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Highly sensitive upregulation of apolipoprotein A-IV by peroxisome proliferator-activated receptor α (PPARα) agonist in human hepatoma cells

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ABSTRACT

Peroxisome proliferator-activated receptor α (PPARα) is a key regulator in hepatic lipid metabolism and a potential therapeutic target for dyslipidemia. However, in humans hepatic PPARα-regulated genes remain unclear. To investigate the effect of PPARα agonism on mRNA expressions of lipid metabolism-related genes in human livers, a potent PPARα agonist, KRP-101 (KRP), was used to treat the human hepatoma cell line, HepaRG cells. KRP did not affect AOX or L-PBE, which are involved in peroxisomal β-oxidation. KRP increased L-FABP, CPT1A, VLCAD, and PDK4, which are involved in lipid transport or oxidation. However, the EC_{50} values (114–2500 nM) were >10-fold weaker than the EC_{50} value (10.9 nM) for human PPARα in a transactivation assay. To search for more sensitive genes, we determined the mRNA levels of apolipoproteins, apoA-I, apoA-II, apoA-IV, apoA-V, and apoC-III. KRP had no or little effect on apoA-I, apoC-III, and apoA-II. Interestingly, KRP increased apoA-IV (EC_{50}, 0.99 nM) and apoA-V (EC_{50}, 0.29 nM) with high sensitivity. We identified apoA-IV as a PPARα-upregulated gene in a study using PPARα siRNA. Moreover, when administered orally to dogs, KRP decreased the serum triglyceride level and increased the serum apoA-IV level in a dose-dependent manner. These findings suggest that apoA-IV, newly identified as a highly sensitive PPARα-regulated gene in human livers, may be one of the mechanisms underlying PPARα agonist-induced triglyceride decrease and HDL elevation.

1. Introduction

Peroxisome proliferator-activated receptors (PPARs) belong to the nuclear hormone receptor superfamily [1] and act as important transcriptional regulators involved in lipid and glucose homeostasis [2]. There are three PPAR subtypes: PPARα (NR1C1), PPARγ (NR1C3), and PPARδ (NR1C2) [3]. PPARα is predominantly expressed in tissues that have a high lipid-catabolic activity such as liver, heart, kidney, and muscle [4–6], and regulates the transcription of numerous genes that encode proteins involved in lipid metabolism [7]. Beneficial effects of fibrate drugs for dyslipidemic subjects have been

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Abbreviations: AOX, acyl-CoA oxidase; apo, apolipoprotein; CHO-K1, Chinese hamster ovary-K1; CPT1A, carnitine palmitoyltransferase 1A; DMEM, Dulbecco's modified Eagle's medium; HDL, high-density lipoprotein; LBD, ligand-binding domain; L-FABP, liver fatty acid binding protein; L-PBE, enoyl-CoA hydratase/3-hydroxyacyl CoA dehydrogenase; LPL, lipoprotein lipase; PDK4, pyruvate dehydrogenase kinase, isozyme 4; PPAR, peroxisome proliferator-activated receptor; PPRE, peroxisome proliferator response element; VLCAD, very long-chain acyl-CoA dehydrogenase.

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explained in part by hepatic PPARα activation [8–11]. Triglyceride-lowering effect of fibrates can be attributed to elevated rates of fatty acid (FA) catabolism through increasing FA β-oxidation and FA transport, increasing catabolism of triglyceride-rich lipoproteins by upregulation of lipoprotein lipase [12] and apoA-V [13] and by downregulation of apoC-III [14–16]. Fibrates-induced elevation level of HDL-cholesterol seems to be associated with increases in serum levels of apoA-I and apoA-II [17–20]. However, direct evidence for PPARα-regulated genes on human livers has been limited because of lacking human liver-derived cell lines highly responsive to PPARα agonists.

To get more insights on the hepatic PPARα-regulated genes in humans, human hepatoma cell lines, HepaRG cells were treated with a potent PPARα agonist, KRP-101 (KRP). In the present study, we examined the expression levels of genes; acyl-CoA oxidase (AOX) and enoyl-CoA hydratase/3-hydroxyacyl CoA dehydrogenase (L-PBE) involved in peroxisomal β-oxidation [21], and liver fatty acid binding protein (L-FABP) [22], carnitine palmitoyltransferase 1A (CPT1A) [23], very long-chain acyl-CoA dehydrogenase (VLCAD) [24], and pyruvate dehydrogenase kinase, isozyme 4 (PDK4) [25] involved in FA transport and mitochondrial β-oxidation, and apolipoproteins, apoA-I and apoA-II, major components of HDL, apoA-IV and apoA-V, components of HDL that are capable of LPL activation [26,13], and apoC-III, a component of VLDL and a known LPL inhibitor [27].

