Fatty-acyl-CoA thioesters inhibit recruitment of steroid receptor co-activator 1 to α and γ isoforms of peroxisome-proliferator-activated receptors by competing with agonists

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Peroxisome-proliferator-activated receptors (PPARs) α and γ are ligand-dependent transcription factors that are key regulators of lipid and carbohydrate homeostasis. Fatty acids bind to the ligand-binding domains (LBDs) of PPAR α and PPAR γ and activate these receptors. To clarify whether fatty-acyl-CoAs interact directly with the LBDs of PPAR α and PPAR γ , we performed a competition binding assay with radiolabelled KRP-297, a known dual agonist for these receptors. We show here that fatty-acyl-CoAs bind directly to PPAR α and PPAR γ . Inter-

estingly, fatty-acyl-CoAs, unlike fatty acids, failed to recruit steroid receptor co-activator 1 (SRC-1), on the basis of conformational changes in the LBDs of PPAR α and PPAR γ . Moreover, fatty-acyl-CoAs also markedly inhibited agonist-induced recruitment of SRC-1. These findings demonstrate that fatty-acyl-CoAs have a novel function in the signalling pathways of PPAR α and PPAR γ .

Key words: antagonist, lipid metabolism, nuclear receptor.

INTRODUCTION

Peroxisome-proliferator-activated receptors (PPARs) α and γ are members of the nuclear receptor superfamily of ligand-activated transcription factors; their activation is achieved through agonist binding to the ligand-binding domain (LBD) located at the C-termini of PPARs [1]. The transcriptional activity of nuclear receptors can be influenced by cofactors such as co-activators or co-repressors. Agonist binding to nuclear receptors induces allosteric alterations in the conformation of LBD proteins, recruitment of transcriptional co-activator proteins, and subsequent target gene transcription [2,3]. Co-activators interact with nuclear receptors through a highly conserved motif, the consensus sequence LXXLL (single-letter amino acid codes) [4,5].

PPARs are characterized by tissue-specific expression and distinct metabolic functions. PPAR α is abundantly expressed in tissues with a high lipid catabolic activity and is activated by a variety of compounds, including fatty acids and a fibrates class of hypolipidaemic drugs [6,7]. PPAR α seems to have a crucial role in the adaptive response to fasting and the regulation of metabolism of fatty acids, triacylglycerols and cholesterol by modulating the expression levels of target genes [8–10]. PPAR γ is a master regulator in adipocyte differentiation and a target molecule of a new class of anti-diabetic drugs, thiazolidinediones, which improve insulin resistance [11–13]. Thus PPAR α and PPAR γ seem to be key regulators in the homeostasis of carbohydrates as well as lipids. Although their biological significance *in vivo* has yet to be clarified, recent studies have suggested that fatty acid analogues, such as fatty acids, oxidized fatty acids, eicosanoids and phytanic acid, function as PPAR α and PPAR γ agonists [6,14–20]. Nevertheless, it remains unknown whether a class of fatty acid metabolites, the fatty-acyl-CoAs, interact with PPAR α and PPAR γ .

In the present study we performed a competition binding assay with the soluble LBD proteins of PPAR α and PPAR γ and [³H]KRP-297, a known dual agonist for these receptors [21]. We found that fatty-acyl-CoAs interact directly with the LBDs of PPAR α and PPAR γ without recruitment of steroid receptor co-activator 1 (SRC-1). These results suggest that fatty-acyl-CoAs might be important in the signalling pathways of PPAR α and PPAR γ .

MATERIALS AND METHODS

Chemicals

KRP-297 and BRL-49,653 were synthesized by Kyorin Pharmaceutical Co. Ltd. (Tochigi, Japan). [³H]KRP-297 was synthesized at Amersham (Little Chalfont, Bucks., U.K.). Wy-14,643, fatty acids and fatty-acyl-CoAs were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). 8(*S*)-Hydroxyeicosatetraenoic acid [8(*S*)-HETE] and 15-deoxy- $\Delta^{12,14}$ -prostaglandin J₂ were obtained from Cayman Chemical Co. (Ann Arbor, MI, U.S.A.). All compounds were dissolved in DMSO at a final concentration of 0.1 % (v/v) in all assays.

Plasmid constructs

cDNA species of the LBDs of human PPAR α (167–468) [22] and PPAR γ (176–478) [23] were amplified by PCR from total RNA of the human hepatoma cell line HepG2 (A.T.C.C., Manassas, VA, U.S.A.); these cDNA species were then introduced into a histidine-tagged pQE-30 expression vector (Qiagen, Hilden, Germany). The cDNA of human SRC-1 (594–714) [24] containing two copies of the consensus sequence LXXLL [4,5] was amplified by the PCR from the total RNA of HepG2 cells, then

Abbreviations used: HETE, hydroxyeicosatetraenoic acid; LBD, ligand-binding domain; PPAR, peroxisome-proliferator-activated receptor; SRC-1, steroid receptor co-activator 1; ZDF rats, Zucker diabetic fatty rats.

