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STRUCTURAL AND METABOLIC REQUIREMENTS FOR ACTIVATORS OF THE PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR

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Abstract—Fatty acids have recently been demonstrated to activate peroxisome proliferator-activated receptors (PPARs) but specific structural requirements of fatty acids to produce this response have not yet been determined. Importantly, it has hitherto not been possible to show specific binding of these compounds to PPAR. To test whether a common PPAR binding metabolite might be formed, we tested the effects of long-chain ω-3 polyunsaturated fatty acids, differentially β-oxidizable fatty acids and inhibitors of fatty acid metabolism. We determined the activation of a reporter gene by a chimaeric receptor encompassing the DNA binding domain of the glucocorticoid receptor and the ligand binding domain of PPAR. The ω-3 unsaturated fatty acids were slightly more potent PPAR activators *in vitro* than saturated fatty acids. 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 P450 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. These data should aid understanding of signal transduction via PPAR and the identification of a receptor ligand.

Peroxisome proliferators, including clofibric acid, nafenopin, WY 14,643 or industrially used phthalate esters, activate nuclear receptors; the cDNAs for such peroxisome proliferator-activated receptors (PPARs‡) have been cloned from mice [1], rats [2], *Xenopus* [3] and humans [4]. The use of chimaeric receptors comprising the amino-terminal trans-activating and DNA binding domains of the glucocorticoid receptor (GR) fused to the putative ligand binding domain of PPAR stably expressed in CHO cells has led to the identification of fatty acids as activators of PPAR [2, reviewed in 5]. Thus, PPARs might not only respond to peroxisome proliferators but also mediate the described effects of disturbances in fatty acid homeostasis on peroxisome proliferation [6, reviewed in 7]. Since DNA target elements for PPAR have been identified in the 5′-flanking regions of the genes for acyl-CoA oxidase and cytochrome P450IVA6 [8, 9], fatty acids could induce their own metabolism by activation of PPAR. The PPARs are members of the steroid and vitamin nuclear receptor superfamily and therefore the existence of a ligand which binds with high affinity and specificity might be expected [10–12]. Importantly, however, activators of the PPARs, such as fatty acids or peroxisomal proliferation-inducing drugs, have not yet been shown to bind specifically to PPAR [1].§ Furthermore, the lack of pronounced structural or steric specificity of PPAR-activating drugs or fatty acids conflicts with the concept of highly specific ligand recognition by members of the nuclear receptor superfamily.

In the present study we have therefore tested: (i) whether PPAR, despite a broad spectrum of activators, can discriminate between fatty acid derivatives exhibiting differential peroxisome proliferation inducing potency *in vivo*, and (ii) which metabolic pathways might be involved in formation of a putative molecule which ultimately will bind to PPAR. In particular, we tested whether our cell model would respond to the peroxisomal proliferation inducing potency of ω-3 unsaturated fatty acids [13] and distinguish them from other saturated or unsaturated fatty acids. The role of β-oxidation was tested by comparing the inducing effect of fatty acids such as arachidonic acid (C20:4) with that of non-β-oxidizable fatty acid derivatives. Inhibition of fatty acid degradation, e.g. by methylation of the β-carbon [14], sulfur substitution of the β-carbon [15] or, in a similar manner, by perfluorination [16], is known to create potent inducers of peroxisomal

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‡ Abbreviations: BSA, bovine serum albumin; CHO, Chinese hamster ovary; FBS, fetal bovine serum; GR, glucocorticoid receptor; Me_2SO, dimethyl sulfoxide; ME, tetradecylthioacetic acid; MP, tetradecylthiopropionic acid; OE, octanoylthioacetic acid; PPAR, peroxisome proliferator-activated receptor; TD, 3,14-dithiahexadecanedioic acid; 1,12-dioic, 1,12-dodecanedioic acid; 1,16-dioic, 1,16-hexadecanedioic acid.

§ Göttlicher, unpublished observation.

proliferation. Thus, we tested pairs of compounds such as tetradecylthioacetic (ME) vs tetradecylthiopropionic (MP) acid and 3,14-dithiahexadecanedioic acid (TD) vs 1,16-hexadecanedioic acid (1,16-dioic), only one of which is accessible to β-oxidation [17].

