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Pharmacological characterization of a human-specific peroxisome proliferater-activated receptor α (PPARα) agonist in dogs

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Abstract

Peroxisome proliferator-activated receptor α (PPARα) is a key regulator in lipid metabolism and a potential therapeutic target for lipid-related metabolic diseases. It has been shown that there are species differences between human and mouse in response to several PPARα agonists in a transactivation assay. In the present study, we cloned a full length of dog PPARα and investigated the effects of a novel and potent agonist (KCL) for human PPARα. In a transactivation assay using the full length of PPARα, agonistic activity of KCL for dog PPARα (EC_{50}: 0.007 μM) was comparable to that for human PPARα (EC_{50}: 0.003 μM), but not that for rat PPARα (EC_{50}: 11.49 μM). Similar results were obtained from a transactivation assay using a GAL4/PPARα ligand-binding domain (LBD) chimera. A point-mutation study showed that I272 on PPARαLBD is a major contributor to species differences in response to KCL between human, dog, and rat PPARα. KCL also induced mRNA levels of HMG-CoA synthase in dog hepatocytes. When administered orally to dogs and rats, KCL significantly decreased plasma triglyceride levels in a dose-dependent manner. The triglyceride-lowering effects of KCL in dogs were >100-fold more potent than those in rats. These results suggest that KCL may induce activation of highly potent PPARα in humans as well as dogs, and that dog is a suitable animal model for studying and predicting the biological actions of potent agonists for human PPARα.

Keywords: Nuclear receptor; PPARα; HMG-CoA synthase; Triglyceride; KCL; Species difference

1. Introduction

Peroxisome proliferator-activated receptors (PPARs) belong to the nuclear hormone receptor superfamily [1] and act as important transcriptional regulators involved in the control of lipid and glucose homeostasis [2]. There are three PPAR subtypes: PPARα (NR1C1), PPARγ (NR1C3), and PPARδ (NR1C2) [3]. PPARα is predominantly expressed in lipid-metabolically active tissues such as liver, heart, kidney, and muscle [4–6], and regulates the transcription of numerous genes encoding proteins involved in lipid metabolism [7]. The beneficial pharmacological actions of fibrate drugs are explained in part by PPARα activation in the liver [8]. The triglyceride-lowering activity of fibrates can be attributed to both the inhibition of hepatic fatty acid synthesis and increased catabolism of triglyceride-rich lipoproteins [9,10]. Fibrates upregulate LPL expression [11] and downregulate apoC-III expression [12–14], leading to increased VLDL catabolism. Elevation of HDL-cholesterol by fibrates seems to correlate with increases in apoA-I expression [15–17]. In addition, it has recently been suggested that PPARα activation improves insulin resistance associated with obesity through a modulation of lipid metabolism [18–20], and mediates the anti-inflammatory actions at the level of the vascular wall [21]. Thus, PPARα is a potential molecular target for the treatment of

Abbreviations: AOX, acyl-CoA oxidase; CHO-K1, Chinese hamster ovary-K1; DMEM, Dulbecco's modified Eagle's medium; KCL, KCL-1999000269; LBD, ligand-binding domain; PPAR, peroxisome proliferator-activated receptor; PPRE, peroxisome proliferator-response element; RACE, rapid amplification of cDNA ends

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coronary heart disease characterized by abnormal lipid metabolism and inflammation.

The PPARα gene has been cloned from mouse [22], frog [23], rat [24], guinea pig [25], chicken [26], rhesus monkey [27], and human [28,29]. The homology of amino acids on the ligand-binding domain (LBD) of PPARα are highly conserved at levels >90% among these species. However, it should be considered that alteration of a single amino acid in PPARα produces a PPARδ character in response to agonists [26]. In fact, in a transcativation assay it has been known that there are species differences in response to several synthetic compounds, GW9578, KRP-297, GI262570, L-796449, for PPARα between human and mouse [30]. Therefore, the results from in vivo studies of PPARα agonists using rodents should be carefully reviewed so as to predict their biological actions in humans. Moreover, it is necessary to investigate species-differences in response to each agonist for a full biological characterization of the new PPARα agonists.

In the present study, dog PPARα was cloned and pharmacologically characterized using a novel and potent agonist for human PPARα, KCL, synthesized by our laboratories [31]. We report herein that dog is useful for investigating the in vivo biological actions of human-specific PPARα agonists.