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Figure 1 Concentration-binding isotherms of [^3H]KRP-297 to PPAR α and PPAR γ

Histidine-tagged proteins of the LBDs of PPAR α (A) and PPAR γ (B) were incubated with various concentrations of [^3H]KRP-297 in the absence (total binding, $\blacklozenge$) or presence (non-specific binding, $\blacksquare$) of 500 μM unlabelled KRP-297. Specific binding ($\blacktriangle$) was calculated by subtracting non-specific binding from total binding. Data are presented as means $\pm$ S.E.M. for four independent experiments.

cloned into a pcDNA3.1(+) expression vector (Invitrogen, San Diego, CA, U.S.A.).

Binding assay

The histidine-tagged proteins of the LBDs of human PPAR α (molecular mass 35248 Da) and PPAR γ (molecular mass 35583 Da) were prepared from bacterial extracts as described previously [21]. The purity of the histidine-tagged proteins of PPAR α and PPAR γ was assessed by SDS/PAGE followed by staining with Coomassie Blue. Histidine-tagged proteins of PPAR α and PPAR γ comprised 85% and 95% of total proteins respectively (Ultrosan XL Laser Densitometer; LKB, Bromma, Sweden). The histidine-tagged proteins of PPAR α (0.12 nM) and PPAR γ (0.13 nM) and [^3H]KRP-297 (specific radioactivity 27 Ci/mmol) were incubated at 25 $^{\circ}\text{C}$ for 30 min in a buffer containing 50 mM Tris/HCl, pH 7.4, 50 mM KCl and 10 mM dithiothreitol. Bound and free [^3H]KRP-297 were immediately separated on a Sephadex G-25 (Pharmacia Biotech, Uppsala, Sweden) spin column and the radioactivity of the bound [^3H]KRP-297 fraction was counted with a liquid-scintillation analyser (2000CA; Packard, Meriden, CT, U.S.A.). Specific binding was calculated by subtracting non-specific binding in the presence of an excess (500 μM) of unlabelled KRP-297 from total binding. For radioligand concentration binding isotherms, ten concentrations of [^3H]KRP-297, in the range 25–800 nM, were used, and apparent K_d values of [^3H]KRP-297 for PPAR α and PPAR γ were calculated from a Scatchard plot. Competition binding assays were performed with 100 nM [^3H]KRP-297; competitors were added to the reaction as indicated in each Figure.

PPAR-co-activator complex formation assay

^{35}S -labelled SRC-1 protein was generated by transcription and translation *in vitro* (TNT[®] Coupled Reticulocyte Lysate System; Promega, Madison, WI, U.S.A.). The total assay volume was 100 μl . The histidine-tagged proteins of the LBDs of PPARs (12–27 pM protein) were incubated for 1 h at 4 $^{\circ}\text{C}$ with ^{35}S -

labelled SRC-1 protein (2.5 μl) in the presence or absence of various ligands in a buffer containing 50 mM Tris/HCl, pH 7.4, 50 mM KCl, 1 mM dithiothreitol and 0.1% BSA. An anti-histidine antibody (2 μg) (Qiagen) was added and the mixture was rotated at 4 $^{\circ}\text{C}$ for 1 h.

After the addition of 20 μl of Protein G-Sepharose beads (Pharmacia Biotech), samples were rotated at 4 $^{\circ}\text{C}$ for 1 h. Samples were centrifuged gently and washed three times. The resin was then suspended in 20 μl of SDS sample buffer and subjected to SDS/PAGE [20% (w/v) gel]. Gels were dried and subjected to autoradiography; the radioactivity of ^{35}S -SRC-1 was quantified with an imaging analyser (BASstation software and Bio-Imaging analyser BAS-2,000II; Fujix, Kanagawa, Japan).

Animals

Male Zucker diabetic fatty/Gmi-*fa/fa* (ZDF) rats and their lean littermates were obtained from Genetic Models International (Indianapolis, IN, U.S.A.) at 5 weeks of age. All institutional guidelines for animal care and use were applied in this study. Rats were housed in individual cages and subjected to a standard light (07:00 h to 19:00 h) and dark (19:00 h to 07:00 h) cycle. They were fed with standard rat chow (CE-2; Japan Clea, Tokyo, Japan) and allowed tap water *ad libitum*. Male ZDF rats ($n = 5$) and their lean littermates ($n = 5$) were used at 8 weeks of age.

Tissue extraction

Rats were killed with a bolus injection of pentobarbital; the livers and rectus femoris muscles were excised and then frozen in liquid nitrogen. Tissues were kept at -80°C until extraction. Determination of fatty-acyl-CoAs was performed basically as described previously [25], with some modifications. Approximately 1 g of frozen tissue was added to 4 ml of ice-cold methanol/chloroform (2/1, v/v) containing 3 μg of heptadecanoyl-CoA as an internal standard, and homogenized with a Polytron homogenizer (Kinematica AG, Luzern, Switzerland).