The induction of cytochrome P450IVA is a primary event in the peroxisome proliferator response and it has been suggested that the induced fatty acid ω-hydroxylation activity of cytochrome P450IVA might be essential for formation of the PPAR-activating molecule [18]. We therefore tested the effect of inhibitors of cytochrome P450-dependent metabolism. Furthermore, the cyclooxygenase pathway was blocked by indomethacin. Since peroxisomal β-oxidation is associated with the production of reactive oxygen species *in vivo* [19], we also tested the effect of antioxidants and radical scavengers as well as the direct effect of H_2O_2 on the activation of the GR–PPAR chimaera. The results indicate that β-oxidation of the inducing fatty acid is not required and, moreover, blocking of β-oxidation increases the potency of a fatty acid derivative for activating PPAR. These findings suggest that the PPAR-activating molecule is formed prior to the degradation of the acyl-CoA ester, and thus this study might support the search for the ultimate ligand to PPAR.

MATERIALS AND METHODS

Animals and treatment. Male Wistar rats from the Möllegaard Breeding Laboratory (Ejby, Denmark), weighing 180–200 g, were housed individually in metal wire cages with a 12 hr light–dark cycle, constant temperature of 20 ± 3°, and free access to water and food composed of 55% (w/w) carbohydrate, 25% (w/w) protein, 2.1% (w/w) fat, and all necessary minerals and vitamins. The animals were acclimatized to these conditions for at least 1 week prior to the start of the experiments. Fatty acids and fatty acid derivatives were suspended in 0.5% sodium carboxymethyl cellulose. The indicated individual doses were administered by gastric intubation in a volume of 1 mL once a day for 5 days. The control animals received the vehicle only. The animals were killed at the start of the sixth day after 12 hr of starvation. The induction of peroxisomal β-oxidation in the liver was measured by palmitoyl-CoA oxidase activity as described previously [17].

Stably transfected reporter cells and transactivation assay. The reporter cell line, culture conditions and cell treatment were as described [2]. Briefly, the cDNA for a secreted form of human placental alkaline phosphatase (AP) under the control of the GR-dependent MMTV promoter was stably integrated into Chinese hamster ovary (CHO) cells as a reporter gene. Additionally, a chimaeric receptor encompassing the N-terminal transactivating and DNA binding domains of GR and the ligand binding domain of PPAR was stably expressed. Thus, activation of the PPAR ligand binding domain becomes detectable by increased reporter enzyme activity in the cell culture supernatant. Polyunsaturated fatty acids were added as liquids to culture medium containing 10% fetal bovine serum (FBS) and were suspended by sonication. Serial dilutions were prepared in cell culture medium. Sulfur-substituted fatty acids were synthesized and checked for >98% purity as described [20] before addition to the cell culture medium as 400–1000-fold concentrated stock solutions in dimethyl sulfoxide (Me_2SO). Where indicated, fatty acid derivatives were dissolved in cell culture medium containing 10% FBS and fatty acid-free bovine serum albumin (Sigma Chemical Co., St Louis, MO, U.S.A.; No. A-6003) at a molar concentration identical to the fatty acid concentration. Further dilutions were prepared in FBS-containing medium while maintaining the 1:1 molar ratio between the additional bovine serum albumin (BSA) and the fatty acid derivative. WY 14,643 (Chemsyn, Lenexa, KA, U.S.A.) and myristic acid (Sigma) were dissolved in Me_2SO. H_2O_2 was added to the culture medium as a 30% solution in water. Heat-stable placental alkaline phosphatase activity in the cell culture supernatant after 48 hr exposure to the test compounds was determined in a colorimetric assay as described previously [2].