We report herein that a potent PPARα agonist, KRP induces apoA-IV expression via PPARα activation. KRP administration increases serum levels of apoA-IV protein in dogs. We suggest that ApoA-IV is a highly sensitive PPARα-regulated gene newly identified in human hepatocytes, and may play a key role in the beneficial pharmacological effects of PPARα agonist, such as triglyceride decrease and HDL elevation in humans.

2. Materials and methods

2.1. Chemicals

A novel PPARα agonist, KRP-101 (KRP) was synthesized by Kyorin Pharmaceutical Co., Ltd., Fenofibric acid was obtained from Tyger Scientific.

2.2. Animals

Male beagle dogs were obtained from Covance Research Products Inc. at 5 months of age. All experiments were followed by the ethical guidelines described in National Institutes of Health Guide for Care and Use of Laboratory Animals. Dogs were housed in individual cages and subjected to a standard light (7 a.m. to 7 p.m.) and dark (7 p.m. to 7 a.m.) cycle. Each dog was fed 300 g/day of a standard dog chow (Certified Canine Diet 5007, PMI Feeds Inc.) and allowed tap water *ad libitum*.

2.3. Cell culture

HepaRG (Biopredic International) cells were cultured in Williams'E medium with Glutamax containing 10% fetal calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 5 μg/ml insulin, 50 mmol/l hydrocortisone and 2% DMSO with 5% CO_2. Chinese hamster ovary-K1 (CHO-K1) cells (ATCC) were cultured in Ham's F12 medium containing 10% fetal calf serum with 5% CO_2.

2.4. Plasmids

The cDNA encoding the LBD of human PPARγ (amino acid 174–475) and PPARδ (amino acid 139–441) were isolated from HepG2 cells by RT-PCR using sense primer 5′-CCCGGATCCTGTCTCATAATGCCATCAGGTTTGGG-3′ and antisense primer 5′-CCCAAGCTTCTAGTACAAGTCCTTGTAGATCTCCTG-3′ for PPARγ, and sense primer 5′-CCCGGATCCTGTCACACAACGCTATCCGTTTTGG-3′ and antisense primer 5′-CCCAAGCTTTAGTACATGTCCTTGTAGATCTCCTG-3′ for PPARδ. These amplified DNA fragments were digested with BamHI and HindIII and inserted into the pM expression vector (CLONTECH) to generate chimeric plasmids with the DNA binding domain of yeast GAL4, GAL4-PPARγ, and δ LBD. The human GAL4-PPARα was prepared as indicated previously [28]. The GAL4-responsive firefly-luciferase plasmid (pFR-Luc) and renilla luciferase plasmid (pRL-Luc) were purchased from Stratagene and Promega, respectively.

2.5. Transactivation assay

CHO-K1 cells were co-transfected with GAL4-PPARα, γ or δ LBD, pFR-Luc and internal standard pRL-luc using Lipofectamine reagent (Invitrogen). Cells were treated with the indicated compounds for 24 h in Ham's F12 medium containing 10% delipided fetal calf serum, and cell extracts were measured and normalized with the respective internal standard luciferase activity. The EC_{50} values of tested compounds were derived by curve-fitting using the Prism program (GraphPad Software).

2.6. Binding assay

Five micrograms of histidine-tagged human PPARα LBD protein and 100 nM [^{3}H]KRP-297 (specific activity, 41 Ci/mmol) were incubated at 25 °C for 30 min in a buffer containing 50 mM Tris (pH 7.4), 50 mM KCl, and 10 mM dithiothreitol [29]. KRP or fenofibric acid was added to the reaction as indicated in Fig. 2C. Bound and free [^{3}H]KRP-297 were immediately separated on a Sephadex G-25 (Pharmacia Biotech) spin column, and the radioactivity of the bound [^{3}H]KRP-297 fraction was counted with a liquid-scintillation analyzer (2000CA, Packard).