This homogenate was centrifuged at 1300 *g* for 15 min and the supernatant was saved. The tissue pellet was rehomogenized in 4 ml of methanol/chloroform (2/1, v/v) and centrifuged, then finally the supernatant was added to the first supernatant. To effect phase separation, 2.1 ml of 10 mM KH_2PO_4 buffer, pH 5.3, and 2.1 ml of chloroform were added, and the samples were vigorously vortex-mixed. The lower chloroform-rich layer was removed and discarded. The upper methanol-rich layer was washed again by the addition of 2.1 ml of chloroform. The lower chloroform layer was again removed and discarded. The upper phase was evaporated under nitrogen to a volume of approx. 1 ml. The resulting buffer was then applied to a C_{18} Sep-Pak® Plus cartridge (Waters Chromatography, Milford, MA, U.S.A.) that had been prewashed with 5 ml of methanol, 5 ml of water and 5 ml of 10 mM KH_2PO_4 buffer, pH 5.3. After application of the sample, the columns were washed with 5 ml of 10 mM KH_2PO_4 buffer (pH 5.3)/acetonitrile (4/1, v/v) and eluted with 2 ml of 10 mM KH_2PO_4 buffer (pH 5.3)/acetonitrile (3/7, v/v) and 2 ml of 10 mM KH_2PO_4 buffer (pH 5.3)/methanol (1/3, v/v). This combined eluate was freeze-dried. The eluate was then dissolved in 0.1 ml of 10 mM KH_2PO_4 buffer, pH 5.3, and subsequently subjected to reverse-phase HPLC.

HPLC

Chromatography was performed on a liquid chromatograph (LC-10 AS; Shimadzu, Kyoto, Japan) with a C_{18} analytical column (250 mm $\times$ 4.6 mm internal diam., TSKgel ODS-80Ts; Tosoh, Tokyo, Japan). The following solvents were delivered at a flow rate of 1.0 ml/min: acetonitrile (A) and 10 mM KH_2PO_4 buffer, pH 5.3 (B). The chromatograph was programmed to deliver the solvents as follows: initially 24% A/76% B with a linear gradient of 4 min to 34% A/66% B; a linear gradient of 14 min to 45% A/55% B; a linear gradient of 23 min to 57% A/43% B; and a linear gradient of 31 min to 70% A/30% B. Fatty-acyl-CoAs were monitored by UV absorbance at 254 nm (UV spectrophotometric detector SPD-10A; Shimadzu). Various fatty-acyl-CoA contents were calculated from standard curves obtained from the authentic fatty-acyl-CoAs.

Statistical analysis

The IC_{50} values of inhibitors in a competition binding assay were derived from curve fitting with the PRISM program (GraphPad Software, San Diego, CA, U.S.A.). Apparent K_i values (means $\pm$ S.E.M.) were calculated from the Cheng and Prusoff equation: $K_i = \text{IC}_{50}/(1 + [\text{KRP-297}]/K_d)$ [26]. The statistical significance of differences between ZDF and lean rats was assessed with the unpaired Student's *t* test.

RESULTS

Fatty-acyl-CoAs are ligands for PPAR α and PPAR γ

$[\text{H}]\text{KRP-297}$ concentration-binding isotherms for the LBDs of PPAR α and PPAR γ are shown in Figures 1(A) and 1(B). Apparent K_d values of KRP-297 for the LBDs of PPAR α and PPAR γ were 298 ± 38 and 325 ± 27 nM respectively, which are consistent with those in our previous report [21]. A competition binding assay was therefore performed at a $[\text{H}]\text{KRP-297}$ concentration of 100 nM. $[\text{H}]\text{KRP-297}$ binding to PPAR α was displaced by 10 μM 8(S)-HETE, Wy-14,643 and unlabelled KRP-297 (Figure 2A). Myristoyl-CoA, palmitoyl-CoA, stearoyl-CoA, oleoyl-CoA, linoleoyl-CoA and arachidonoyl-CoA were effective in displacing $[\text{H}]\text{KRP-297}$ binding to PPAR α at a concentration of 10 μM . However, HS-CoA and acetyl-CoA did not displace

Figure 2 Effects of fatty-acyl-CoAs on $[\text{H}]\text{KRP-297}$ binding to PPAR α and PPAR γ

Histidine-tagged proteins of the LBDs of PPAR α (A) and PPAR γ (B) were incubated with 100 nM $[\text{H}]\text{KRP-297}$ in the presence of competitors at 10 μM ; 100% binding indicates specific binding of $[\text{H}]\text{KRP-297}$. The radioactivity applied was 599400 d.p.m. Specific bindings were calculated by subtracting non-specific binding [62.55 ± 4.71 d.p.m. in (A); 83.68 ± 23.35 d.p.m. in (B)], determined in the presence of 500 μM unlabelled KRP-297, from total binding [11812 ± 1038.09 d.p.m. in (A); 16765.84 ± 4539.11 d.p.m. in (B)]. Data are presented as means $\pm$ S.E.M. for three independent experiments. Abbreviation: 15d- $\Delta^{12,14}$ -PGJ $_2$, 15-deoxy- $\Delta^{12,14}$ -prostaglandin J $_2$.

