

Research Article

Activating Effect of Benzbromarone, a Uricosuric Drug, on Peroxisome Proliferator-Activated Receptors

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Benzbromarone, a uricosuric drug, reportedly causes hepatic hypertrophy accompanied by proliferation of peroxisomes in rats. To elucidate the mechanisms underlying induction of peroxisome proliferation by benzbromarone, we examined binding affinity for peroxisome proliferator-activated receptor α (PPAR $_{\alpha}$) and γ (PPAR $_{\gamma}$), and effects on the binding activity of PPARs with peroxisome proliferation-responsive element (PPRE) and expression of the PPARs target protein. Binding affinity of benzbromarone for PPAR $_{\alpha}$ and PPAR $_{\gamma}$ was examined by reporter gene assay. Binding activity of PPARs with PPRE was determined by electric mobility shift assay, and expression of lipoprotein lipase (LPL) and acyl-CoA synthetase (ACS) by Western blot method. Benzbromarone displayed affinity for PPAR $_{\alpha}$ and PPAR $_{\gamma}$, and promoted binding of PPARs to PPRE. Furthermore, cultured cells with benzbromarone added showed upregulated expression of LPL and ACS. These results suggest that benzbromarone induces peroxisome proliferation in hepatocytes by binding to PPARs, and controls expression of proteins related to lipid metabolism.

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1. INTRODUCTION

Benzbromarone is a uricosuric drug that has been widely used for more than 25 years as a therapeutic agent for hyperuricemia. Recently, urate transporter 1 (URAT1) was identified in the luminal membrane of the proximal renal tubule, and the mechanism of action for benzbromarone was identified as suppression of uric acid resorption via inhibition of URAT1 [1]. Regarding the toxicity of benzbromarone, hypertrophy of the liver accompanied by proliferation of peroxisomes has been reported in a repeated-dose toxicity study in rats [2]. Similar changes have also been observed in studies on clofibrate and fenofibrate, both of which are antihyperlipemic drugs [2, 3]. Clofibrate and fenofibrate represent activators of peroxisome

proliferator-activated receptor α (PPAR $_{\alpha}$), and benzbromarone may similarly cause hypertrophy of the liver via actions on PPAR $_{\alpha}$.

We have previously demonstrated that benzbromarone has affinity for PPAR $_{\alpha}$ and PPAR $_{\gamma}$, and upregulates expression of each protein [4]. In this study, to clarify the actions of benzbromarone on PPARs, the affinity of benzbromarone for PPAR $_{\alpha}$ and PPAR $_{\gamma}$ was reexamined in comparison with a representative activator of each receptor. In addition, regarding the mechanism of gene expression induced by benzbromarone, binding between PPARs and peroxisome proliferator-responsive element (PPRE), and protein expressions of lipoprotein lipase (LPL), and acyl-CoA synthetase (ACS) encoded by the target gene of PPAR were examined.

Based on the findings, hepatic hypertrophy due to benzobromarone and the influence of benzobromarone on lipid metabolism are discussed.

2. MATERIALS AND METHODS

2.1. Chemicals

Benzobromarone, an active pharmaceutical ingredient of Uri-norm marketed by Torii Pharmaceutical Co., Ltd. (Tokyo, Japan), was used as a test compound in this study. Troglitazone, pioglitazone and fenofibric acid were synthesized at the Central Research Pharmaceutical Institute of Japan Tobacco Inc. (Osaka, Japan). Clofibrac acid was purchased from Sigma-Aldrich Japan (Tokyo, Japan), nuclear and cytoplasmic extraction reagents from Pierce (Ill, USA), and a nucleic acid labeling kit from Molecular Probes (Ore, USA).

2.2. Cell culture

NIH/3T3 cells and human kidney 293T cells were maintained in D-MEM containing 10% fetal bovine serum, 50 U/mL penicillin and 50 $\mu\text{g}/\text{mL}$ streptomycin at 37°C in a humidified 95% air and 5% CO₂ atmosphere.

2.3. Reporter gene analysis

NIH/3T3 cells (0.4×10^5 cells/well) were cultured in 24-well plates for 20 hours and then transfected with a receptor plasmid for the chimera of PPAR γ or the PPAR α ligand-binding domain and GAL4 DNA-binding domain, together with a receptor plasmid containing the GAL4-responsive promoter driving expression of luciferase. After 6 hours, cells were given fresh D-MEM containing test compounds at final concentrations of 0.001–100 μM , and were cultured for an additional 2 days. Benzobromarone, clofibrac acid, fenofibric acid, troglitazone, and pioglitazone were suspended in 0.1% DMSO solution. After cells had been lysed, luciferase activity was determined using a CT-9000D luminometer (Dia-iatron, Tokyo, Japan).

