Peroxisome proliferator-activated receptor α-independent peroxisome proliferation

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Abbreviations: AOX, acyl-CoA oxidase; DAB, 3,3'-diaminobenzidine; DLP1, dynamin-like protein 1; FP, forward primer; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HCC, hepatocellular carcinoma; PCR, polymerase chain reaction; PEX, peroxisome biogenesis factor; PPAR, peroxisome proliferator-activated receptor; RP, reverse primer.

Abstract

Hepatic peroxisome proliferation, increases in the numerical and volume density of peroxisomes, is believed to be closely related to peroxisome proliferator-activated receptor α (PPARα) activation; however, it remains unknown whether peroxisome proliferation depends absolutely on this activation. To verify occurrence of PPARα-independent peroxisome proliferation, fenofibrate treatment was used, which was expected to significantly enhance PPARα dependence in the assay system. Surprisingly, a novel type of PPARα-independent peroxisome proliferation and enlargement was uncovered in PPARα-null mice. The increased expression of dynamin-like protein 1, but not peroxisome biogenesis factor 11α, might be associated with the PPARα-independent peroxisome proliferation at least in part.

Keywords: peroxisome proliferator-activated receptor α; peroxisome proliferation; morphometry; dynamin-like protein 1.

Peroxisome proliferator-activated receptor (PPAR) belongs to the nuclear receptor superfamily, and three isoforms (α, δ and γ) have been identified [1]. PPARα, abundantly expressed in liver, kidney, and heart, plays an important role in the regulation of fatty acid catabolism [2]. The administration of PPARα agonists such as fibrate drugs to rats and mice not only enhances the expression of fatty acid-metabolizing enzymes, but also causes massive hepatic peroxisome proliferation and the development of hepatocellular carcinoma (HCC) [3, 4]. In previous studies, after treatment with PPARα agonists such as clofibrate and Wy-14,643, PPARα-null mice showed neither hepatic peroxisome proliferation nor the development of HCC [5, 6]. These findings indicate that hepatic peroxisome proliferation and hepatocarcinogenesis by PPARα agonist treatment occur exclusively through PPARα.

On the other hand, earlier studies have reported that treatment with rosiglitazone, a selective PPARγ agonist, led to hepatic peroxisome proliferation in *ob*/*ob* mice [7], and that highly selective PPARγ or PPARγ/δ dual agonists induced peroxisome proliferation in mice lacking functional PPARα [8]. These results suggest the existence of PPARα-independent mechanisms that affect peroxisome proliferation. In the present study, we aimed to identify and characterize a novel type of PPARα-independent peroxisome proliferation by using a specific PPARα agonist.

Materials and methods

Animals and fenofibrate treatment

PPARα-null mice on a Sv/129 genetic background were generated as described elsewhere [5]. Twelve-week-old wild-type Sv/129 male mice (n = 12) and age- and sex-matched PPARα-null mice (n = 12) were each divided into two groups, one of

which was treated with 25 mg/kg (4 ml/kg corn oil) fenofibrate daily for 10 days by gavage, and the other of which was treated with the same amount of corn oil for 10 days. After treatment, the mice were killed and their livers were excised and subjected to serial analyses. All animal experiments were conducted in accordance with animal study protocols approved by the Shinshu University School of Medicine.

Cytochemical staining of peroxisomes

Cytochemical staining for peroxisomal catalase was performed following the method described by Novikoff and Goldfischer [9]. Small pieces of liver from each mouse were fixed with 3% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) for 1 h at 4°C, cut into 100-μm sections using an Oxford Vibratome (Oxford Laboratories, Foster City, CA, USA) and post-fixed with 3% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) for 1 h. The sections were incubated in a 3,3'-diaminobenzidine (DAB) reaction medium (0.2% DAB tetrahydrochloride, 50 mM propanediol, pH 9.7, 5 mM KCN, 0.05% H_2O_2) for 1 h at room temperature, then post-fixed with aqueous 1% osmium tetroxide in 0.1 M sodium cacodylate buffer, dehydrated with a graded series of ethanol and acetone, and embedded in Epok 812 (Oken, Tokyo, Japan). The sections (2 μm) were cut with a glass knife, counterstained with 0.1% toluidine blue solution, and examined by light microscopy. Ultrathin sections (0.1 μm) were cut with a diamond knife, collected on grid meshes, stained with lead citrate and uranyl acetate, and examined with a JEM 1200EX II electron microscope (JEOL, Tokyo, Japan) at an accelerating voltage of 80 keV.