Enzyme inhibitors and antioxidants. SKF525A (Calbiochem, San Diego, CA, U.S.A.), metyrapone, 1-aminobenzotriazole, pyrrolidinedithiocarbamate ammonium salt and indomethacin (all Sigma) were dissolved as 1000-fold concentrated stock solutions in Me_2SO and a 200 mM stock solution of *N*-acetylcysteine (Sigma) was prepared in water. In preliminary experiments the maximum concentrations of inhibitors were determined which the cells would tolerate without any effect on GR-dependent induction of the MMTV-AP reporter. In the inhibition experiments, cells were seeded in half the final culture volume (0.5 mL) and grown for 24 hr. The inhibitors were added to the cell cultures in a quarter of the final culture volume (0.25 mL) 15 min prior to the addition of fatty acid in an additional 0.25 mL of culture medium. Alkaline phosphatase activity was determined essentially as in the experiments without inhibitors.

Activation of the GR–PPAR chimaera in primary rat hepatocytes. Primary hepatocytes were prepared by non-recirculating collagenase perfusion through the portal vein of ether-anesthetized male Sprague–Dawley rats essentially as described previously [21]. Cells were seeded on 60 mm diameter culture dishes at a density of 1×10^6/dish in 3 mL of Dulbecco's modified Eagle medium (4.5 mg/mL glucose) and Ham's F12 medium (1:1). The medium was supplemented with insulin (0.1 μg/mL), T_3 (1 nM), vitamin C (280 μM), penicillin (100 IU/mL), streptomycin (100 μg/mL) and 5% FBS. After the first 24 hr in culture the FBS content was reduced to 2.5% and after an additional 24 hr to 1.25%. Cultures were maintained at 37° in a humidified atmosphere containing 5% CO_2. After 48 hr in culture cells were transfected with 1 μg of the reporter plasmid pMMTV-AP [2] and 3 μg of the expression vector pMT-GR–PPAR [2] using 25 μL of the cationic lipid DOTAP (Boehringer Mannheim, F.R.G.) in 3 mL of cell culture medium containing 1.25% FBS. Cells were exposed to the liposomes for 12 hr before the culture medium was replaced by fresh medium containing, unless otherwise stated, 1.25% FBS, 0.1% BSA, 0.1% Me_2SO and the fatty acids.

Table 1. Induction of peroxisomal β-oxidation in rat liver by ω-3 unsaturated or thia-substituted fatty acids

Compound	Fatty acid dose (mg/kg body wt/day)				
	0	250	600	1000	1500
Carboxymethyl cellulose (control)	3.8 ± 0.3*†				
Palmitic acid		3.9 ± 0.4*‡	3.6 ± 0.3	4.2 ± 0.3	4.0 ± 0.4
C20:5§		4.4 ± 0.3‖	4.6 ± 0.2‖	5.2 ± 0.2‖	5.4 ± 0.4‖
C22:6		4.3 ± 0.4	4.7 ± 0.3‖	5.3 ± 0.4‖	5.8 ± 0.5‖
ME		24.8 ± 7.5‖	—¶	—	—
MP		7.2 ± 3.8	—	—	—
OE		11.1 ± 4.2		—	—
Sulfoxide		3.1 ± 0.3‖	3.5 ± 0.4	—	—
TD		31.5 ± 3.8‖	—	—	—
1,12-Dioic		4.4 ± 0.2‖	3.8 ± 0.8	4.0 ± 0.2	—
1,16-Dioic		3.5 ± 0.3	3.6 ± 0.4	3.7 ± 0.4	—

* Peroxisomal β-oxidation in liver was determined as palmitoyl-CoA oxidase activity (nmol/min/mg protein) after treatment of rats for 5 days with the indicated doses of fatty acids or fatty acid derivatives.

† The value is the mean ± SD of determinations from six control animals.

‡ Values are means ± SD of determinations from at least three animals treated with fatty acids or fatty acid derivatives.

§ For abbreviations see legend to Fig. 1.

‖ Values significantly ($P < 0.05$) different from control in the Student's *t*-test.

¶ —Concentration not tested.

Alkaline phosphatase activity in the cell culture supernatant was determined 30 hr after addition of fatty acids.