2. Materials and methods

2.1. Chemicals

A novel PPARα agonist, KCL-1999000269 (KCL), was synthesized by Kyorin Pharmaceutical Co. Ltd. Wy-14,643 was purchased from Cayman Chemical Co. Fenofibric acid was obtained from LaboTest. All compounds were dissolved in dimethyl sulfoxide at a final concentration of 0.1% in all assays.

2.2. Animals

Male beagle dogs were obtained from Genetic Models International at 5 months of age. All institutional guidelines for animal care and use were applied in this study. Dogs were housed in individual cages and subjected to a standard light (7.00 a.m. to 7.00 p.m.) and dark (7.00 p.m. to 7.00 a.m.) cycle. They were fed standard dog chow (Certified Canine Diet 5007, PMI Feeds Inc.) and allowed tap water ad libitum. Total RNA from tissues was prepared with ISOGEN (Nippon Gene) according to the manufacturer's instructions.

2.3. Dog PPARα cDNA

cDNA clones of dog PPARα were isolated as mentioned below. The nucleotide sequence was determined by the dideoxynucleotide chain termination procedure. cDNA (636–2032) was isolated from the dog liver 5′-STERTCH λgt11 cDNA library (CLONTECH) by screening with a probe for a 0.9-kb *Bam*HI–*Hin*dIII fragment from GAL4-chimeric human PPARα LBD plasmid [32]. The inserted cDNA fragment was subcloned into pBluescript-II SK+ (Stratagene). cDNA (395–738) was isolated from the dog liver cDNA library by PCR using sense primer 5′-GGT GGC GAC GAC TCC TGG AGC CCG-3′ and antisense primer 5′-GCC GGA TCC CGA CCG AAA GGC ACT TGTG-3′. cDNA (1–662) was isolated from dog liver total RNA by the 5′-rapid amplification of cDNA ends (RACE) system (Gibco BRL) using primers 5′-GCC GGA TCC TGT TTT TTC TGA TCG TGG CAT TC-3′ for first-strand cDNA synthesis, 5′-GCC GGA TCC CGA CCG AAA GGC ACT TGTG-3′ for first PCR, and 5′-GCC GGA TCC GGT CAC ATT TGT CAT AGG CCA GC-3′ for second PCR. The overlapping clone (636–1790) was isolated from dog liver total RNA by RT-PCR using sense primer 5′-CGG GAT CCG ACC GCA GCT GCA AAA TTC AG-3′ and antisense primer 5′-CCC AAG CTT AAG CAG GCA TTG CTC CCA GT-3′. cDNA (739–1647) to construct GAL4-chimeric dog PPARαLBD plasmid was isolated from dog liver total RNA by RT-PCR using sense primer 5′-CCC GGA TCC TGT CCC ATA ATG CCA TCC GCT TTG-3′ and antisense primer 5′-CCC AAG CTT TCA GTA CAT GTC CCT GTA GAT TTC CTG-3′. These amplified DNA fragments were digested with *Bam*HI and *Hin*dIII and inserted into the GAL4 (pM) expression vector (CLONTECH). cDNA (241–1647) to construct a full length of the dog PPARα expression vector, and cDNA (107–1647) were isolated from dog liver total RNA by RT-PCR using sense primer 5′-GCC GAA TTC ATG GTA GAC ACA GAA AGC CCG ATT TGC C-3′ and antisense primer 5′-CAG CAT CCC GTC TTT GTT CAT C-3′, and sense primer 5′-GCC GAA TTC ACC TCC GAA CTG CCA AGG CTG CAG-3′ and antisense primer 5′-CAG CAT CCC GTC TTT GTT CAT C-3′, respectively. The amplified DNA fragments were digested with *Eco*RI and *Sac*I, and inserted into the pcDNA3.1 expression vector (Invitrogen) with *Sac*I–*Hin*dIII fragments from GAL4-dog PPARα LBD plasmid.