$[\text{H}]\text{KRP-297}$ binding to PPAR α . $[\text{H}]\text{KRP-297}$ binding to PPAR γ was displaced by known agonists for PPAR γ at 10 μM , including 15-deoxy- $\Delta^{12,14}$ -prostaglandin J $_2$ and BRL-49,653 (Figure 2B). Palmitoyl-CoA, stearoyl-CoA, oleoyl-CoA and linoleoyl-CoA displaced more than 50% of $[\text{H}]\text{KRP-297}$ binding to PPAR γ . Neither HS-CoA nor acetyl-CoA influenced the displacement of $[\text{H}]\text{KRP-297}$ binding to PPAR γ .

We next compared the abilities of fatty-acyl-CoAs to displace $[\text{H}]\text{KRP-297}$ binding to PPAR α and PPAR γ with those of their corresponding fatty acids (Figure 3). Displacements by palmitoyl-CoA, oleoyl-CoA and linoleoyl-CoA were comparable ($K_i = 2.36 \pm 0.31$, 0.77 ± 0.21 and 1.16 ± 0.02 μM) to those of their corresponding fatty acids ($K_i = 4.88 \pm 0.77$, 0.41 ± 0.07 and 0.50 ± 0.17 μM) (Figures 3A, 3B and 3C). Displacement by oleoyl-CoA ($K_i = 7.90 \pm 1.44$ μM) for PPAR γ was nearly equal

Figure 3 Effects of fatty acids and their corresponding fatty-acyl-CoAs on [^3H]KRP-297 binding to PPAR α and PPAR γ

Histidine-tagged proteins of the LBDs of PPAR α (A–C) and PPAR γ (D–F) were incubated with 100 nM [^3H]KRP-297 in the presence of various concentrations of fatty acids or fatty-acyl-CoAs: (A, D) palmitic acid (●) and palmitoyl-CoA (○); (B, E) oleic acid (▲) and oleoyl-CoA (△); (C, F) linoleic acid (■) and linoleoyl-CoA (□). Data are presented as means $\pm$ S.E.M. for three independent experiments.

to that by oleic acid ($K_i = 7.30 \pm 2.06 \mu\text{M}$) (Figure 3E). Palmitoyl-CoA and linoleoyl-CoA more effectively displaced binding ($K_i = 4.44 \pm 0.38$ and $5.33 \pm 0.54 \mu\text{M}$) to PPAR γ than did their corresponding fatty acids ($K_i = 29.5 \pm 16.8$ and $15.4 \pm 2.11 \mu\text{M}$) (Figures 3D and 3F).

Fatty-acyl-CoAs inhibit agonist-induced SRC-1 recruitment to PPAR α and PPAR γ

To assess the agonistic action of fatty-acyl-CoAs on PPAR α and PPAR γ , the histidine-tagged PPAR-LBD proteins were incubated with ^{35}S -labelled SRC-1 in the presence or absence of ligands (Figure 4). SRC-1 was recruited to the LBD of PPAR α by KRP-297, 8(*S*)-HETE and linoleic acid in a concentration-dependent manner (Figures 4A, 4B and 4C), whereas linoleoyl-CoA failed to recruit SRC-1 to the LBD of PPAR α (Figure 4D). KRP-49,653, 15-deoxy- $\Delta^{12,14}$ -prostaglandin J_2 and linoleic acid

recruited SRC-1 to the LBD of PPAR γ in a concentration-dependent manner (Figures 4E, 4F and 4G). Linoleoyl-CoA failed to recruit SRC-1 to the LBD of PPAR γ (Figure 4H).

We next examined the effect of fatty-acyl-CoAs on the agonist-induced recruitment of SRC-1 to the LBDs of PPAR α and PPAR γ (Figure 5). Linoleoyl-CoA inhibited, in a concentration-dependent manner, the KRP-297- and linoleic acid-induced recruitment of SRC-1 to the LBDs of PPAR α (Figures 5A and 5B). Recruitment of SRC-1 to the LBDs of PPAR γ induced by KRP-297 and linoleic acid was also inhibited by linoleoyl-CoA in a concentration-dependent manner (Figures 5C and 5D). Linoleoyl-CoA at 3 and 10 μM shifted concentration–response curves rightwards in SRC-1 recruitment to the LBDs of PPAR α and PPAR γ induced by KRP-297 and linoleic acid without altering the maximal effects of these agonists, whereas higher concentrations (30 and 100 μM) of linoleoyl-CoA decreased

Figure 4 Effects of fatty acids and fatty-acyl-CoAs on recruitment of SRC-1 to PPAR α and PPAR γ

Histidine-tagged proteins of the LBDs of PPAR α (A–D) and PPAR γ (E–H) were incubated with 35 S-SRC-1 (594–714) protein in the presence of various concentrations of the compounds indicated. The insets show representative autoradiograms of the gels.

the maximal effect of these agonists on SRC-1 recruitment to the LBDs of PPAR α and PPAR γ . HS-CoA had no effect on SRC-1 recruitment to the LBDs of PPAR α and PPAR γ in either the absence or the presence of fatty acids (results not shown).