2.7. Analysis of mRNA expression by real-time quantitative PCR

HepaRG cells were treated with the indicated compounds for 24 h. Total RNA was isolated and treated with DNase by RNeasy mini kit (Qiagen) according to the manufacturer's instructions. Total RNA (200 ng) was reverse transcribed in 20 μl reaction using Superscript™ II reverse transcriptase (Invitrogen) according to manufacturer's instructions. Quantitative gene expression analysis was performed on AB7500

(Applied Biosystems) using TaqMan gene expression assay systems. PCR were performed using Hs00157347_m1 for L-PBE, Hs00244515_m1 for AOX, Hs00157079_m1 for CPT1A, 00817723_g1 for VLCAD, Hs00176875_m1 for PDK4, Hs00163641_m1 for apoA-I, Hs00155788_m1 for apoA-II, Hs00166636_m1 for apoA-IV, Hs00364830_m1 for apoA-V, Hs00163644_m1 for apoC-III, Hs99999901_s1 for 18S ribosomal RNA (Applied Biosystems).

2.8. PPARα siRNA study

HepaRG cells were transfected with 10 nM control siRNA (12935-200, Invitrogen) or 10 nM PPARα siRNA (HSS108289, Invitrogen) for 48 h using Lipofectamine RNAiMAX (Invitrogen) according to manufacturer's instructions. After transfection, cells were treated with DMSO or 10^{-8} M KRP for 24 h, then total RNA was isolated and treated with DNase by RNeasy mini kit (Qiagen) according to the manufacturer's instructions. The mRNA expression levels of each gene were measured by real-time quantitative PCR method as described above. The significance of differences were assessed by the Student's *t*-test. $P < 0.05$ was considered to be statistically significant.

2.9. Immunoblotting analysis

One microlitre of dog serum were separated in 10% SDS-polyacrylamide gels (29:1) and transferred to an Immobilon-P transfer membrane (Millipore). Immunoblotting was performed using 1:200 goat anti-human apoA-IV polyclonal antibody (N-20, Santa Cruz Biotechnology), followed by incubation with 1:2000 polyclonal rabbit anti-goat immunoglobulins/HRP (Dako Cytomation) or using 1:1000 goat anti-dog IgG polyclonal antibody/HRP (sc-2433, Santa Cruz Biotechnology) and ECL detection (Amersham Biosciences).

2.10. In vivo studies

Male beagle dogs (2-year-old, Covance Research Products Inc.) were orally dosed for 7 days with KRP at various dosages: 0, 0.03, and 0.1 mg/kg ($N = 3$). On the 8th day, serum samples were collected. ApoA-IV levels were measured by immunoblotting analysis as mentioned above. Triglyceride levels were measured using Liquiteck TG II reagents (Roche Diagnostic). The significance of differences from values obtained in control animals was assessed by the Dunnet's multiple comparison test. $P < 0.05$ was considered significant.

3. Results

3.1. KRP is a potent and subtype-selective agonist for human PPARα

KRP-101 (KRP) is a novel PPARα agonist synthesized in our laboratory. The chemical structure of KRP is a phenylpropionic acid derivative but quite different from that of the fibrate class (Fig. 1). KRP-activated PPARα, γ, and δ in a transactivation assay using GAL4/ human PPAR ligand-binding domain (LBD) (Fig. 2A). EC_{50} values of KRP for human PPARα, γ, and δ were 10.9, 1110, and 1810 nM, respectively. In a competition binding assay using a human PPARα LBD and a radiolabeled PPARα agonist [29], KRP displayed binding of a known PPARα ligand in a concentration-dependent manner (Fig. 2C). The IC_{50} value was 8.8 nM. Fenofibric acid was a weak PPARα agonist (Fig. 2B and C), compared with KRP.

3.2. Effect of PPARα agonists on mRNA expression of lipid transport, FA oxidation-related genes, and apolipoproteins

To explore the major PPARα-regulated genes in human hepatocytes, we measured the mRNA expression levels of genes correlated with lipid transport and FA oxidation in human HepaRG cells treated with KRP and fenofibric acid for 24 h (Fig. 3). KRP did not affect expression levels of genes, AOX or L-PBE mRNA, involved in peroxisomal β-oxidation (Fig. 3). KRP increased mRNA levels of L-FABP, CPT1A, VLCAD, and PDK4, involved in lipid transport and oxidation, in a concentration-dependent manner (Fig. 3). However, the EC_{50} values (114–2500 nM, Table 1) were >10-fold weaker than the EC_{50} value (10.9 nM, Fig. 2A) for human PPARα in a transactivation assay. Next, we measured the mRNA expression levels of apolipoproteins in HepaRG cells treated with KRP and fenofibric acid for 24 h (Fig. 4). Interestingly, KRP increased mRNA levels of apoA-IV and apoA-V with higher potencies (EC_{50}, 0.99 and 0.29 nM, respectively, Table 1) that were apparently >100-fold more sensitive, compared to those of other genes. However, KRP did not affect apoA-I or apoC-III, while it increased apoA-II with a weak potency (EC_{50},