2.4. Rat PPARα cDNA

The upstream region of rat PPARα cDNA from the *Sac*I recognition site [24] was isolated from rat liver total RNA by RT-PCR using sense primer 5′-GCG AAT TCA TGG TGG ACA CAG AGA GCC CCA TC-3′ and antisense primer 5′-CAG CAT CCC GTC TTT GTT CAT C-3′. To construct a full length of rat PPARα expression vector, the amplified DNA fragments were digested with *Eco*RI and *Sac*I, and inserted into the pcDNA3.1 expression vector with *Sac*I–*Hin*dIII fragments from GAL4-rat PPARα LBD plasmid [32] and the overlapping oligonucleotide pair, 5′-TCG AGA TGG TGG ACA CAG AGA GCC CCA TCT

GTC CTC TCT CCC CAC TTG AAG CAG ATG ACC TGG AAA GTC CCT TAT CTG AAG-3′ and 5′-AAT TCT TCA GAT AAG GGA CTT TCC AGG TCA TCT GCT TCA AGT GGG GAG AGA GGA CAG ATG GGG CTC TCT GTG TCC ACC ATC-3′.

2.5. Human PPARα cDNA

A full length of human PPARα cDNA was isolated from HepG2 cells (ATCC) by RT-PCR using sense primer 5′-CCC GGA TCC GCG ATG GTG GAC ACG GAA AG-3′ and antisense primer 5′-CCC AAG CTT CAG TAC ATG TCC CTG TAG ATC TCC TG-3′. To construct a full length of human PPARα expression vector, the amplified DNA fragments were digested with *Bam*HI and *Sma*I, and inserted into the pcDNA3.1 expression vector with *Sma*I–*Bam*HI fragments from GAL4-human PPARα LBD plasmid in which the *Hinc*II recognition site of multiple cloning sites was replaced by a *Bam*HI site.

2.6. Mutagenesis

Chimeric constructs encoding dog/rat, rat/dog, human/rat, and rat/human PPARα LBD were prepared by digestion of GAL4-PPARα LBDs at the *Sac*I recognition site (nucleotide 1096, amino acid 286). Point-mutants of PPARα were created by the QuikChange site-directed mutagenesis kit (Stratagene).

2.7. Northern blotting

Total RNA (30 μg) was subjected to formalin-denatured agarose electrophoresis and transferred to a nylon membrane (Hybond N+, Amersham Biosciences Corp.). Hybridization was performed with the cDNA probes for rat mitochondrial HMG-CoA synthase [33] and acyl-CoA oxidase (AOX) derived from rat [34] and dog. Dog AOX probe was prepared by PCR using sense primer 5′-CCG GAA TTC CTG AAC GAC CCA GAC TTC CAG CAT GAG GAC-3′ and antisense primer 5′-CCC AAG CTT GAA GGC ATA GGC AGT GGC CAG GAG-3′.

2.8. Transactivation assay

Chinese hamster ovary-K1 (CHO-K1) cells (ATCC) were maintained in Ham's F-12 medium supplemented with 10% delipided fetal calf serum. Cells were cotransfected with the full length of PPARα, the firefly-luciferase (Stratagene) reporter containing three copies of rat AOX peroxisome proliferator-response element (PPRE) [34], and internal standard renilla-luciferase (Promega) plasmids using LipofectAMINE reagent (Gibco BRL). In a GAL4-chimeric system, cells were cotransfected with GAL4-PPARα LBD, the GAL4-responsive firefly-luciferase reporter, and internal standard renilla-luciferase plasmids. Cells were treated with the indicated compounds for 24 h, 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).

2.9. Binding assay

Five micrograms of histidine-tagged human PPARα LBD protein [18] and 100 nM [^{3}H]KRP-297 (specific activity, 27 Ci/mmol) were incubated at 25 °C for 30 min in a buffer containing 50 mM Tris (pH7.4), 50 mM KCL, and 10 mM dithiothreitol. KCL was added to the reaction as indicated in Fig. 4. 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).