Morphometry of hepatic peroxisomes

Morphometric analysis of DAB-stained peroxisomes was carried out using electron

microscopy [10]. For each experimental group, 60 independent fields in the pericentral area of liver lobuli were photomicrographed at an original magnification of x 3,900. At this magnification, peroxisomes smaller than 450 nm were clearly identified. The peroxisomes were easily detected because of their high contrast due to the positive DAB reaction. In each frame, the number of peroxisomal profiles and the area of each individual profile were determined. The numerical density and volume density of the peroxisomes were calculated using the following equations: numerical density (number/μm^2) = N_P / (A_T – A_{empty}), and volume density (%) = A_{TP} / (A_T – A_{empty}) x 100, where N_P is the peroxisome number in the test area, A_T is the test area, A_{empty} is the area of the vascular and biliary lumens and that of the hepatocyte nuclei, and A_{TP} is the area of total peroxisomal profiles in the test area. The area was measured with a Luzex AP image analyzer (Nireco, Tokyo, Japan). It is well known that the shape of peroxisomes changes from spherical to oval or tubular after treatment with peroxisome proliferators [11]. For simplification, the diameter of the area-equivalent circle of the peroxisomal profiles was calculated as the diameter of the peroxisomes. These morphometric parameters were expressed as the means $\pm$ S.D.

mRNA analysis

Mouse livers were homogenized and total RNA was extracted using an RNeasy Mini Kit (Qiagen, Tokyo, Japan). One μg of RNA was reverse transcribed with SuperScript II reverse transcriptase (Gibco BRL, Paisley, Scotland), and real-time quantitative polymerase chain reaction (PCR) was performed and analyzed with the ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). The following primer sequences were selected with Primer Express software (Applied

Biosystems) and the primer pairs were designed as follows: forward primer (FP) 5'-CCTCAGGGTACCACTACGGAGT-3' and reverse primer (RP) 5'-GCCGAATAGTTCGCCGAA-3' for PPARα (GenBank accession number **NM011144**) [12]; FP 5'-TCAACATGGAATGTCGGGTG-3' and RP 5'-ATACTCGAGCTTCATGCGGATT-3' for PPARδ (**NM011145**) [13]; FP 5'-TTCCACTATGGAGTTCATGCTTGT-3' and RP 5'-TCCGGCAGTTAAGATCACACCTA-3' for PPARγ (**NM011146**) [14]; FP 5'-TGGTATGGTGTCGTACTTGAATGAC-3' and RP 5'-AATTTCTACCAATCTGGCTGCAC-3' for acyl-CoA oxidase (AOX) (**NM015729**) [15]; FP 5'-ACTGGCCGTAAATGGTTCAGA-3' and RP 5'-CGGTTGAGGTTGGCTAATGTC-3' for peroxisome biogenesis factor (PEX) 11α (**NM011068**) [16]; and FP 5'-CGCCTATTGATGGAACAAGAGACT-3' and RP 5'-TCCAGGTCCCACAGTTTCTACTC-3' for PEX11β (**NM011069**) [16]. Each mRNA expression level was normalized to the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA expression level, and subsequently normalized to that of wild-type mice fed a control diet. In the case of analysis of dynamin-like protein 1 (DLP1) mRNA, Northern blotting was performed as described previously [2] and hybridized with a ^{32}P-labeled rat cDNA probe [17] because the mouse cDNA sequence has not yet been fully confirmed. The band intensity of DLP1 mRNA was quantified densitometrically, normalized to that of GAPDH mRNA, and subsequently normalized to that of wild-type mice fed a control diet.

Statistical analysis

Statistical analysis was performed using SPSS software 11.5J for Windows (SPSS

Inc., Chicago, IL, USA). Results were compared using the Student's t-test. A probability value of less than 0.05 was considered to be statistically significant.