RESULTS

Peroxisomal β-oxidation in vivo

PPAR potentially plays a key role in the induction of peroxisome proliferation *in vivo*, and thus compounds with differential peroxisome proliferation inducing potency *in vivo* might be expected to provide useful tools to delineate requirements for activators of PPAR. Therefore, we compared the induction of peroxisomal proliferation in the rat by the model compounds which we intended to use in the cell model. The ω-3 polyunsaturated fatty acids eicosapentaenoic (C20:5) and docosahexaenoic (C22:6) acid and sulfur-substituted derivatives of saturated fatty acids induce palmitoyl-CoA oxidase activity (Table 1). The ω-3 unsaturated fatty acids increase peroxisomal β-oxidation by less than 2-fold, whereas the saturated palmitic acid does not affect peroxisomal activity at all. ME, which is blocked for β-oxidation by sulfur substitution of the β-carbon, induces peroxisomal β-oxidation by more than 6-fold *in vivo*. In contrast, β-oxidation is increased by less than 2-fold by the corresponding γ-substituted MP, which undergoes at least one round of β-oxidation. Shortening of the aliphatic tail of ME to eight carbon atoms (octanoylthioacetic acid, OE) reduces induction to less than 3-fold. The sulfoxide derivative of ME (referred to as sulfoxide) does not induce peroxisomal proliferation *in vivo*. The saturated dicarboxylic acids, 1,12-dodecanedioic acid (1,12-dioic) and 1,16-dioic do not induce peroxisomal β-oxidation. However, the homologous derivative (TD), which carries sulfur substitutions of both β-carbon atoms in hexadecanedioic acid, is a similarly potent inducer as the non-β-oxidizable monocarboxylic acid ME.

Activation of the hGR–PPAR chimaera

Fatty acids such as arachidonic acid (C20:4) have previously been shown to activate a chimaeric protein consisting of the amino-terminal and DNA binding domains of hGR and the putative ligand binding domain of PPAR to the same extent as the potent peroxisome proliferator WY 14,643, although higher concentrations of C20:4 or myristic acid (C14:0) are required (Fig. 1 upper panel). Compared to C20:4 or C14:0, lower concentrations of C22:6 are sufficient for activation of the GR–PPAR chimaera indicating that the long-chain ω-3 unsaturated fatty acid C22:6, and, to a lesser extent, C22:5 are more potent than C20:4 or C14:0. Concentrations of fatty acids higher than those indicated in Fig. 1 are toxic and cause cell detachment.

The concentrations required for activation of the GR–PPAR chimaera are different for the homologous pair of thia-substituted fatty acids with sulfur atoms in either the β- or γ- position. The non-β-oxidizable ME induces the reporter activity at concentrations comparable to those of the potent peroxisomal proliferator WY 14,643. The β-oxidizable homologue, MP, however is only as potent as a non-sulfur-substituted fatty acid such as C14:0 or C20:4 (Fig. 1, upper and center panels). Shortening of the fatty acid backbone of the non-β-oxidizable alkylthioacetic acid to $n = 8$ in OE increases the concentrations required for PPAR activation compared to ME ($n = 14$), but the cells tolerate higher concentrations so that the maximum