2.10. Analysis of PPARα mRNA expression by real time quantitative PCR

Total RNA was isolated from liver of male beagle dogs (2-year-old, Covance Research Products Inc.) fasted for 20 h and male ICR mice (9-week-old, Clea Japan Inc.) fasted for 20 h using TriZol reagent (Invitrogen) according to the manufacture's instructions. Male human liver total RNA (24-, 25-, 26-, 30-, and 64-year-old) was purchased from BioChain. Total RNA (1 μg) was reverse transcribed in 20 μl reaction using SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen) according to the manufacture's instructions. Quantitative gene expression analysis was performed on a SmartCycler (Takara Bio Inc.) using SYBR Green technology. PCR were performed using sense primer 5′-TCT TCC ACT GCT GCC AGT GC-3′ and antisense primer 5′-CAG CAT CCC GTC TTT ATT CAT C-3′ for dog PPARα, sense primer 5′-TCT TTC ACT GCT GCC AGT GC-3′ and antisense primer 5′-CAG CAT CCC GTC TTT GTT CAT C-3′ for human PPARα, and sense primer 5′-TCT TCC ACT GCT GCC AGT GC-3′ and antisense primer 5′-CAG CAT CCC GTC TTT GTT CAT C-3′ for mouse PPARα. In a PCR tube, 15 μl of SYBR Green master mix including 10 pmol sense and antisense primer was added to 10 μl of cDNA (corresponding to 100 ng of total RNA input). Subsequently, 40 PCR cycles consisting of 20 s at 94 °C, 20 s at 55 °C, and 30 s at 72 °C were applied. Full length of dog, human, and mouse PPARα cDNA fragments were used as standard to calculate the number of copies of PPARα mRNA.

2.11. Primary culture of hepatocytes

Dog and rat hepatocytes were isolated by collagenase perfusion of livers from male beagle dogs and Wistar rats, respectively. Hepatocytes were cultured in Dulbecco's

modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, dexamethasone, insulin, and antibiotics. After 4 h, hepatocytes were treated with the indicated compounds in DMEM supplemented with 10% fetal calf serum, dexamethasone, and antibiotics for 24 h, and total RNA was then prepared.

2.12. In vivo studies

Male Wistar rats (6-week-old, Charles River, Japan) and ICR mice (6-week-old, Clea Japan Inc.) were orally dosed for 7 days with KCL at various dosages; 0, 0.03, 0.3, 3, and 30 mg/kg ($n = 6$). Male beagle dogs (2-year-old, Covance Research Products Inc.) were orally dosed for 7 days with KCL at various dosages; 0, 0.03, and 0.3 mg/kg ($n = 3$). On the eighth day, plasma samples were collected. Triglyceride levels were measured by using Liquiteck TG II reagents (Roche Diagnostic). The statistical significance of differences from control animals was assessed by the Dunnett's multiple comparison test. $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Isolation of dog, rat, and human PPARα genes

To explore the species differences in response to PPARα activation by agonists, we cloned dog, rat, and human PPARα cDNAs. Dog PPARα cDNA (636–2032) was initially identified by the screening of a dog liver cDNA library with a human PPARα cDNA as a probe under high-stringency conditions. The entire primary structure and the amino acid sequence of dog PPARα were deduced by using the cDNA clone (636–2032) and other overlapping cDNA clones, (395–738), (1–662), (739–1647), (636–1790), (241–1647), and (107–1647), obtained from dog liver RNA by 5′RACE or RT-PCR (Fig. 1). The initiation codon is assigned to the first methionine residue found in a large open reading frame. Dog PPARα was found to be composed of 468 amino acids with a calculated molecular mass of 52,122, sharing 94.7 and 91.0% amino acid sequence identity with the human [28] and rat PPARα [24], respectively, and 64.6–68.6% identity with other PPAR subtypes. In the regions coding for the putative LBD, the amino acid sequence of dog PPARα was found to be 97.0 and 92.4% identical in human and rat PPARα, respectively. Because almost no information about dog PPARα has been available, we next evaluated the tissue distribution of dog PPARα by Northern blotting (Fig. 2A). Dog PPARα mRNA was approximately 7.9 kb and detected in the tissues possessing high catabolic activity such as skeletal muscle, kidney, liver, and heart. Additionally, we estimated expression levels of PPARα mRNA in dog, human, and mouse livers by real time quantitative PCR. As shown in Fig. 2B, expression levels of PPARα mRNA were markedly less in dog and human livers than in mouse livers.

3.2. Species difference in response to synthetic PPARα agonists

KCL is a novel compound to activate PPARα (Fig. 3) [31]. In this study, the binding affinity of KCL was measured by a competitive binding assay using [^{3}H]KRP-297 [18]. The displacement of [^{3}H]KRP-297 by KCL was concentration-dependent and IC_{50} value was 0.19 μM (Fig. 4), indicating that KCL has an ability to directly bind to human PPARα LBD.