Hepatic and muscle fatty-acyl-CoA contents in ZDF rats

To understand the physiological involvement of fatty-acyl-CoAs in the signalling pathways of PPAR α and PPAR γ , we determined

fatty-acyl-CoA levels in the tissues of an animal model of hyperlipidaemia and insulin resistance, the ZDF rat (Figure 6). ZDF rats exhibited increased hepatic levels of myristoyl-CoA, palmitoyl-CoA, palmitoleoyl-CoA and stearoyl-CoA but not of linoleoyl-CoA (Figure 6A). In skeletal muscles, palmitoyl-CoA, stearoyl-CoA and oleoyl-CoA levels were increased in ZDF rats in comparison with those in lean rats (Figure 6B). The total levels of fatty-acyl-CoAs in the livers and skeletal muscles of

Figure 5 Fatty-acyl-CoAs inhibit agonist-induced recruitment of SRC-1 to PPAR α and PPAR γ

Histidine-tagged proteins of the LBDs of PPAR α (A, B) and PPAR γ (C, D) were incubated with 35 S-SRC-1 protein, and the indicated concentrations of KRP-297 (A, C) and linoleic acid (B, D) and various concentrations (○, 0 μ M; ■, 3 μ M; ▲, 10 μ M; ◆, 30 μ M; ●, 100 μ M) of linoleoyl-CoA. Data are presented as means $\pm$ S.E.M. for three or four independent experiments.

ZDF rats were significantly increased by 1.5-fold and 2.0-fold respectively (Figures 6C and 6D).

DISCUSSION

These findings are the first evidence that fatty-acyl-CoAs interact directly with the LBDs of PPAR α and PPAR γ in a competition binding assay with a known dual agonist for these receptors, [3 H]KRP-297. The displacement of [3 H]KRP-297 binding by fatty-acyl-CoAs was comparable to that by their corresponding fatty acids. HS-CoA and acetyl-CoA had no effect on the displacement of [3 H]KRP-297 binding to the LBDs of PPAR α and PPAR γ , demonstrating that the hydrophobic region, but not the CoA region, of fatty-acyl-CoA is involved in the displacement of [3 H]KRP-297 binding. Fatty-acyl-CoAs, unlike fatty acids, failed to recruit SRC-1 to the LBDs of PPAR α and PPAR γ , despite the fact that the K_i values of fatty-acyl-CoA were comparable to those of fatty acids. Forman et al. [6] reported fatty-acyl-CoAs to be incapable of inducing complex formations with target DNA in PPAR α and PPAR δ , which seems to be consistent with our results suggesting that fatty-acyl-CoAs do not have agonistic activity against PPAR α and PPAR γ . More interestingly, fatty-acyl-CoAs inhibited agonist-induced SRC-1 recruitment to the LBDs of PPAR α and PPAR γ . The agonist-induced recruitment of nuclear co-activators was mediated through a conformational change in the LBDs of PPAR α and PPAR γ [4,5,7]. Our results therefore suggest that fatty-acyl-CoAs might inhibit agonist-induced conformational change in

the LBDs of PPARs. Linoleoyl-CoA at 3 and 10 μ M produced a concentration-dependent parallel rightwards shift of the curves for KRP-297 and linoleic acid without altering the maximal effects of these agonists (Figure 6), suggesting that the inhibition by fatty-acyl-CoAs might be attributable to competition with agonists. However, we cannot exclude the possibility that the inhibitory effect observed at higher concentrations (more than 10 μ M) of fatty-acyl-CoAs is also mediated by non-competitive mechanisms, because linoleoyl-CoA significantly decreased the maximal effects of these agonists on SRC-1 recruitment to the LBDs of PPAR α and PPAR γ .

In this study, fatty acids recruited SRC-1 to the LBDs of PPAR α and PPAR γ in the micromolar range. Fatty acids as agonists have been characterized in a cell-based transactivation assay [6,7,15,20,27]. A recent report showed that fatty acids bind to PPAR α in the nanomolar range in a competition binding assay with a synthetic fluorescent ligand [19]. Nevertheless, the biological function of fatty acids for PPARs *in vivo* has not yet been established. We report here that, in contrast with fatty acids, fatty-acyl-CoAs function as antagonists, but not as agonists, for PPAR α and PPAR γ *in vitro*.