Results

mRNA analysis of PPARs and AOX

The hepatic mRNA levels of PPARα and AOX, a representative PPARα target gene, were increased by fenofibrate treatment in wild-type mice, but not in PPARα-null mice (Figs. 1A and D), demonstrating a typical PPARα-dependent response in the present experimental system. The mRNA level of PPARδ was lower in PPARα-null mice than in wild-type mice, and remained unchanged in both genotypes after treatment (Fig. 1B). The mRNA level of PPARγ mRNA was much lower, approximately one-hundredth, than that of PPARα or PPARδ, when calculated relative mRNA copy numbers toward GAPDH, and remained unchanged in both genotypes after treatment (Fig. 1C). These results suggest that treatment with fenofibrate, a specific PPARα agonist, induced PPARα target genes.

Morphometric analysis of hepatic peroxisomes

To investigate the relationship between fenofibrate-induced PPARα activation and peroxisome proliferation in the liver, we performed cytochemical DAB staining for peroxisomal catalase. Light microscopic analysis revealed that the number of hepatic peroxisomes was significantly increased in wild-type mice after treatment (Fig. 2, upper panel). Additionally, the number of hepatic peroxisomes seemed to be slightly increased by the treatment in PPARα-null mice as well; electron microscopic analysis showed a similar tendency (Fig. 2, lower panel). Interestingly, large-sized peroxisomes were

found in both genotypes after fenofibrate treatment (Fig. 2, lower panel). To more accurately evaluate the degree of peroxisome proliferation, we carried out morphometric analysis of peroxisomes and compared parameters such as numerical density and volume density. The numerical density and volume density were significantly increased in the fenofibrate-treated wild-type mice compared with those in control wild-type mice (0.244 $\pm$ 0.063 /μm^2 vs. 0.038 $\pm$ 0.003 /μm^2, P = 0.007; and 10.7 $\pm$ 2.4% vs. 1.4 $\pm$ 0.2%, P = 0.005, respectively) (Figs. 3A and B). Unexpectedly, in the PPARα-null mice, numerical density was increased approximately 2.5-fold after treatment (0.086 $\pm$ 0.025 /μm^2 vs. 0.037 $\pm$ 0.009 /μm^2, P = 0.004) (Fig. 3A). The volume density, the most reliable parameter of peroxisome proliferation, was also increased approximately 2-fold in the PPARα-null mice after treatment (3.3 $\pm$ 0.9% vs. 1.6 $\pm$ 0.4%, P = 0.008) (Fig. 3B). These parameters did not differ between the wild-type and PPARα-null mice treated with a control diet. The average diameter of peroxisomes remained unchanged in all groups (Fig. 3C). These results indicate that treatment with fenofibrate causes hepatic peroxisome proliferation, even in mice lacking functional PPARα, and furthermore, peroxisome enlargement occurs PPARα-independently.

mRNA analysis of PEX11 and DLP1

The number of peroxisomes is controlled by several peroxisome membrane proteins [18]. PEX11 protein, encoded by the *PEX11* gene, is one of the major regulators of peroxisome proliferation or division in mammals [18]. To clarify the molecular mechanism of fenofibrate-induced peroxisome proliferation in PPARα-null mice, we first examined the expression of PEX11. The hepatic mRNA level of PEX11α, whose expression is induced by peroxisome proliferators [16, 19], was markedly

increased in the wild-type mice by fenofibrate treatment, but not in similarly-treated PPARα-null mice (Fig. 4A). The hepatic mRNA level of PEX11β, another isoform of PEX11, remained constant under treatment in both genotypes (Fig. 4B). Next, we examined the mRNA level of DLP1, a mammalian dynamin-related protein required for peroxisome division [18, 20]. The DLP1 mRNA level was increased approximately 2.5-fold in the fenofibrate-treated wild-type mice (Fig. 4C). Interestingly, it was also increased approximately 2.4-fold in the PPARα-null mice under the same treatment (Fig. 4C). These results demonstrate that the increased expression of DLP1, but not PEX11, might be one of the factors associated with the peroxisome proliferation observed in the fenofibrate-treated PPARα-null mice.

Discussion

Detailed high resolution morphometric analysis of hepatic peroxisomes was carried out using electron micrographs of DAB-stained sections. This method has proved to be a suitable and accurate means for analysis of peroxisome proliferation because cytochemical DAB staining can prevent the common underestimation of peroxisomes smaller than 450 nm [10], and because electron microscopic analysis can minimize error caused by overlapping of peroxisomes found in thick sections [10]. The volume density of peroxisomes in the control wild-type mice was estimated to be 1.4%, which is in good agreement with previously reported values of 1.4% [21] and 1.2% [22]. Thus, the results of morphometric analysis are considered to be reasonable.