To evaluate the agonistic activity of KCL, Wy-14,643, and fenofibric acid (Fig. 3) for dog, human, and rat PPARα, a transactivation assay using the full length of PPARα was carried out. Wy-14,643 is a known PPARα agonist that has been used widely as an experimental tool. Fenofibric acid is a pharmacologically active metabolite of fenofibrate that is clinically used as a hypolipidemic drug. As shown in Fig. 5A, KCL was found to be the most potent agonist for dog and human PPARα, but not for rat PPARα. EC_{50} values of KCL for dog, human, and rat PPARα were 0.007, 0.003, and 11.49 μM, respectively. In contrast, Wy-14,643 was found to be the most potent agonist for rat PPARα among the tested compounds, but its agonistic activity was reduced for dog and human PPARα. These data suggest that KCL is the human- and dog-selective and that Wy-14,643 is rat-selective in agonistic activity for PPARα, and the KCL and Wy-14,643 have species differences in response to PPARα activation, whereas fenofibric acid is species-independent.

To clarify whether the LBD of PPARα is the critical region causing species differences, we next performed a transactivation assay using GAL4-PPARα LBDs (Fig. 5B). KCL potently activated dog and human PPARα, while the agonistic activity of KCL for rat PPARα was profoundly less effective. EC_{50} values of KCL for dog, human, and rat PPARα were 0.48, 0.1, and 10.01 μM, respectively. Wy-14,643 was found to be the most effective agonist for rat PPARα among the test compounds, but a weak agonist for dog and human PPARα. These data are consistent with the results shown in Fig. 5A, suggesting that differences in amino acids in the LBD of PPARα contribute to species differences in response to PPARα activation by KCL and Wy-14,643. In contrast, there were no species differences observed in the agonistic activity of fenofibric acid in transactivation assays using a GAL4-PPARα LBD as well as the full length of PPARα, suggesting that the interaction mode of weak agonists such as fenofibric acid with PPARα LBD is different from those of KCL and Wy-14,643.

3.3. Identification of amino acids involved in species differences

Fig. 6A shows the alignment of PPARα LBDs among dog, human, and rat. To specify the region involved in a

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GCAGGTTTCCCATTGCCGCTCAAGTAGGGTCACCGTGATTTCCTCTTCCAAGGTGGTGGAGAGTTGGGGAGAATCCAGAGGACATATCGT      90

AACCGTTTCCTCCTTTACCTCCGAACTGCCAAGGCTGCAGGGAAGGGACCGGCCCTGCCTCGGCCTGGGCTCCCGGTGAGGCGAGCTCAG     180

AGGTCGGCCGGGCCGCCACAGGTTGGGGCTCAGCTACATTTCCAGAACCATCCAGTCAAGATGGTAGACACAGAAAGCCCGATTTGCCCC     270
                                                          M  V  D  T  E  S  P  I  C  P       10

CTCTCCCCCCTTGAGGCTGATGACCTGGAAAGCCCGTTATCTGAAGAATTTCTACAAGAAATGGGAAACATCCAAGAGATTTCTCAGTCC     360
L  S  P  L  E  A  D  D  L  E  S  P  L  S  E  E  F  L  Q  E  M  G  N  I  Q  E  I  S  Q  S       40

ATTGGTGAGGATAGTTCTGGAAGTTTTAGTTTTACAGAATACCAGTATTTAGGAAGTGGCCCGGGATCAGATGGATCTGTTATCACAGAC     450
I  G  E  D  S  S  G  S  F  S  F  T  E  Y  Q  Y  L  G  S  G  P  G  S  D  G  S  V  I  T  D       70

ACGCTCTCACCAGCTCCCAGCCCCTCGTCGGTCACGCACCCGGCGGCTCCCGGTGGTGCCGAGGAACCTTCCAGCGTAGCCTTGAACATT     540
T  L  S  P  A  P  S  P  S  S  V  T  H  P  A  A  P  G  G  A  E  E  P  S  S  V  A  L  N  I      100

GAATGCAGAATCTGCGGGGACAGAGCCTCGGGCTACCATTACGGAGTCCACGCGTGCGAAGGCTGCAAGGGTTTCTTCCGGCGAACCATC     630
E  C  R  I  C  G  D  R  A  S  G  Y  H  Y  G  V  H  A  C  E  G  C  K  G  F  F  R  R  T  I      130