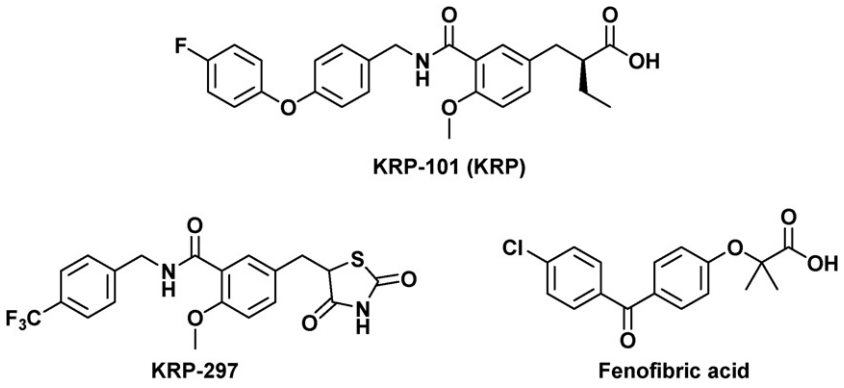

Fig. 1 – Chemical structure of PPARα agonist.

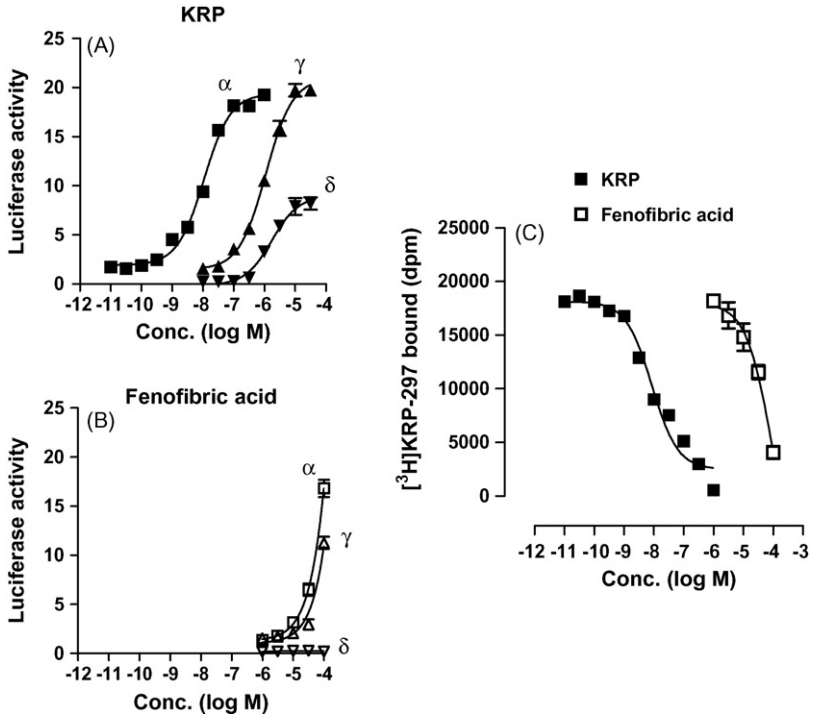

Fig. 2 – Effect of KRP and fenofibric acid on agonistic activity for human PPARα. (A) Dose-response curves of KRP on transcriptional activity for human PPARα (■), PPARγ (▲), and PPARδ (▼). (B) Dose–response curves of fenofibric acid on transcriptional activity for human PPARα (□), PPARγ (△), and PPARδ (▽). CHO-K1 cells were co-transfected with chimeric GAL4-PPARα, γ, δ expression plasmid and pFR-luc, as a reporter plasmid. Transfected cells were treated with KRP for 24 h. Data are presented as the means ± S.E. of three independent experiments. (C) Dose–response curve of displacement of [^{3}H]KRP-297 binding to human PPARα by KRP or fenofibric acid. Competitive binding assays using the purified LBD protein of human PPARα and 100 nM [^{3}H]KRP-297 were performed in the presence of various concentrations of KRP. Data are presented as the means ± S.E. of three independent experiments.