A total of 3 measurements were performed for each batch of cultured cells per test, and a total of 3 tests were performed.

2.4. Electric mobility shift assay (EMSA)

First, following addition of benzobromarone at a final concentration of 1 μM , 293T cells were cultured for 24 hours. Cells were washed with TBS and pelleted by centrifugation at 1500 \times g for 5 minutes. Pellets were suspended in 1 mL TBS and transferred into an Eppendorf tube, then centrifuged again at 1000 \times g for 5 minutes to obtain pellets. Double-stranded oligonucleotides for sequences of the PPRE and mutant of PPRE were purchased from Santa Cruz Biotechnology (Calif, USA). Oligonucleotides were labeled with Alexa Flour 488 using a ULYSIS Nucleic Acid Labeling Kit (Molecular Probes). Labeled oligonucleotides (500 ng) were added to nuclear extracts. The binding assay buffer comprised 20 mM HEPES (pH 7.5) containing 40 mM KCl, 5% glycerol, and 1 μg dl-dC. Assay was performed at room temperature for 10 minutes at a final volume of 25 μL . Products

of the binding reaction were separated on 5% polyacrylamide gel run in Tris-borate-EDTA at room temperature at 100 V for 60 minutes. Gels were visualized using a Fluorimager SI (Molecular Dynamics, Tokyo, Japan). This test was performed 6 times.

2.5. Western blot analysis

Benzobromarone was added to 293T cells to achieve a final concentration of 1 or 10 μM , and cells were cultured for 24 hours. Western blots for LPL and ACS proteins were then performed according to established procedures. Briefly, after cells (10^8 cells/well) had been suspended in lysis buffer (10 mM sodium phosphate, 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% SDS, 100 $\mu\text{g}/\text{mL}$ PMSEF, 30 $\mu\text{g}/\text{mL}$ aprotinin, and 1 mM sodium orthovanadate, pH 7.4), aliquots of the cell suspension were obtained by repetitive pipetting and lysed by incubation at 4°C for 15 minutes. Lysed cells were centrifuged at 400 \times g for 10 minutes, and the supernatant was separated by SDS-polyacrylamide gel electrophoresis in 10% acrylamide gel. The protein was transferred onto a nitrocellulose membrane and blocked for 60 minutes. The membrane was then incubated with anti-ACS (Japan Ram, Tokyo, Japan) or anti-LPL (Daichi Pure Chemicals, Tokyo, Japan) antibodies. After washing, the membrane was incubated with horseradish peroxidase-conjugated anti-rabbit secondary antibodies (The Binding Site, Birmingham, UK). Chemiluminescence of bound antibodies was detected using a kit (Amersham BioSciences, NJ, USA). Protein contents were determined by the generally established method of Lowry using BSA as a standard. This test was performed 4 times.

3. RESULTS

The binding affinity of benzobromarone for PPAR α and PPAR γ was examined by reporter gene assay using transcriptional activity of the PPAR target gene as a marker, in comparison with those of clofibrac acid, fenofibric acid, troglitazone, and pioglitazone (Figures 1 and 2). At 100 μM , benzobromarone induced an increase in luciferase activity, representing transcriptional activation of the target gene via binding of benzobromarone to PPAR α , and the magnitude was nearly equal to that induced by clofibrac acid, while fenofibric acid and pioglitazone induced transcriptional activation of the target gene at concentrations of ≥ 10 μM . Against PPAR γ , benzobromarone induced an increase in luciferase activity at a concentration of 100 μM , troglitazone at ≥ 1 μM , and pioglitazone at ≥ 0.1 μM .

After adding benzobromarone to 293T cells, the nuclear extract was incubated with fluorescence-labeled PPRE oligonucleotide, and binding activity between PPARs and PPRE was determined by EMSA. Binding between PPARs and PPRE increased in cells with benzobromarone as compared to cells without benzobromarone (Figure 3). In addition, when mutant PPRE oligonucleotide was incubated with PPARs, no evidence of binding was found.