CGGCTGAAGCTGGCCTATGACAAATGTGACCGCAGCTGCAAAATTCAGAAAAAGAACCGGAATAAGTGCCAATATTGTCGTTTCCACAAG     720
R  L  K  L  A  Y  D  K  C  D  R  S  C  K  I  Q  K  K  N  R  N  K  C  Q  Y  C  R  F  H  K      160

TGCCTTTCGGTCGGGATGTCCCATAATGCCATCCGCTTTGGAAGAATGCCACGATCAGAAAAAGCAAAACTGAAAGCGGAAATTCTCACC     810
C  L  S  V  G  M  S  H  N  A  I  R  F  G  R  M  P  R  S  E  K  A  K  L  K  A  E  I  L  T      190

TGTGAACAGGACCCAGAAGACGCAGAGACAGCAGATCTCAAGTCCCTGGCCAAGAGAATTTACGAGGCCTACCTCAAGAACTTCAACATG     900
C  E  Q  D  P  E  D  A  E  T  A  D  L  K  S  L  A  K  R  I  Y  E  A  Y  L  K  N  F  N  M      220

AACAAAGTCAAAGCCCGGGTCATCCTCGCAGGGAAGGCCAGCAACAACCCGCCCTTTGTCATACATGACATGGAGACGTTGTGCATGGCT     990
N  K  V  K  A  R  V  I  L  A  G  K  A  S  N  N  P  P  F  V  I  H  D  M  E  T  L  C  M  A      250

GAGAAGACTCTGGTGGCCAAGCTGGTGGCCAATGGCATCCAGAACAAGGAGGCAGAGGTGCGCATCTTCCACTGCTGCCAGTGCACATCT    1080
E  K  T  L  V  A  K  L  V  A  N  G  I  Q  N  K  E  A  E  V  R  I  F  H  C  C  Q  C  T  S      280

GTGGAGACCGTCACGGAGCTCACGGAGTTCGCCAAGTCCATCCCTGGCTTCGCGAACTTGGACTTGAATGATCAGGTCACTCTACTGAAA    1170
V  E  T  V  T  E  L  T  E  F  A  K  S  I  P  G  F  A  N  L  D  L  N  D  Q  V  T  L  L  K      310

TATGGAGTGTATGAGGCCATATTTGCGATGCTGTCTTCTGTGATGAATAAAGACGGGATGCTGGTAGCCTATGGAAATGGCTTTATAACT    1260
Y  G  V  Y  E  A  I  F  A  M  L  S  S  V  M  N  K  D  G  M  L  V  A  Y  G  N  G  F  I  T      340

CGTGAGTTCCTAAAGAGCCTAAGGAAACCGTTCTGTGATATCATGGAACCAAAGTTTGATTTTGCGATGAAGTTCAATGCACTGGAGCTA    1350
R  E  F  L  K  S  L  R  K  P  F  C  D  I  M  E  P  K  F  D  F  A  M  K  F  N  A  L  E  L      370

GATGACAGCGACATCTCCCTTTTTGTGGCTGCTATCATTTGCTGTGGAGATCGCCCCGGCCTTCTAAACGTGGGACACATTGAAAAAATG    1440
D  D  S  D  I  S  L  F  V  A  A  I  I  C  C  G  D  R  P  G  L  L  N  V  G  H  I  E  K  M      400

CAGGAGGGCATTGTGCATGTGCTCAAACTTCACCTGCAGACCAACCACCCGGACAACATCTTTCTCTTCCCAAAACTCCTTCAAAAAATG    1530
Q  E  G  I  V  H  V  L  K  L  H  L  Q  T  N  H  P  D  N  I  F  L  F  P  K  L  L  Q  K  M      430

GCAGACCTCCGGCAGCTGGTCACCGAGCACGCACAGCTGGTGCAGGTCATCAAAAAGACGGAGTCGGACGCAGCCCTGCACCCCCTGCTG    1620
A  D  L  R  Q  L  V  T  E  H  A  Q  L  V  Q  V  I  K  K  T  E  S  D  A  A  L  H  P  L  L      460

CAGGAAATCTACAGGGACATGTACTGACGGCTTGAGAGCGAACCGCACGACAAGCAGCTCTTCGAGGACTTTTAAAGTCGACAGCACTAC    1710
Q  E  I  Y  R  D  M  Y  *                                                                      468