>22500 nM, Table 1). These findings suggest that apoA-IV and apoA-V are highly sensitive to PPARα agonist.

3.3. *ApoA-IV is one of PPARα-regulated genes*

There has not yet been any evidence to show whether apoA-IV expression is regulated by PPARα. Therefore, study using a human PPARα-specific siRNA (siPPARα) was performed and showed that siPPARα suppressed the expression level of PPARα mRNA by 70% in HepaRG cells. These knock down levels of PPARα mRNA were not affected by the presence of 0.1% DMSO (vehicle) and 10^{-8} M KRP (Fig. 5, left panel) for 24 h. KRP-induced upregulations of apoA-IV were dramatically abrogated by siPPARα treatment (Fig. 5, middle panel). KRP-induced upregulations of apoA-V were also abrogated by siPPARα treatment (Fig. 5, right panel). These findings strongly suggest that KRP-induced apoA-IV expression in HepaRG cells is mediated by PPARα activation.

3.4. *KRP administration increases serum apoA-IV level in dogs*

We previously suggested that the dog is a suitable animal model for studying and predicting the biological actions of a highly potent agonist for human PPARα [30]. We next attempted to determine whether KRP increases serum apoA-IV levels and acts as a potent lipid-lowering agent in dogs. When administered orally to beagle dogs (0.03 and 0.1 mg/kg) once daily for 7 days, KRP significantly increased serum apoA-IV levels (Fig. 6B and C) and decreased serum triglyceride levels in dogs (Fig. 6A). Immunoblot of dog IgG (heavy chain) shows equal protein levels (Fig. 6B). The body weights at day 7 on KRP-101 treatments of 0.03 mg/kg (10.52 ± 0.34 kg) and 0.1 mg/kg (11.00 ± 1.19) were significantly unaltered from their pretreatment values (0.03 mg/kg, 10.60 ± 0.39 kg; 0.1 mg/kg, 11.05 ± 1.17 kg).

4. Discussion

In HepaRG cells, we found that a potent agonist for human PPARα, KRP, upregulated apoA-IV and apoA-V mRNA expressions with higher sensitivities, compared to those for other PPARα-regulated genes. The EC_{50} values for apoA-IV and apoA-V mRNA expressions were close to the EC_{50} value for human PPARα in a transactivation assay, even though we cannot exclude the possibility that a small amount of lipids in culture medium of HepaRG cells may affect background regulation of genes. In human hepatocytes, apoA-V is upregulated by PPARα agonists via a putative PPRE [13]. However, it remains unclear