After addition of benzobromarone to 293T cells at final concentrations of 1 μM and 10 μM , LPL and ACS protein

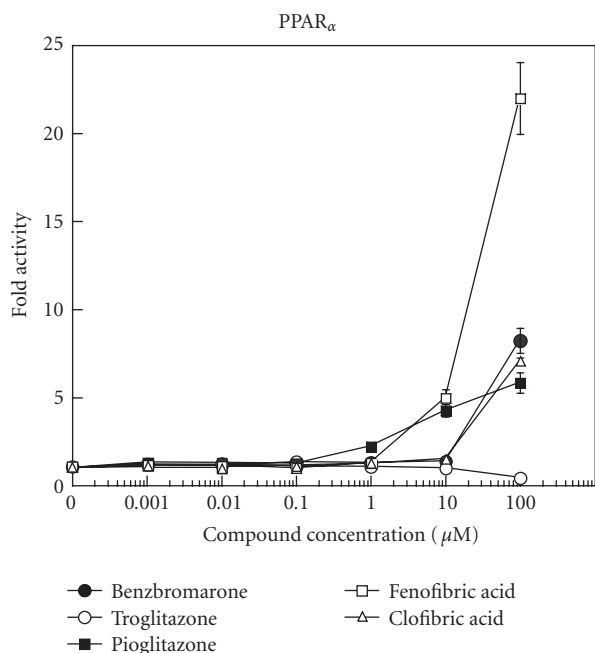


FIGURE 1: Effects of benzbromarone (●), troglitazone (○), pioglitazone (■), fenofibric acid (□), and clofibrac acid (Δ) on PPAR_α activation. Cells were transfected with the receptor plasmid for the chimera of the PPAR_α ligand-binding domain and GAL4 DNA-binding domain, together with a reporter plasmid containing a GAL4-responsive promoter driving the expression of luciferase. Each point represents mean \pm standard deviation (SD).

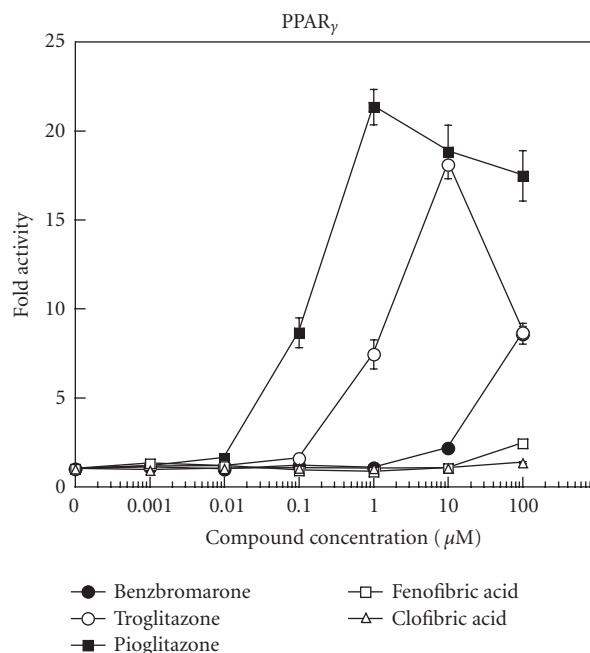


FIGURE 2: Effects of benzbromarone (●), troglitazone (○), pioglitazone (■), fenofibric acid (□), and clofibrac acid (Δ) on PPAR_γ activation. Cells were transfected using the receptor plasmid for the chimera of the PPAR_γ ligand-binding domain and GAL4 DNA-binding domain, together with a reporter plasmid containing a GAL4-responsive promoter driving the expression of luciferase. Each point represents mean \pm SD.

expressions were determined using the western blot method, demonstrating a dose-dependent increase in each protein (Figure 4).

4. DISCUSSION

PPAR regulates transcriptional activation of the target gene by forming a heterodimer with the retinoid X receptor, and recognizing and binding to PPRE via a specific gene sequence [5]. Various proteins such as LPL, ACS, fatty acid transporter, and fatty acid binding protein have been reported to be expressed as a result of transcriptional activation of target genes by PPARs [6–8].

Clofibrate and fenofibrate as PPAR_α activators and troglitazone and pioglitazone as PPAR_γ activators are considered to affect the metabolism of lipids and carbohydrates in the living body through these processes [9–11]. The present studies were performed to elucidate the details of PPAR activation by benzbromarone, which has been shown to induce hepatic hypertrophy associated with peroxisomal proliferation in rats [2].