AAATGAAAACTGGGAGCAATGCCTGCTTTGCACAAATTCCACCATTTTCACCTTAGAGAATGGGACGGATGAGCCCTAGGTAACCGGAAA    1800

CCTAGACCACATTTCTTTCTTTTTCACATCAGTACTTGTAAGAGCGTGCCAAGTGTCGAAACGCCGGTGACAGGTAGCTGATAGGCAGTT    1890

CTTGGAAAATTAAGGGGAATGATGCCCAGCAGTCTGATTTTAACTCACTTGATGTTAATCCATGCAGATTTCTTTATATCACATTAGCCG    1980

TGTAGCCTTGAATTAACTGGTGACCTTGAAAAAAAAAAAAAAAAAAAAAAA                                           2032
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Fig. 1. Nucleotide and predicted amino acid sequences of dog PPARα cDNA. Uppercase letters indicate the nucleotide sequences. The initiation codon ATG (underlining) and the stop codon TGA (*) are shown. The predicted amino acid sequences are shown below the nucleotide sequence. Nucleotide and amino acid sequences have been submitted to GenBank (accession number AF350327).

species-specific response to agonists between dog and rat PPARα, transactivation assays using several chimeric PPARα LBDs among species (Fig. 6B) were performed. Changing the upstream region from the *Sac*I recognition site, including helix 3 of dog PPARα LBD, to those derived from rat PPARα (rat/dog) diminished the agonistic activity of KCL (Fig. 7A) and increased the agonistic activity of Wy-14,643 (Fig. 7B). In contrast, changing the upstream

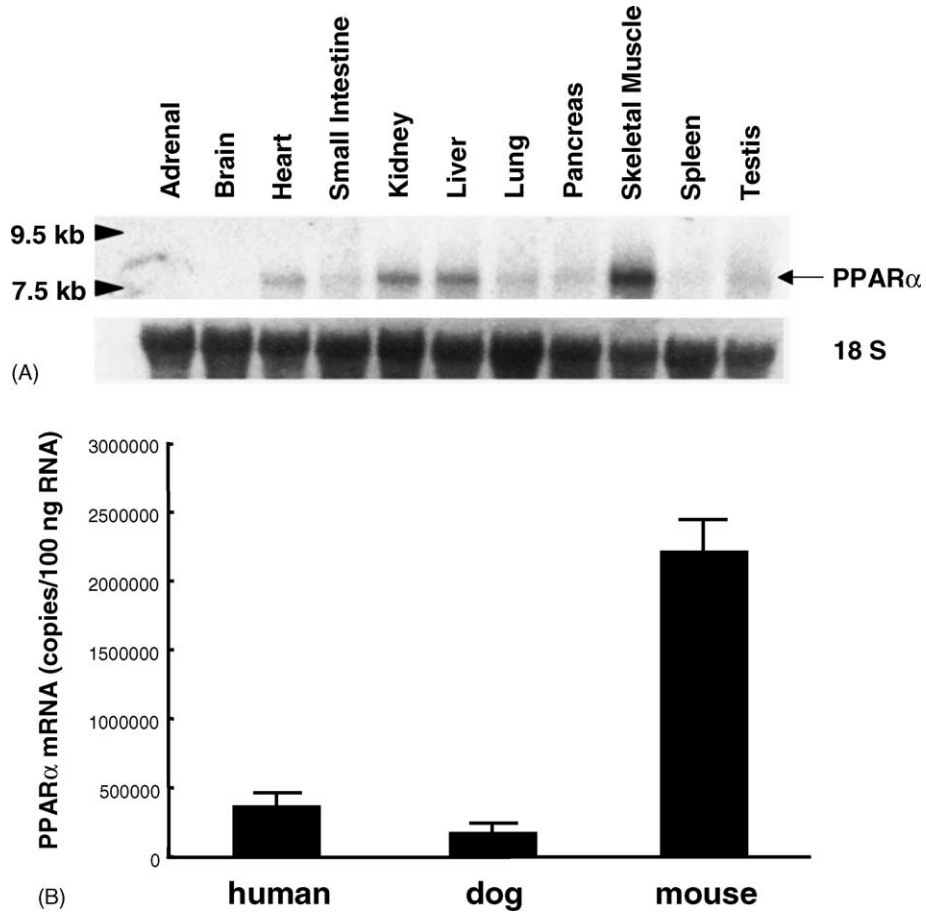

Fig. 2. (A) Expression of PPARα mRNA in dogs. Total RNA from the indicated tissues was equally loaded on the gel, and the mRNA levels were then detected by Northern blotting. Similar results were obtained from independent experiments. (B) Expression of PPARα mRNA in dog ($n = 3$), human ($n = 5$), and mouse ($n = 5$) liver as determined by real time quantitative PCR. The mRNA values are expressed by the number of copies calculated from full length of PPARα cDNA fragments. Data are presented as the means ± S.E.M.