For the present mouse treatment, fenofibrate, known as a PPARα ligand, was used. Surprisingly, peroxisome proliferation was observed in the absence of PPARα (Figs. 2 and 3), demonstrating the existence of a newly identified type of PPARα-independent

peroxisome proliferation. It is also noteworthy that enlargement of peroxisomes occurs PPARα-independently. Although the precise molecular mechanism of this phenomenon remains unclear, the following explanation may be helpful in understanding it. One of the key molecules associated with peroxisome proliferation is PEX11α, which is closely correlated with PPARα activation (Fig. 4A) [16, 19] and which probably promotes fenofibrate-induced peroxisome proliferation in wild-type mice; this protein is not induced in PPARα-null mice. This observation might be explained by the fact that PEX11α is a PPARα target gene, but that it is dispensable for peroxisome proliferation according to the phenotype of PEX11α-null mice [23]. Another key mediator of peroxisome proliferation is DLP1, which demonstrates increased expression after treatment with fenofibrate in both wild-type and PPARα-null mice (Fig. 4C). DLP1 is believed to be required for the division step of peroxisomes [18, 20]. The PPARα-independent increase in DLP1 is consistent with the possibility that this protein causes peroxisome proliferation in both genotypes. DLP1 expression is increased in rat livers treated with bezafibrate [20], a potent PPARδ agonist and a moderate PPARα agonist [24]. However, it remains unclear whether DLP1 contributes to peroxisome proliferation in either genotype through PPARδ activation, because fenofibrate is PPARα agonist and is not known to activate PPARδ. On the other hand, it is clear that PPARα-independent enlargement of peroxisomes has little relevance to PPARα-dependent increases in peroxisomal matrix proteins such as AOX (Fig. 1D) or peroxisome-forming membrane proteins such as PEX11α (Fig. 4A). Further experimentation is required in order to understand the detailed molecular mechanisms of PPARα-independent peroxisome proliferation.

Hepatic peroxisome proliferation is considered to be strong proof of PPARα

activation in rats and mice [25], and has been used as an important standard to estimate susceptibility to rodent HCC in the development of new candidate compounds with PPARα activation and serum lipid-lowering activity. However, the present results indicate that peroxisome proliferation is not necessarily a suitable biological marker of continuous PPARα activation. Therefore, caution should be exercised when attempting to assess the risk of hepatocarcinogenesis based solely on the presence of peroxisome proliferation in short-term bioassays.

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Figure legends

Fig. 1. Analysis of PPARs and AOX mRNA levels. The hepatic mRNA expression of PPARα□ (A), δ (B) and γ (C), and of AOX (D) was determined by quantitative real-time PCR and normalized to that of the control wild-type mice. Data are expressed as the means ± S.D. (n = 6 in each group). *, $P<0.05$ compared with control wild-type mice.

Fig. 2. Light and electron micrographs of DAB-stained liver tissues of wild-type and PPARα-null mice. The pericentral area of liver lobuli was photomicrographed. Peroxisomes appear as darkly stained particles. The bars in the light and electron micrographs of fenofibrate-treated PPARα-null mice indicate 20 μm and 2 μm, respectively. C, central vein.

Fig. 3. Morphometric analysis of hepatic peroxisomes. The number of peroxisomes and the area of each individual peroxisome profile were measured, and morphometric parameters such as the numerical density (A), volume density (B) and average diameter (C) of peroxisomes were calculated. Data are expressed as the means ± S.D. (n = 6 in each group). *, $P<0.05$ compared with control wild-type mice; #, $P<0.05$ compared with control PPARα-null mice.

Fig. 4. Analysis of mRNAs encoding proteins affecting peroxisome proliferation. The hepatic mRNA expression of PEX11α (A) and β (B) was determined by quantitative real-time PCR, normalized to that of GAPDH, and subsequently normalized to that of control wild-type mice. DLP1 mRNA expression was examined using Northern blotting,

quantified densitometrically, normalized to that of GAPDH, and finally normalized to that of control wild-type mice. Data are expressed as the means $\pm$ S.D. (n = 6 in each group). *, $P<0.05$ compared with control wild-type mice; #, $P<0.05$ compared with control PPARα-null mice.