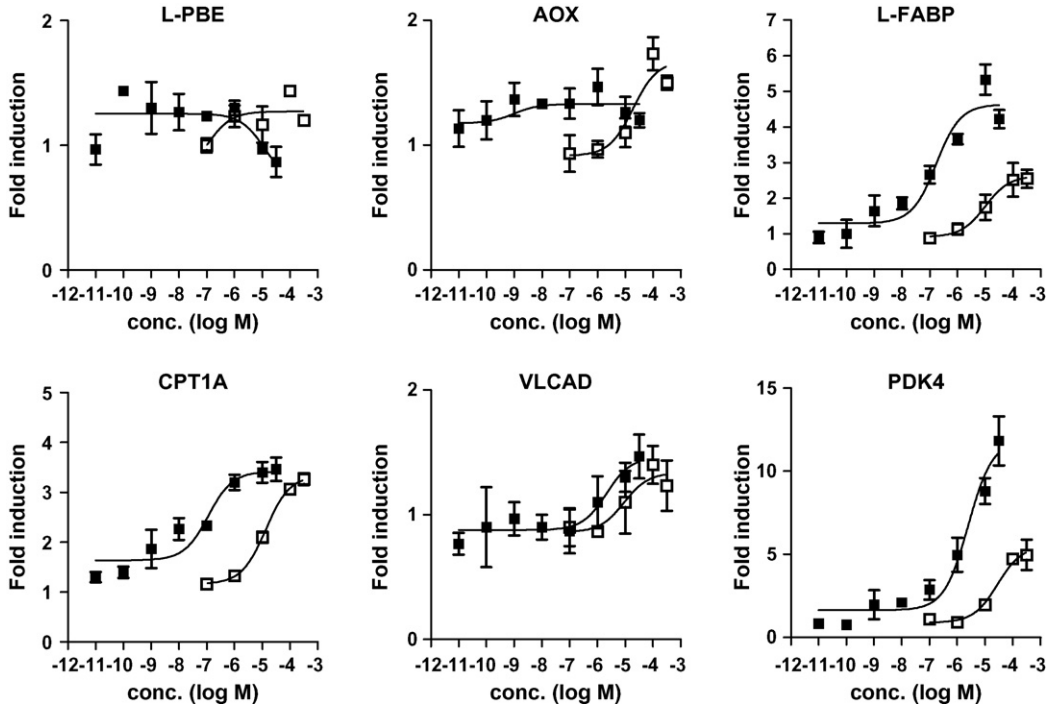

Fig. 3 – Effect of KRP and fenofibric acid on mRNA expression of lipid transport and oxidation-related genes. HepaRG cells were treated with the indicated concentrations of KRP (■) or fenofibric acid (□) for 24 h. The mRNA expression levels of each gene were measured by real-time quantitative PCR method, normalized to 18S rRNA expression and shown as the fold induction to the vehicle. Data are presented as the means ± S.E. of three independent experiments.

whether apoA-IV is a PPARα-regulated gene. This paper provides the evidence that apoA-IV is a highly sensitive- and PPARα-regulated gene in human hepatoma cells, HepaRG, and simultaneously proposes the importance of apoA-IV as one of the pharmacological biomarkers for PPARα agonists.

Consistent with the results obtained from HepaRG cells, KRP significantly increased serum levels of apoA-IV protein in dogs in a dose-dependent manner (Fig. 6). In contrast, previous studies using rodent livers demonstrated that PPARα agonist tends to decrease apoA-IV mRNA expression [31,32]. We also found that apoA-IV expression in the mouse liver was decreased by oral administration of a potent agonist for rodent PPARα, Wy-14,643 (data not shown). Thus, apparent species difference was observed in regulation of apoA-IV gene in response to PPARα agonist between human/dog and rodent. Similarly, it has been reported that PPARα agonist causes apoA-I expression to decrease in rodents but increase in human species [17,19]. To clarify the mechanism of species difference observed in apoA-IV regulation, further study will be needed to investigate the promoter regulation of apoA-IV.

Table 1 – Effects and the EC50 values of KRP on mRNA expression of β-oxidation-related genes and apolipoproteins

Gene name	mRNA expression	
	Effect	EC_{50} (nM)
Peroxisomal β-oxidation		
AOX	→	N.C.
L-PBE	→	N.C.
Fatty acid transport and mitochondrial-β-oxidation		
L-FABP	Up	169
CPT1A	Up	114
VLCAD	Up	2100
PDK4	Up	2500
Apolipoprotein		
apoA-I	→	N.C.
apoA-II	→	N.C.
apoA-IV	Up	0.99
apoA-V	Up	0.29
apoC-III	→	N.C.

N.C.: not calculable.

ApoA-IV is a 46 kDa glycoprotein synthesized in intestine and liver and present in plasma, interstitial fluid, and lymph [33–36], and circulates freely or is associated with chylomicrons and HDLs [33]. Transgenic mice overexpressing mouse apoA-IV have increased HDL levels [37] and apoA-IV knockout mice have decreased HDL levels [38], suggesting that apoA-IV is a key determinant of plasma HDL levels. In addition, it has been reported that apoA-IV increases LPL activity [26] and controls appetite in response to fat in the diet [39,40]. A recent report suggested that ApoA-IV polymorphism has significant associations with lower levels of HDL cholesterol in type 2 diabetes mellitus [41]. Furthermore, rodent studies demonstrated the protective role of apoA-IV in the development and progression of atherosclerosis [37,42]. Taken together, upregulation of apoA-IV induced by PPARα agonism may be one of the beneficial effects of therapy for atherosclerosis as well as dyslipidemia.