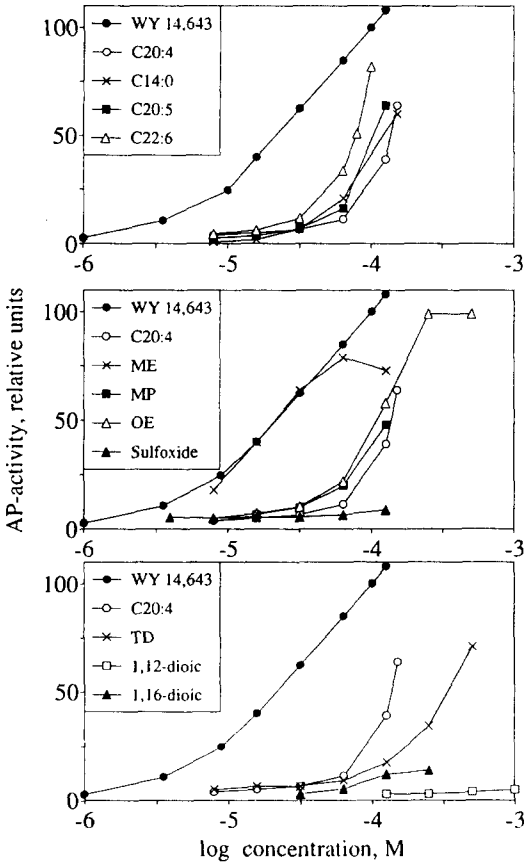

Fig. 1. Activation of the GR–PPAR chimaeric receptor by the ω-3 fatty acids eicosapentaenoic (C20:5) and docosahexaenoic (C22:6) acid (upper panel), the sulfur-substituted fatty acids ME, MP, OE and tetradecylsulfoxyacetic acid (center panel) and the dicarboxylic acids (TD), 1,12-dioic and 1,16-dioic (lower panel) as compared to the peroxisomal proliferator WY 14,643, arachidonic acid (C20:4) and myristic acid (C14:0). Reporter cells stably expressing the GR–PPAR chimera were seeded at a density of 10,000/cm^2 and grown for 24 hr. Cells were exposed to fatty acids by the addition of a 2-fold concentrated suspension/solution of the fatty acid in culture medium and grown for an additional 48 hr prior to determination of AP activity in the culture supernatant. Values represent the means of at least four individual cultures in at least two independent experiments. SDs were less than 15%.

achievable activation by OE is comparable to that of ME. Oxidation of the thioether ME to the corresponding sulfoxide, which does not induce peroxisomal proliferation and which is thought not to be activated to its acyl-CoA thioester [22], abolishes its ability to induce the reporter gene. Like other fatty acids and their derivatives, however, the sulfoxide appears to be taken up by the cells and is toxic at concentrations exceeding 125 μM (Fig. 1, center panel).

Dicarboxylic acids such as 1,12-dodecanedioic (1,12-dioic) or 1,16-hexadecanedioic (1,16-dioic) acid do not substantially activate the GR–PPAR

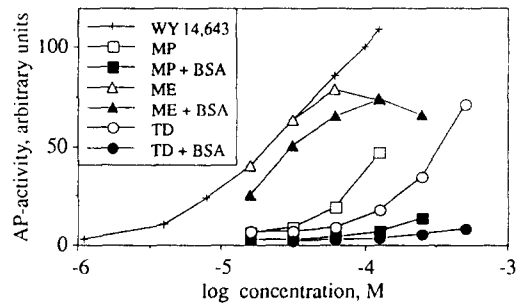

Fig. 2. Activation of the GR–PPAR chimaera by sulfur-substituted fatty acids complexed with BSA. Activation of the chimaeric receptor was analyzed as described in the legend of Fig. 1 with the exception that the fatty acid derivatives were dissolved in cell culture medium as a 1:1 molar complex with essentially fatty acid-free BSA (Materials and Methods). Values are means of duplicate determinations which did not differ by more than 10%.

chimaera in CHO cells (Fig. 1, lower panel). Blocking of both carboxylic groups for β-oxidation by sulfur substitution of the β-carbon atoms (TD) markedly increases the PPAR-activating potential although the required concentrations of TD still exceed those of non-substituted monocarboxylic acids (Fig. 1, lower panel).

The two highest concentrations of fatty acids which are tolerated by the GR–PPAR chimaera-expressing cells were tested in parallel in reporter cells which overexpress the full-length GR in place of the chimaera. In control experiments none of the fatty acids activates the full-length GR indicating that their effects are mediated by the ligand binding domain of PPAR (data not shown).

Effect of BSA on activation of the GR–PPAR chimaera

BSA might affect activation of the GR–PPAR chimaera by fatty acids by two distinct mechanisms: (i) exposure to endogenous BSA-associated levels of fatty acids in the culture medium or (ii) binding the exogenously added fatty acids. The BSA present in culture medium containing 10% FBS (30 μM according to batch analysis of the supplier) may be expected to harbor approximately 30 μM fatty acids. The slight activation of the reporter gene by an additional 30 μM of a fatty acid results in an overestimation of the effect which one might expect of the constitutively present fatty acids [2]. Furthermore, additional serum-free albumin in the culture medium will bind the fatty acid derivatives and thus reduce their "freely available" concentrations [14]. Therefore, activation of the GR–PPAR chimaera by different sulfur-substituted fatty acid derivatives was also compared to a situation where the fatty acid derivatives were added to the culture medium as a 1:1 molar complex with serum-free BSA (Fig. 2). The dose–response curves for TD and MP were markedly shifted to higher concentrations whereas the potency of ME was not substantially affected (Fig. 2). The dose–response curve for palmitic acid in the presence of BSA