PPAR proteins are evenly distributed between the cytosol and nucleus [28,29]. A recent report showed fatty-acyl-CoAs to be detectable in the nucleus as well as in the cytosol [30]. There is therefore a possibility that fatty-acyl-CoAs interact with the LBDs of PPAR α and PPAR γ in the cytosol and nucleus. The $K_m^{\text{palmitoyl-CoA}}$ of carnitine palmitoyltransferase-I (CPT-I) has been reported to be 30–60 μ M [31]. Our results showed that total

Figure 6 Hepatic and muscle fatty-acyl-CoA contents in lean and ZDF rats

Fatty-acyl-CoAs were measured in livers (**A, B**) and skeletal muscles (**C, D**) from 8-week-old ZDF (filled columns) and lean (open columns) rats. The samples were extracted and analysed as described in the Materials and methods section. The total fatty-acyl-CoA content (**B, D**) represents the sum of myristoyl-CoA, palmitoyl-CoA, palmitoleoyl-CoA, stearoyl-CoA, oleoyl-CoA and linoleoyl-CoA. Data are presented as means $\pm$ S.E.M. for five rats. * $P < 0.05$, ** $P < 0.01$ compared with lean rats.

fatty-acyl-CoA levels were $24 \mu\text{M}$ in the livers and $8 \mu\text{M}$ in the skeletal muscles of lean rats, assuming 80% of the tissue to be water [32]. In this study, the K_i values of fatty-acyl-CoAs for PPAR α and PPAR γ were within physiological concentration ranges in the tissues. Nevertheless, intracellular non-bound concentrations of fatty acids and fatty-acyl-CoAs could be decreased by being bound to fatty-acid-binding protein ('FABP') and acyl-CoA-binding protein ('ACBP') [33,34]. Significantly, fatty-acyl-CoA levels were notably increased in the livers and skeletal muscles of an insulin-resistant and hyperlipidaemic animal, the ZDF rat (Figure 6). Elevated fatty-acyl-CoA levels in these tissues are thought to be involved in the induction of insulin resistance through the activation of protein kinase C isoforms that inhibit insulin signalling [35,36]. The actions of fatty-acyl-CoAs in PPAR α and PPAR γ might therefore have more important roles in obesity, insulin resistance and hyperlipidaemia, which are characterized by increased intracellular levels of fatty-acyl-CoAs. Fatty-acyl-CoAs have been reported to interact with other nuclear receptors [37–39], although their biological significance *in vivo* has yet to be clarified. To obtain a better understanding of the biological role of fatty-acyl-CoAs in these nuclear receptors including PPAR α and PPAR γ , subsequent studies must focus on the relationship between the intracellular concentration of fatty-acyl-CoAs and their transcriptional responses.

In conclusion, our studies *in vitro* have provided evidence that naturally occurring fatty-acyl-CoAs interact with the LBDs of PPAR α and PPAR γ and function as antagonists for these receptors. Further studies will be needed to investigate the biological significance of fatty-acyl-CoAs *in vivo* in the signalling pathways of PPAR α and PPAR γ . Nevertheless, the present findings might also provide new insights into the molecular

mechanisms underlying lipid metabolism disorders such as obesity, insulin resistance and hyperlipidaemia.