First, the ligand affinity of benzbromarone for PPAR_α was equivalent to that of clofibrac acid, a representative activator of PPAR_α. The affinity of benzbromarone for PPAR_γ was weak and clearly inferior to the affinities of troglitazone and pioglitazone, which are representative activators of

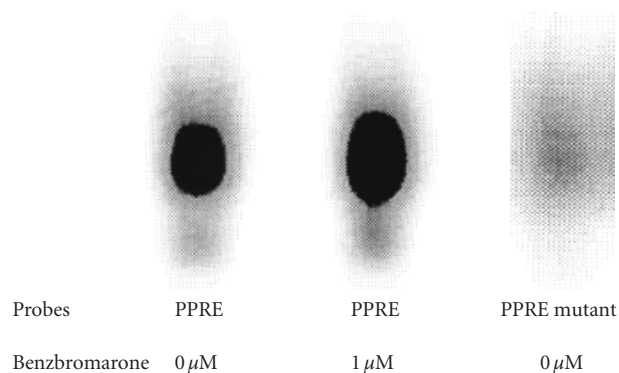


FIGURE 3: Comparative analysis of PPARs DNA-labeled PPRES complex by EMSA in nuclear extracts of 293T cells with or without benzbromarone treatment for 24 hours. Presence of PPRES was verified by incubation with mutant PPRES oligonucleotide.

PPAR_γ. Second, benzbromarone added to 293T cells elicited an increase in specific binding between PPARs and PPRES. Third, benzbromarone enhanced protein expression of LPL and ACS in a concentration-dependent manner.

In addition, the last experiment revealed more marked enhancement of PPAR_α expression than PPAR_γ expression by benzbromarone [4]. Moreover, based on the results of *in vitro* and *in vivo* expression analyses of various

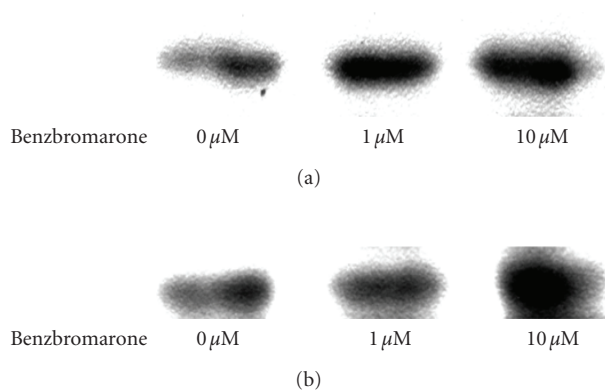


FIGURE 4: Expression levels of (a) LPL protein and (b) ACS protein as determined by Western blot analysis in 293T cells with or without benzbromarone treatment for 24 hours. The optimal density for each band measured using NIH Image. 1.6 is as follows: (a) LPL protein: 64.14 (0 μM), 98.16 (1 μM), 113.37 (10 μM). (b) ACS protein: 51.05 (0 μM), 63.29 (1 μM), 94.66 (10 μM).

PPAR $_{\alpha}$ -regulated genes in rats, benzbromarone has recently been classified as a PPAR $_{\alpha}$ agonist [12, 13]. The present findings support these reports from the perspective of intracellular gene transcription.

In patients receiving benzbromarone and in those receiving troglitazone, pioglitazone, clofibrate, fenofibrate, or other PPARs activators, hepatic impairment with an undeniable causal relationship to these drugs has been reported [14–17].

On the basis of the above findings and taking into account the mechanisms underlying increases in PPAR-mediated gene expression by PPAR $_{\alpha}$ activators such as clofibrate and fenofibrate and PPAR $_{\gamma}$ activators such as troglitazone and pioglitazone, we concluded that benzbromarone can be classified as a PPAR $_{\alpha}$ activator. The hepatic hypertrophy caused by benzbromarone in rats may thus be induced via enhancement of PPAR $_{\alpha}$ -mediated target gene expression and the resultant changes in lipid metabolism. In this context, the newly elucidated pharmacological features of benzbromarone could provide some hints that benzbromarone and other PPAR activators might display common mechanisms with relation to hepatic adverse effects.

The present study demonstrated that benzbromarone, a uricosuric drug, displays affinity for PPARs despite having a different chemical structure from fibrate-class antihyperlipemic drugs (fenofibrate, clofibrate) and thiazolidine-class antidiabetic drugs (troglitazone, pioglitazone), by comparison to these PPAR activators.

Considering that C_{\max} following oral administration of 100 mg benzbromarone in humans is approximately 5 μM [18], which is lower than levels in the present study, the present in vitro results may be difficult to simply extrapolate to clinical use. However, further investigations must be carefully conducted using the present findings as reference, into the relationships between benzbromarone and glucose and lipid metabolism, to reconfirm the clinical value of benzbromarone.

ABBREVIATIONS

ACS: Acyl-CoA synthetase
 EMSA: Electric mobility shift assay
 LPL: Lipoprotein lipase
 PPAR $_{\alpha}$: Peroxisome proliferator-activated receptor α
 PPAR $_{\gamma}$: Peroxisome proliferator-activated receptor γ
 PPRE: Peroxisome proliferator-responsive element
 URAT1: Urate transporter 1

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