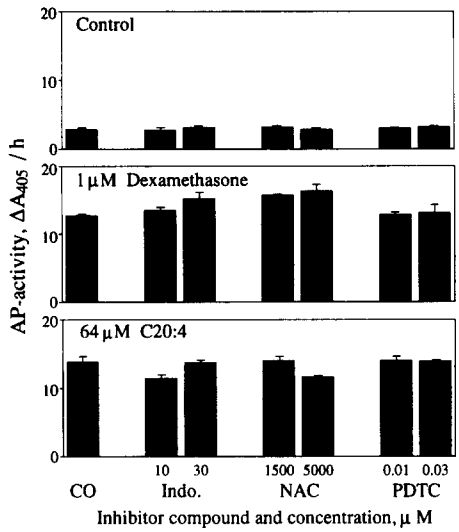

Fig. 3. Effect of enzyme inhibitors on PPAR activation. Experimental design and scaling of ordinate were as described as in Fig. 1. Two different concentrations of indomethacin (Indo.) or radical scavengers/antioxidants, e.g. *N*-acetyl-cysteine and pyrrolidinedithiocarbamate, were added to the cultures prior to addition of control medium (upper panel), 1 μM dexamethasone (center panel) or 65 μM C20:4 (lower panel). Values are means ± SD from triplicate determinations. Similar results were obtained in three independent experiments.

coincides approximately with that for MP (data not shown). 1,16-dioic in the presence of BSA activates the reporter gene to 65% of the activation by TD (data not shown).

Inhibitors of GR–PPAR activation

In order to investigate whether metabolic pathways other than β-oxidation of the fatty acids might generate an ultimately PPAR-activating molecule, we examined the effect of inhibitors of cyclo-oxygenase-dependent metabolism, as well as scavengers for reactive oxygen intermediates. These compounds were added to the cells prior to arachidonic acid at the weakly inducing concentration of 64 μM (Fig. 3, lower panel). The concentrations of the inhibitors chosen were as high as the cells would tolerate without affecting background levels of reporter gene activity or altering its inducibility by dexamethasone via the low levels of endogenous glucocorticoid receptor present in CHO cells (Fig. 3, upper and center panel). None of the tested compounds interferes with activation of the GR–PPAR chimaera by arachidonic acid (Fig. 3, lower panel).

It has been proposed that induction of cytochrome P450IVA is a primary event in the peroxisome proliferator response [18]. Hence, we also tested the effect of cytochrome P450 inhibitors on activation of the GR–PPAR chimaera. The non-specific inhibitors metyrapone and SKF525A do not significantly affect the response to fatty acids up to inhibitor concentrations which do not interfere with reporter gene induction by dexamethasone (data not shown). Cytochrome P450IVA-dependent fatty acid ω-hydroxylation is more specifically inhibited by 1-aminobenzotriazole [23], but this compound does not antagonize GR–PPAR activation by fatty acids at a concentration of 1 mM (data not shown).

