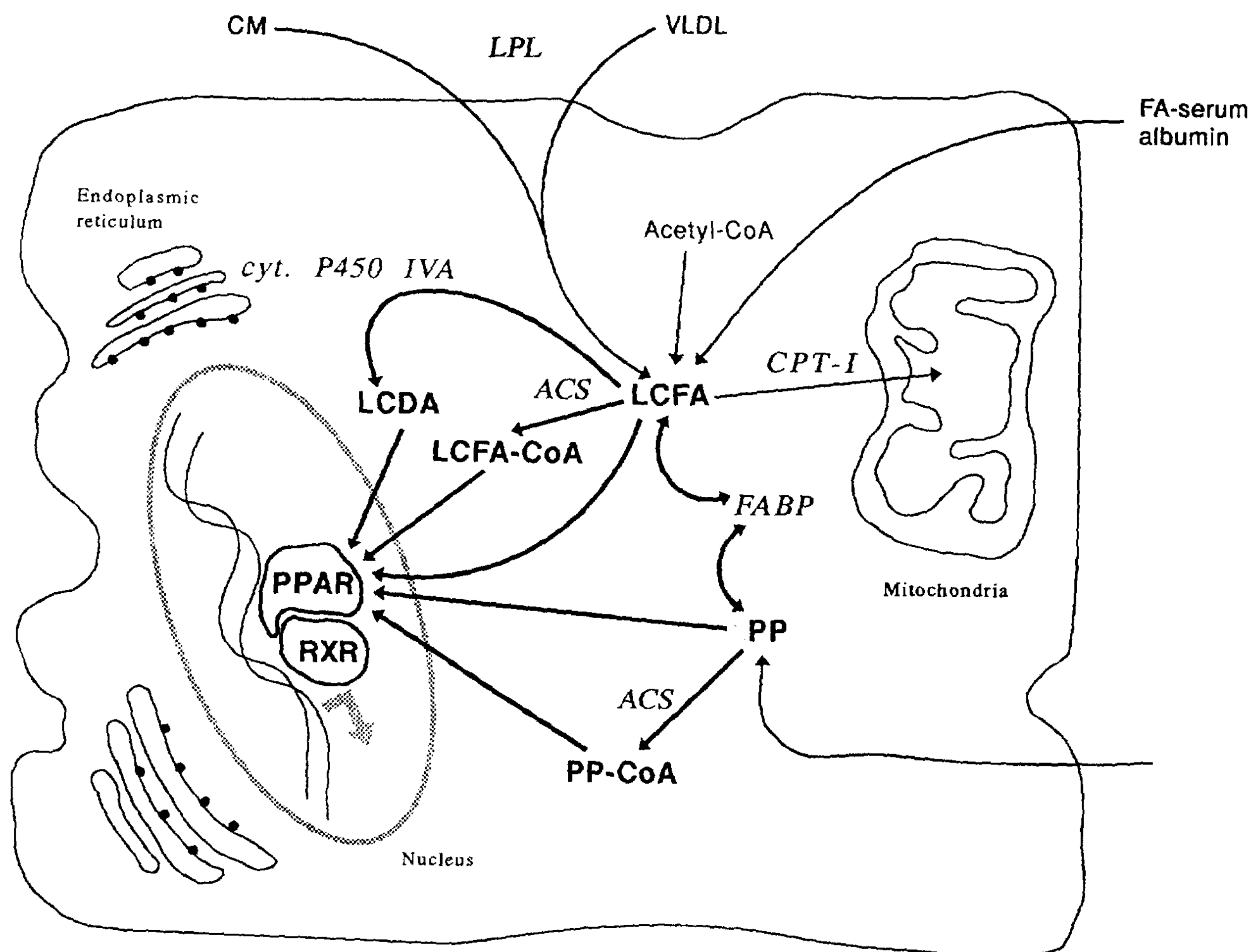

Fig. 1. Candidates for the ultimate ligand to PPAR. Long-chain fatty acids (LCFA) are taken up by the cells either from circulating triglycerides (in lipoproteins, i.e. VLDL and chylomicrons, CM) by lipoprotein lipase (LPL) action, or as complexes with serum albumin. In the cytosol LCFA can be converted to long-chain dicarboxylic acids (LCDA) by cytochrome *P*450 IVA dependent ω-hydroxylation. Peroxisome proliferators (PP) can compete with or displace LCFA for binding to FABP (fatty acid-binding protein). Both PPs and fatty acids can be esterified with coenzyme A. Among all these compounds the natural ligand to PPAR might be found. CPT-I carnitine palmitoyltransferase I; ACS, acyl-CoA synthetase.