REFERENCES

- Mangelsdorf, D. J., Thummel, C., Beato, M., Herrlich, P., Schutz, G., Umesono, K., Blumberg, B., Kastner, P., Mark, M., Chambon, P. and Evans, R. M. (1995) The nuclear receptor superfamily: the second decade. *Cell* **83**, 835–839
- Oate, S. A., Tsai, S. Y., Tsai, M. J. and O'Malley, B. W. (1995) Sequence and characterization of a coactivator for the steroid hormone receptor superfamily. *Science* **270**, 1354–1356
- Kamei, Y., Xu, L., Heinzel, T., Torchia, J., Kurokawa, R., Glass, B., Lin, S. C., Heyman, R. A., Rose, D. W., Glass, C. K. and Rosenfeld, M. G. (1996) A CBP integrator complex mediates transcriptional activation and AP-1 inhibition by nuclear receptors. *Cell* **85**, 403–414
- Nolte, R. T., Wisely, G. B., Westin, S., Cobb, J. E., Lambert, M. H., Kurokawa, R., Rosenfeld, M. G., Willson, T. M., Glass, C. K. and Milburn, M. V. (1998) Ligand binding and co-activator assembly of the peroxisome proliferator-activated receptor- γ . *Nature (London)* **395**, 137–143
- Westin, S., Kurokawa, R., Nolte, R. T., Wisely, G. B., McInerney, E. M., Rose, D. W., Milburn, M. V., Rosenfeld, M. G. and Glass, C. K. (1998) Interactions controlling the assembly of nuclear-receptor heterodimers and co-activators. *Nature (London)* **395**, 199–202
- Forman, B. M., Chen, J. and Evans, R. M. (1997) Hypolipidemic drugs, polyunsaturated fatty acids, and eicosanoids are ligands for peroxisome proliferator-activated receptors α and δ . *Proc. Natl. Acad. Sci. U.S.A.* **94**, 4312–4317
- Krey, G., Braissant, O., L'Horsset, F., Kalkhoven, E., Perroud, M., Parker, M. G. and Wahli, W. (1997) Fatty acids, eicosanoids, and hypolipidemic agents identified as ligands of peroxisome proliferator-activated receptors by coactivator-dependent receptor ligand assay. *Mol. Endocrinol.* **11**, 779–791
- Kersten, S., Seydoux, J., Peters, J. M., Gonzalez, F. J., Desvergne, B. and Wahli, W. (1999) Peroxisome proliferator-activated receptor α mediates the adaptive response to fasting. *J. Clin. Invest.* **103**, 1489–1498
- Peters, J. M., Hennuyer, N., Staels, B., Fruchart, J. C., Fievet, C., Gonzalez, F. J. and Auwerx, J. (1997) Alterations in lipoprotein metabolism in peroxisome proliferator-activated receptor α -deficient mice. *J. Biol. Chem.* **272**, 27307–27312
- Costet, P., Legendre, C., More, J., Edgar, A., Galtier, P. and Pineau, T. (1998) Peroxisome proliferator-activated receptor α -isoform deficiency leads to progressive dyslipidemia with sexually dimorphic obesity and steatosis. *J. Biol. Chem.* **273**, 29577–29585
- Tontonoz, P., Hu, E. and Spiegelman, B. M. (1994) Stimulation of adipogenesis in fibroblasts by PPAR γ 2, a lipid-activated transcription factor. *Cell* **79**, 1147–1156
- Lehmann, J. M., Moore, L. B., Smith-Oliver, T. A., Wilkison, W. O., Willson, T. M. and Kliewer, S. A. (1995) An antidiabetic thiazolidinedione is a high affinity ligand for peroxisome proliferator-activated receptor γ (PPAR γ). *J. Biol. Chem.* **270**, 12953–12956
- Forman, B. M., Tontonoz, P., Chen, J., Brun, R. P., Spiegelman, B. M. and Evans, R. M. (1995) 15-Deoxy- $\Delta^{12,14}$ -prostaglandin J_2 is a ligand for the adipocyte determination factor PPAR γ . *Cell* **83**, 803–812
- Kliewer, S. A., Sundseth, S. S., Jones, S. A., Brown, P. J., Wisely, G. B., Koble, C. S., Devchand, P., Wahli, W., Willson, T. M., Lenhard, J. M. and Lehmann, J. M. (1997) Fatty acids and eicosanoids regulate gene expression through direct interactions with peroxisome proliferator-activated receptors α and γ . *Proc. Natl. Acad. Sci. U.S.A.* **94**, 4318–4323
- Kliewer, S. A., Lenhard, J. M., Willson, T. M., Patel, I., Morris, D. C. and Lehmann, J. M. (1995) A prostaglandin J_2 metabolite binds peroxisome proliferator-activated receptor γ and promotes adipocyte differentiation. *Cell* **83**, 813–819
- Nagy, L., Tontonoz, P., Alvarez, J. G., Chen, H. and Evans, R. M. (1998) Oxidized LDL regulates macrophage gene expression through ligand activation of PPAR γ . *Cell* **93**, 229–240
- Xu, H. E., Lambert, M. H., Montana, V. G., Parks, D. J., Blanchard, S. G., Brown, P. J., Sternbach, D. D., Lehmann, J. M., Wisely, G. B., Willson, T. M. et al. (1999) Molecular recognition of fatty acids by peroxisome proliferator-activated receptors. *Mol. Cell* **3**, 397–403
- Murakami, K., Ide, T., Suzuki, M., Mochizuki, T. and Kadowaki, T. (1999) Evidence for direct binding of fatty acids and eicosanoids to human peroxisome proliferator-activated receptor α . *Biochem. Biophys. Res. Commun.* **260**, 609–613
- Lin, Q., Ruuska, S. E., Shaw, N. S., Dong, D. and Noy, N. (1999) Ligand selectivity of the peroxisome proliferator-activated receptor α . *Biochemistry* **38**, 185–190
- Ellinghaus, P., Wolfrum, C., Assmann, G., Spener, F. and Seedorf, U. (1999) Phytanic acid activates the peroxisome proliferator-activated receptor α (PPAR α) in sterol carrier protein 2/sterol carrier protein x-deficient mice. *J. Biol. Chem.* **274**, 2766–2772