Activation of the GR–PPAR chimaera in primary hepatocytes

TD activates the chimaeric receptor only weakly in CHO cells although it induces peroxisomal β-oxidation approximately as effectively *in vivo* as ME. Furthermore, TD induces cytochrome P4504A1 more potently than a regular monocarboxylic fatty acid in primary hepatocytes.* To test whether this discrepancy is attributable to the cell type or to our artificial model system using the chimaeric receptor, we also tested activation of the chimaeric receptor by the sulfur-substituted fatty acid derivatives in primary hepatocytes (Table 2). Preliminary experiments showed that the dose–response relationship obtained for ME in primary hepatocytes is comparable to that detected in CHO cells. Thus, the capacity of the different fatty acids to activate the chimaeric receptor was compared at 50 μM (Table 2) and was confirmed in a single experiment at a concentration of 250 μM (data not shown). The results in transiently transfected primary hepatocytes are identical to those in stably transfected CHO cells with regard to more potent activation of the receptor chimaera by ME as compared to MP and with regard to the lack of activation by the non-sulfur-substituted 1,16-hexadecanedioic acid. However, the activation of GR–PPAR by TD lies between MP and ME in primary hepatocytes. Similar results were obtained when reporter gene activity was determined after up to 72 hr of exposure of the cells to the fatty acid derivatives.

DISCUSSION

Previously we have shown that fatty acids can activate PPAR but we could not determine stringent structural requirements for a fatty acid to activate PPAR [2] and could not show specific binding of radiolabeled myristic or arachidonic acid by PPAR.† Therefore, we tested the hypothesis that a common metabolite of the inducing fatty acid might be formed that will ultimately activate PPAR. Most of the peroxisome proliferation-inducing compounds share the structural characteristics of a non-β-oxidizable carboxylate [reviewed in 7]. The concept of non-β-oxidizable carboxylates as inducers of peroxisomal proliferation has gained further support from the synthesis of non-β-oxidizable fatty acid derivatives [14–16] (Table 1). Based on the assumption that PPAR mediates at least in part induction of peroxisomal proliferation, we first tested the requirement of fatty acid β-oxidation for the activation of PPAR. We found that the non-β-oxidizable ME activates the GR–PPAR chimaera and is as potent as the strong peroxisome proliferator WY 14,643. In contrast, the homologous γ-substituted and β-oxidizable MP is only as potent as a regular fatty acid. The lower activity of the non-β-oxidizable OE compared to that of ME might

* Tollet *et al.*, manuscript in preparation.
† Göttlicher, unpublished observation.

Table 2. Activation of the hGR–PPAR chimaera by sulfur-substituted fatty acids in primary hepatocytes

Inducer	AP activity (% of induction by WY 14,643)	Fold induction
Control*	2.7 ± 1.3†	1
WY 14,643 (100 μM)	100.0	37
MP (50 μM)	6.8 ± 1.7	2.5
ME (50 μM)	71.1 ± 5	26
TD (50 μM)	17.7 ± 0.1	6.6
1,16-Dioic (50 μM)	5.8 ± 1.3	2.1

* Primary hepatocytes were isolated, cultured and cotransfected with the pMMTV-AP reporter gene together with the pMT-hGR–PPAR expression vector for the chimaeric receptor as described in Materials and Methods. Fatty acids were applied in medium containing 1.25% FBS, 0.1% BSA and 0.1% Me_2SO. Control cultures were exposed to BSA and Me_2SO only in the standard medium.

† Values are means ± range from two experiments in independent preparations of primary hepatocytes.

indicate the requirement of a minimal hydrophobic backbone. This is consistent with the finding that short-chain saturated fatty acids with six carbon atoms do not activate the GR–PPAR chimaera [2]. Comparison of the potencies of MP and ME when the fatty acid derivative was in excess of BSA or in a 1:1 molar ratio reveals that ME is similarly potent when applied as a complex with BSA or as the non BSA-bound, "free" acid. The potencies of MP and a regular fatty acid such as palmitic acid are severely decreased when the fatty acids are applied as a complex with BSA. Thus, PPAR appears to be activated when high concentrations of an as yet unknown fatty acid derivative are present. The data indicate two means by which high concentrations of such a PPAR-activating intermediate might be obtained, e.g. by exposure of the test cells to high "free" non-protein-bound concentrations of a metabolizable fatty acid or by exposure to substantially lower concentrations of a non-β-oxidizable derivative. Taken together, β-oxidation is apparently not required for the formation of the PPAR-activating molecule and, furthermore, blocking of β-oxidation potentiates the PPAR-activating potential of a fatty acid.