find the ultimate ligand to PPAR (see Fig. 1), but since these derivatives are easily interconverted it will be difficult to assess the specific nature of the true receptor-binding species. Accordingly, PPAR still belongs to the orphan receptor group, a growing number of nuclear receptors whose putative activating ligands remain to be identified. Moreover, there is a need to define whether the natural PPAR-activating molecule is derived from the fatty acyl-CoA ester rather than the free fatty acid prior to its β-oxidation (Fig. 1). Thus, whereas the sulfoxide formed through oxidation of the sulphur group of the tetradecylthioacetic acid did not induce peroxisomal proliferation *in vivo* and, at least *in vitro*, cannot be esterified with CoA, and was unable to induce the GR–PPAR chimera [21], perfluorinated fatty acids, which cannot either be activated to their CoA esters, are able to induce peroxisomal proliferation *in vivo* [26]. Furthermore, by using an approach to induce expression of a reporter plasmid under the control of the acyl-CoA oxidase promoter in cells cotransfected with expression vectors for the PPAR and the long-chain-acyl-CoA synthetase, Hertz *et al.* [27] have recently demonstrated that transcriptional activation of acyl-CoA oxidase promoter construct by peroxisomal proliferators or fatty acids was inhibited in the presence of transfected functional acyl-CoA synthetase, arguing for a role of the free fatty acid rather than its CoA ester in activating PPAR.

Search for the ultimate ligand to PPAR might be even more complicated since it has been demonstrated that this receptor forms heteromeric complexes with other partner proteins, a phenomenon which may influence the ligand binding of PPAR. By using PPAR overexpressed in insect cells by a recombinant baculovirus, it was shown that this receptor bound to a cognate PPRE from the acyl-CoA oxidase gene. Surprisingly, as soon as PPAR was purified, it lost its ability to bind to DNA, although binding could be restored by addition of extracts from insect cells [28]. Therefore, a factor present in insect cells was necessary for efficient binding of PPAR to its response element. Since the behaviour of this purified overexpressed receptor appeared to be similar to that of the RAR (retinoic acid receptor) [29], which required the presence of an RXR-like activity in the insect cells to enable it to bind to DNA, it was tested whether the complementing factor for DNA binding in the case of PPAR could also be retinoid X receptor (RXR). Besides, it had recently been demonstrated that other receptors structurally related to PPAR, i.e. thyroid hormone and vitamin D receptors, required RXR to bind efficiently to their respective target DNA sequences [29, 30]. Thus, in a gel shift experiment it could be demonstrated that purified overexpressed PPAR or *in vitro* translated RXR (using a rat RXRα cDNA cloned by our group [28]) alone were unable to bind the PPRE, whereas together these proteins were able to form a specific complex with this DNA element. The same conclusion was reached by Kliewer *et al.* [31] by means of a similar approach, and it was later corroborated by other authors [15, 16]. The fact that RXR might be involved in the activation of PPAR might help to explain the fact that retinoic acid is capable of inducing peroxisomal β-oxidation genes [32]. As expected, it has also been shown that acyl-CoA oxidase gene expression may be stimulated by either a peroxisome proliferator (for instance, clofibric acid or WY-14,643) or the retinoid X receptor ligand, 9-*cis* retinoic acid, an all *trans* retinoic acid metabolite, and furthermore, that simultaneous exposure to both activators results in a synergistic induction of gene expression [15, 16, 31]. These results suggest a coupling and cross-talk between the retinoic acid and peroxisome proliferator signalling pathways in the cell.