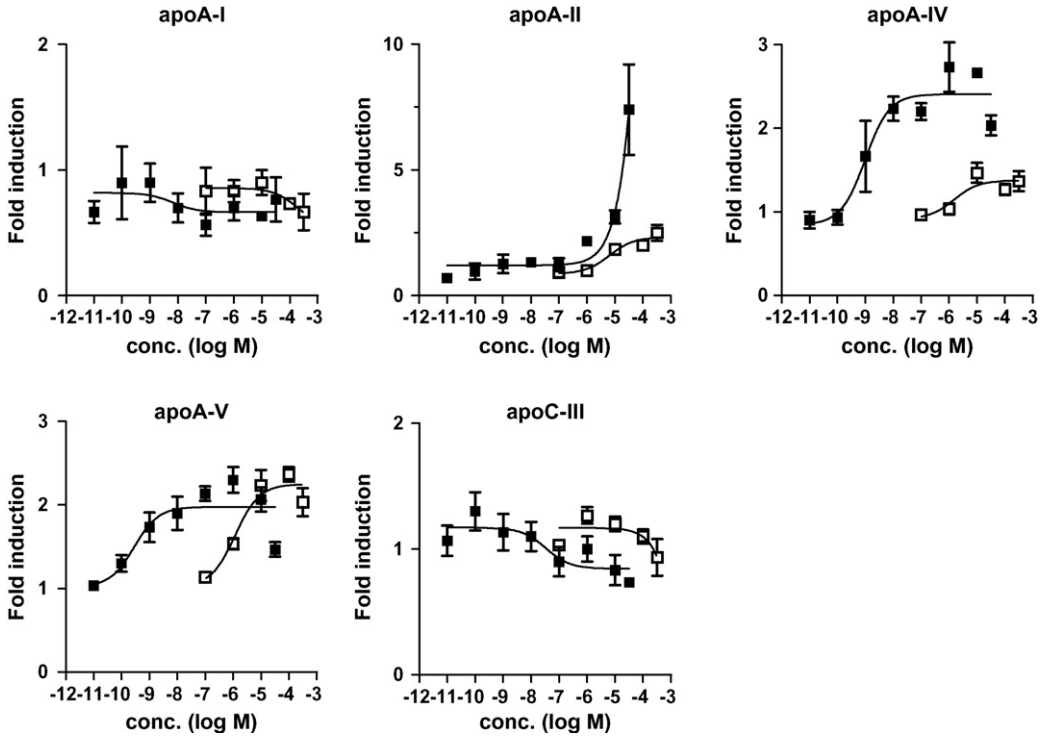

Fig. 4 – Effect of KRP and fenofibric acid on mRNA expression of apolipoproteins. HepaRG cells were treated with the indicated concentrations of KRP (■) or fenofibric acid (□) for 24 h. The mRNA expression levels of each gene were measured by real-time quantitative PCR method, normalized to 18S rRNA expression and shown as the fold induction to the vehicle. Data are presented as the means ± S.E. of three independent experiments.

High concentration (500 μM) of fenofibric acid increased apoA-I [17] and apoA-II [20] mRNA expression in human primary hepatocytes, and 0.5% fenofibrate-supplemented chow for 7 days increased liver apoA-I mRNA in human apoA-I transgenic mice [17]. On the contrary, treatment with fenofibrate (100 mg/(kg day)) for 7 days increased serum HDL cholesterol levels, but did not significantly increase hepatic apoA-I mRNA in human apoA-I transgenic mice [45]. Five hundred micromolars of fenofibric acid decreased apoC-III [15] mRNA in human primary hepatocytes. In the present study, KRP and fenofibric acid had no significant effects on apoA-I and apoC-III mRNA in HepaRG cells. Upregulation of apoA-II