An increased PPAR-activating potency is also discernible when comparing sulfur-substituted, non-β-oxidizable dicarboxylic acids with non-substituted dicarboxylic acids. For instance, TD activates the GR–PPAR chimaera while 1,16-hexadecanedioic acid is virtually inactive. The *in vitro* data, however, do not show the higher potency of the sulfur-substituted dicarboxylic acid as peroxisome proliferator over the monocarboxylic acids *in vivo*. It appears unlikely that the construction of a chimaeric receptor in contrast to the native receptor might have specifically affected the responsiveness to dicarboxylic, but not to monocarboxylic acids, since in primary hepatocytes TD is clearly more potent than MP (Table 2). This finding is in agreement with the current concept of independent function of the different domains of receptors in the nuclear receptor superfamily [24] and it would be a novel finding that the DNA binding domain or potential protein heteromerization partner [25, 26] of a nuclear receptor alters the ligand specificity. Thus, the difference in the activity of TD might depend on cell-specific differences between CHO cells and for example primary hepatocytes which might cause differential uptake of dicarboxylic acids or differential metabolism to the ultimately active derivative. Nevertheless, in all the tested combinations of fatty acid derivatives the β-oxidizable and non-β-oxidizable homologues appear chemically sufficiently similar to allow the conclusion that the non-β-oxidizable derivatives are truly more potent activators of PPAR, and that this higher potency depends on the formation of a specific PPAR-activating intermediate rather than non-specific effects caused by sulfur substitution.

The role of other fatty acid-metabolizing pathways besides β-oxidation was assessed by using enzyme inhibitors or radical scavengers. The lack of any effect of indomethacin on activation of the GR–PPAR chimaera, together with the finding that even saturated fatty acids can activate the chimaeric receptor, suggest that the cyclooxygenase pathway does not play a major role in the formation of the ultimate PPAR-activating species. Reactive oxygen might play a similar role in PPAR activation as in the activation of the NF-κB transcription factor [27]. Antioxidants and radical scavengers, such as *N*-acetyl-cysteine and pyrrolidinedithiocarbamate [27], however, do not substantially affect the activation of the GR–PPAR chimaera. Furthermore, direct application of H_2O_2 does not activate the reporter gene (data not shown) so that it appears unlikely that reactive oxygen species mediate the activation of PPAR. A role of cytochrome P450IV-dependent activation of the fatty acid is also unlikely since cytochrome P450 inhibitors did not substantially affect induction of the reporter gene by fatty acids. Moreover, if oxidation products of the fatty acids are the PPAR-activating species, then ω-hydroxylation and subsequent dehydrogenation to the dicarboxylic acid would represent the obvious pathway. The lower activity of TD versus monocarboxylic acids in CHO cells, however, does not

support either requirement of ω-hydroxylation or formation of the dicarboxylic acid for the response to occur.

In conclusion, we can now delineate one predominant characteristic of fatty acids to be potent activators of PPAR. The intermediates of the β-oxidation spiral appear not to account for activation of PPAR. The sulfur-substituted fatty acid derivatives [22] and some of the peroxisome proliferators of the fibrate and phthalate type [28, 29, reviewed in 7] are activated to their esters with CoA which cannot enter the β-oxidation spiral. Thus, one might expect accumulation of the inducing compounds, their CoA-esters or any derivative thereof, such as the acyl-carnitine esters [30], esters with glycerol or cholesterol, or even more complex lipids. Amongst these compounds which are likely to accumulate it might be possible to find the ultimate ligand for PPAR. However, it is difficult to discriminate between the different possibilities on the basis of cell assays since most of the intermediates are easily interconverted. The lack of GR–PPAR activation by myristoylsulfoxyacetic acid (Fig. 1, center panel), which, at least *in vitro*, cannot be esterified with CoA [22], supports the view that the ultimate PPAR-activating molecule is derived from the fatty acyl-CoA ester rather than the free fatty acid. However, perfluorinated fatty acids cannot be converted to their CoA esters by isolated hepatocytes, but they induce peroxisomal proliferation *in vivo* [31]. Thus, this study utilizing a cell model may provide helpful information in the search for the putative, ultimate ligand for PPAR and increase understanding of the biology of PPAR.

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