A growing number of examples indicate that the PPAR and retinoid-dependent signalling pathways are truly converging. For instance, the PPAR–RXR heterodimer seems to be implicated in regulation of cellular retinol-binding protein II (CRBP II) gene expression, which also responds to high-fat diet [33], through a PPRE [31]. It is thought that the related cytosolic protein, fatty acid-binding protein (FABP), is regulated in a similar way [22, 34]. These findings would be in line with the parallelism existing between fatty acid and retinoic acid actions. Thus, CRBP and FABP might control intracellular concentrations of free retinoic acid and fatty acid, respectively, whereas RAR and PPAR would mediate the biological actions of retinoic acid and fatty acids, respectively. Furthermore, it has been shown that PPAR activators and retinoids co-regulate preadipocyte proliferation, differentiation and survival [35]. Thus, culturing 3T3-L1 preadipocytes in medium containing delipidated bovine calf serum induced their proliferation, an effect which was reversed by addition of PPAR activators, including clofibrate, WY-14,643, and ETYA. Continued exposure of the cells to PPAR activators led to adipose conversion, indicating that PPAR activators may promote adipocyte differentiation in the same way as "normal" differentiating conditions (i.e. fetal calf serum, dexamethasone, isobutylmethylxanthine, and insulin). In fact, both the adipocyte-specific gene aP2, C/EBPα, PPAR and RXR genes were similarly upregulated both in the presence of PPAR activators or using the standard differentiation protocol. A novel member of the peroxisome proliferator activated receptor family, designated mPPARγ2, which is almost exclusively expressed in adipose tissue, has recently been cloned [36, 37]. Induction of mPPARγ2 expression occurs earlier during adipogenesis and at greater magnitude than that of other PPAR types found in this tissue (PPARα and NUCI). Besides, the fast induction of RXRα expression during adipose conversion might enable PPAR to form a functional heterodimer [35]. Accordingly, early expression of PPARγ in adipose tissue might serve to control levels of fatty acids that

accumulate during adipogenesis. In fact, two recent findings seem to favour this notion. On the one hand, long-chain fatty acids have been shown to regulate expression of genes involved in triacylglycerol synthesis, such as adipocyte fatty acid binding protein (aP2) [38], glycerophosphate dehydrogenase, and acyl-CoA synthetase [39] and this adipogenic action of fatty acids did not require their metabolism since 2-bromopalmitate, which is not metabolized by preadipocytes, was more effective than palmitate in inducing differentiation. Furthermore, the transcriptional activation of aP2 (and probably of other adipocyte-specific genes) by fatty acids has been shown to be mediated by mPPARγ2 [37], and these results identify this receptor as the first adipocyte-specific transcription factor, directly implicated in regulation of adipocyte gene expression and differentiation.

Taken together, these results might attribute two important roles, up to now, to PPAR in response to lipid overload, caused for instance by high-fat feeding: one would be carried out by PPAR expressed in peroxisome proliferator responsive tissues such as liver and kidney, and its function might be the activation of a non-energy-generating fatty acid oxidation system (peroxisomal β-oxidation) in order to safeguard the cell against toxic effects of free fatty acids [40]; the other one would be performed in tissues responsible for lipid storage and maintenance of energy balance, i.e. white adipose tissue, and its assignment would be to support lipid homeostasis by stimulation of corresponding adipose development [37]. It remains to be established whether the differential expression of, to date, cloned PPARs may be responsible for distinct regulatory roles of this receptor in different organs, since it has been suggested that the regulation of PPAR-interacting pathways might be a consequence of a complex interplay among the multiple PPAR and RXR isoforms and the ligands for these receptors, determining tissue-specific expression of target genes [18].

Ultimately, then, it would seem that fatty acids are not only utilized as fuel, structural components of the cells, or eicosanoid, triglyceride, and phospholipid precursors, but also serve as important regulators of gene expression by means of activation of (and possibly binding to) the nuclear transcription factor, PPAR, thus representing one of the first examples of nutrient-controlled gene expression in eukaryotes.

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