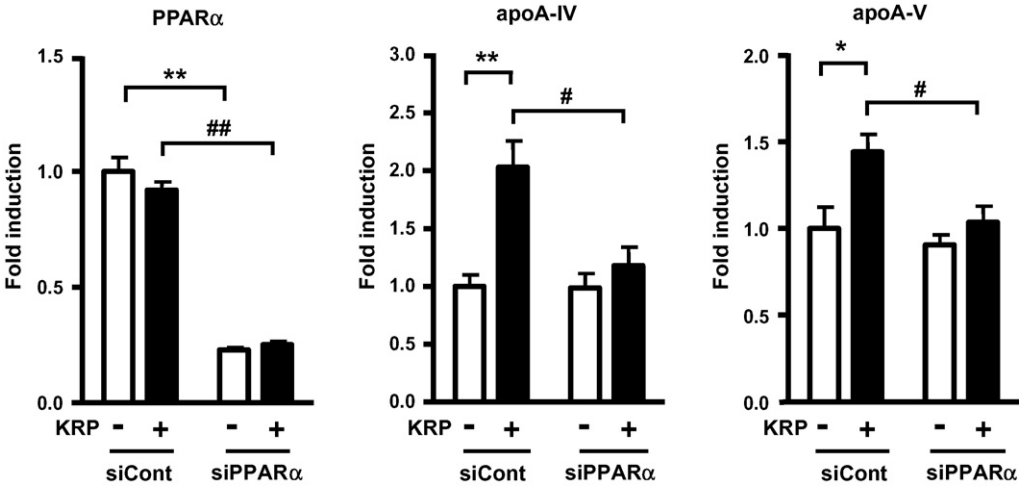

Fig. 5 – Effect of PPARα siRNA on KRP-induced apoA-IV expression. HepaRG cells were transfected with control siRNA (siCont) or PPARα siRNA (siPPARα) for 48 h and then treated with 10^{-8} M KRP for 24 h. The mRNA expression levels of apoA-IV were measured by real-time quantitative PCR method, normalized to 18S rRNA expression and shown as the fold induction to the siCont treated with vehicle. Data are presented as the means ± S.E. of four independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. siCont treated with vehicle; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. siCont treated with KRP.

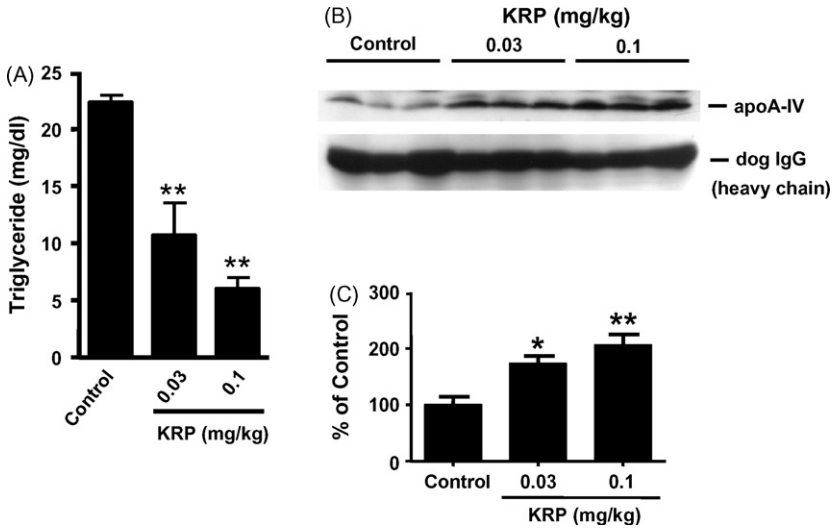

Fig. 6 – Effects of KRP on serum triglyceride and apoA-IV protein levels in dogs. KRP was administrated orally once a day for 1 week to dogs (N = 3). (A) Triglyceride levels in dog serum. (B) Immunoblott analysis of apoA-IV protein in dog serum. The same membrane was stripped and reprobed with anti-dog IgG antibody. (C) Quantitative result of panel (B). Data are presented as the means ± S.E. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. vehicle-treated control group.

mRNA was detected only by high concentrations of KRP and fenofibric acid. Thus, our study using HepaRG cells provides the evidence that apoA-IV and apoA-V rather than apoA-I, apoA-II, and apoC-III are genes with a highly sensitive response to PPARα agonists. We showed that apoA-IV is a PPARα-regulated gene in humans. Based on our results, it will be noted that measurement of serum apoA-IV levels is a highly sensitive biomarker in human subjects treated by PPARα agonists.

In the present study, we found that KRP significantly increased mRNA levels of L-FABP, CPT1A, VLCAD, and PDK4 involved in lipid transport and oxidation, in a concentration-dependent manner (Fig. 3). However, their EC_{50} values (114–2500 nM, Table 1) were over 10-fold higher than that for human PPARα (10.9 nM in a transactivation assay, Fig. 2A). KRP did not affect expression levels of AOX genes or L-PBE mRNA involved in peroxisomal β-oxidation in HepaRG cells (Fig. 3), although in rodent livers these are well known as PPARα agonist-regulated genes [43]. Consistent with our findings, it is reported that PPARα agonists failed to profoundly induce peroxisomal genes in human hepatocytes [44].

In conclusion, using the human-derived cell line, HepaRG cells, we newly identified and characterized the apoA-IV gene as highly sensitive to PPARα agonist. In addition, administration of PPARα agonist increased the serum level of apoA-IV protein in dogs. Upregulation of apoA-IV may be one of the key mechanisms underlying PPARα agonist-induced triglyceride decrease and HDL elevation.

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