- 21 Murakami, K., Tobe, K., Ide, T., Mochizuki, T., Ohashi, M., Akanuma, Y., Yazaki, Y. and Kadowaki, T. (1998) A novel insulin sensitizer acts as a coligand for peroxisome proliferator-activated receptor- α (PPAR- α) and PPAR- γ : effect of PPAR- α activation on abnormal lipid metabolism in liver of Zucker fatty rats. *Diabetes* **47**, 1841–1847
- 22 Sher, T., Yi, H. F., McBride, O. W. and Gonzalez, F. J. (1993) cDNA cloning, chromosomal mapping, and functional characterization of the human peroxisome proliferator activated receptor. *Biochemistry* **32**, 5598–5604
- 23 Greene, M. E., Blumberg, B., McBride, O. W., Yi, H. F., Kronquist, K., Kwan, K., Hsieh, L., Greene, G. and Nimer, S. D. (1995) Isolation of the human peroxisome proliferator activated receptor γ cDNA: expression in hematopoietic cells and chromosomal mapping. *Gene Expr.* **4**, 281–299
- 24 Takeshita, A., Yen, P. M., Misiti, S., Cardona, G. R., Liu, Y. and Chin, W. W. (1996) Molecular cloning and properties of a full-length putative thyroid hormone receptor coactivator. *Endocrinology* **137**, 3594–3597
- 25 Mangino, M. J., Zografakis, J., Murphy, M. K. and Anderson, C. B. (1992) Improved and simplified tissue extraction method for quantitating long-chain acyl-coenzyme A thioesters with picomolar detection using high-performance liquid chromatography. *J. Chromatogr.* **557**, 157–162
- 26 Cheng, Y. and Prusoff, W. H. (1973) Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50 per cent inhibition (I_{50}) of an enzymatic reaction. *Biochem. Pharmacol.* **22**, 3099–3108
- 27 Keller, H., Dreyer, C., Medin, J., Mahfoudi, A., Ozato, K. and Wahli, W. (1992) Fatty acids and retinoids control lipid metabolism through activation of peroxisome proliferator-activated receptor–retinoid X receptor heterodimers. *Proc. Natl. Acad. Sci. U.S.A.* **90**, 2160–2164
- 28 Tuillier, P., Baillie, R., Sha, X. and Clarke, S. D. (1998) Cytosolic and nuclear distribution of PPAR γ 2 in differentiating 3T3-L1 preadipocytes. *J. Lipid Res.* **39**, 2329–2338
- 29 Chinetti, G., Griglio, S., Antonucci, M., Torra, I. P., Delerive, P., Majd, Z., Fruchart, J. C., Chapman, J., Najib, J. and Steals, B. (1998) Activation of proliferator-activated receptors ϵ and θ induces apoptosis of human monocyte-derived macrophages. *J. Biol. Chem.* **273**, 25573–25580
- 30 Elholm, M., Garras, A., Neve, S., Tornehave, D., Lund, T. B., Skorge, J., Flatmark, T., Kristiansen, K. and Berge, R. K. (2000) Long-chain acyl-CoA esters and acyl-CoA binding protein are present in the nucleus of rat liver cells. *J. Lipid Res.* **41**, 538–545
- 31 McGarry, J. D., Mills, S. E., Long, C. S. and Foster, D. W. (1983) Observations on the affinity for carnitine, and malonyl-CoA sensitivity, of carnitine palmitoyltransferase I in animal and human tissues. Demonstration of the presence of malonyl-CoA in non-hepatic tissues of the rat. *Biochem. J.* **214**, 21–28
- 32 Corkey, B. E. (1988) Analysis of acyl-coenzyme A esters in biological samples. *Methods Enzymol.* **166**, 55–70
- 33 Rolf, B., Oudenampsen-Krüger, E., Börchers, T., Færgeman, N. J., Knudsen, J., Lezius, A. and Spener, F. (1995) Analysis of the ligand binding properties of recombinant bovine liver-type fatty acid binding protein. *Biochim. Biophys. Acta* **1259**, 245–253
- 34 Frolov, A. and Schroeder, F. (1998) Acyl coenzyme A binding protein. Conformational sensitivity to long chain fatty acyl-CoA. *J. Biol. Chem.* **273**, 11049–11055
- 35 Schmitz-Peiffer, C., Browne, C. L., Oakes, N. D., Watkinson, A., Chisholm, D. J., Kraegen, E. W. and Biden, T. J. (1997) Alterations in the expression and cellular localization of protein kinase C isozymes ϵ and θ associated with insulin resistance in skeletal muscle of the high-fat-fed rat. *Diabetes* **46**, 169–178
- 36 Schmitz-Peiffer, C., Oakes, N. D., Browne, C. L., Kraegen, E. W. and Biden, T. J. (1997) Reversal of chronic alterations of skeletal muscle protein kinase C from fat-fed rats by BRL-49653. *Am. J. Physiol.* **273**, E915–E921
- 37 Li, Q., Yamamoto, N., Inoue, A. and Morisawa, S. (1990) Fatty acyl-CoAs are potent inhibitors of the nuclear thyroid hormone receptor *in vitro*. *J. Biochem. (Tokyo)* **107**, 699–702
- 38 Hertz, R., Magenheimer, J., Berman, I. and Bar-Tana, J. (1998) Fatty acyl-CoA thioesters are ligands of hepatic nuclear factor-4 α . *Nature (London)* **392**, 512–515
- 39 DiRusso, C. C., Heimert, T. L. and Metzger, A. K. (1992) Characterization of FadR, a global transcriptional regulator of fatty acid metabolism in *Escherichia coli*. Interaction with the fadB promoter is prevented by long chain fatty acyl coenzyme A. *J. Biol. Chem.* **267**, 8685–8691

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