region in rat PPARα LBD to those derived from dog PPARα (dog/rat) increased the agonistic activity of KCL (Fig. 7A) and reduced the agonistic activity of Wy-14,643 (Fig. 7B). Similar results were obtained from a transactivation assay using chimeric PPARα LBDs between humans and rats (Fig. 7C and D), indicating that the upstream region from the *Sac*I recognition site, including helix 3, involved in species differences.

Amino acids in helix 3 of PPARαLBD between dogs and humans were found to be completely identical (Fig. 6A).

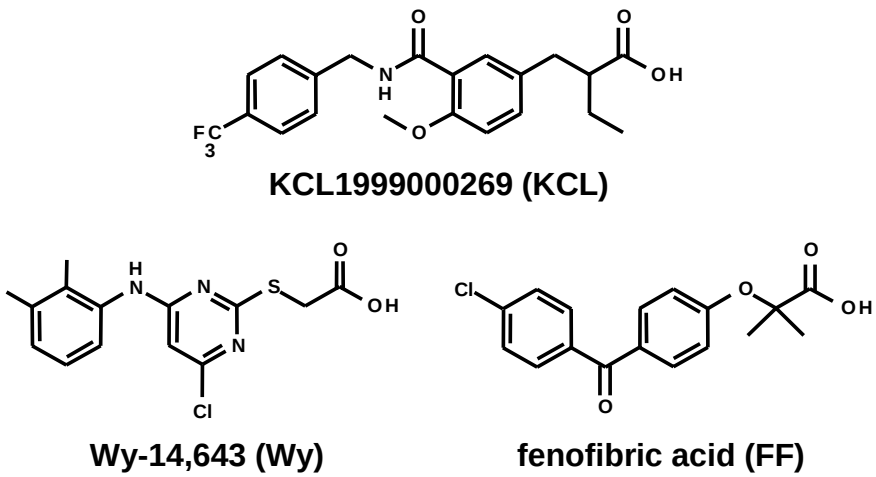

Fig. 3. Chemical structure of PPARα agonists.

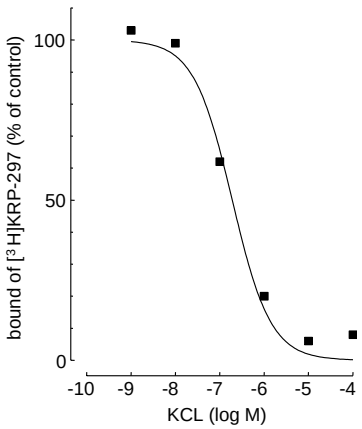

Fig. 4. Dose–response curve of displacement of [^{3}H]KRP-297 binding to human PPARα by KCL. Competitive binding assays using the purified LBD protein of human PPARα and 100 nM [^{3}H]KRP-297 were performed in the presence of various concentrations of KCL. One hundred percent binding indicates the total binding of [^{3}H]KRP-297 in the absence of competitors. Data represent the mean of duplicate points.

There are differences in helix 3 of PPARα LBD between rats and humans at the amino acids 272 and 279. The 272-amino acid is isoleucine (I) in dog and human PPARα, but phenylalanine (F) in rat PPARα. The 279-amino acid is threonine (T) in dog and human PPARα, but methionine (M) in rat PPARα. It has been suggested that these two amino acids contribute to species differences in PPARα-dependent transcriptional responses by agonists between rodents and humans [35]. To confirm the important role of these amino acids in species differences, we constructed point-mutants I272F (I–F at position 272) and T279M (T–M at position 279) of PPARα LBD in dog and human, and carried out a transactivation assay. In dog (Fig. 8A and B) and human (Fig. 8C and D) PPARα, the agonistic activity of KCL was completely diminished in I272F, and was slightly decreased in T279M. Agonistic activity of Wy-14,643 for dog and human PPARα was increased in T279M, but not in I272F.

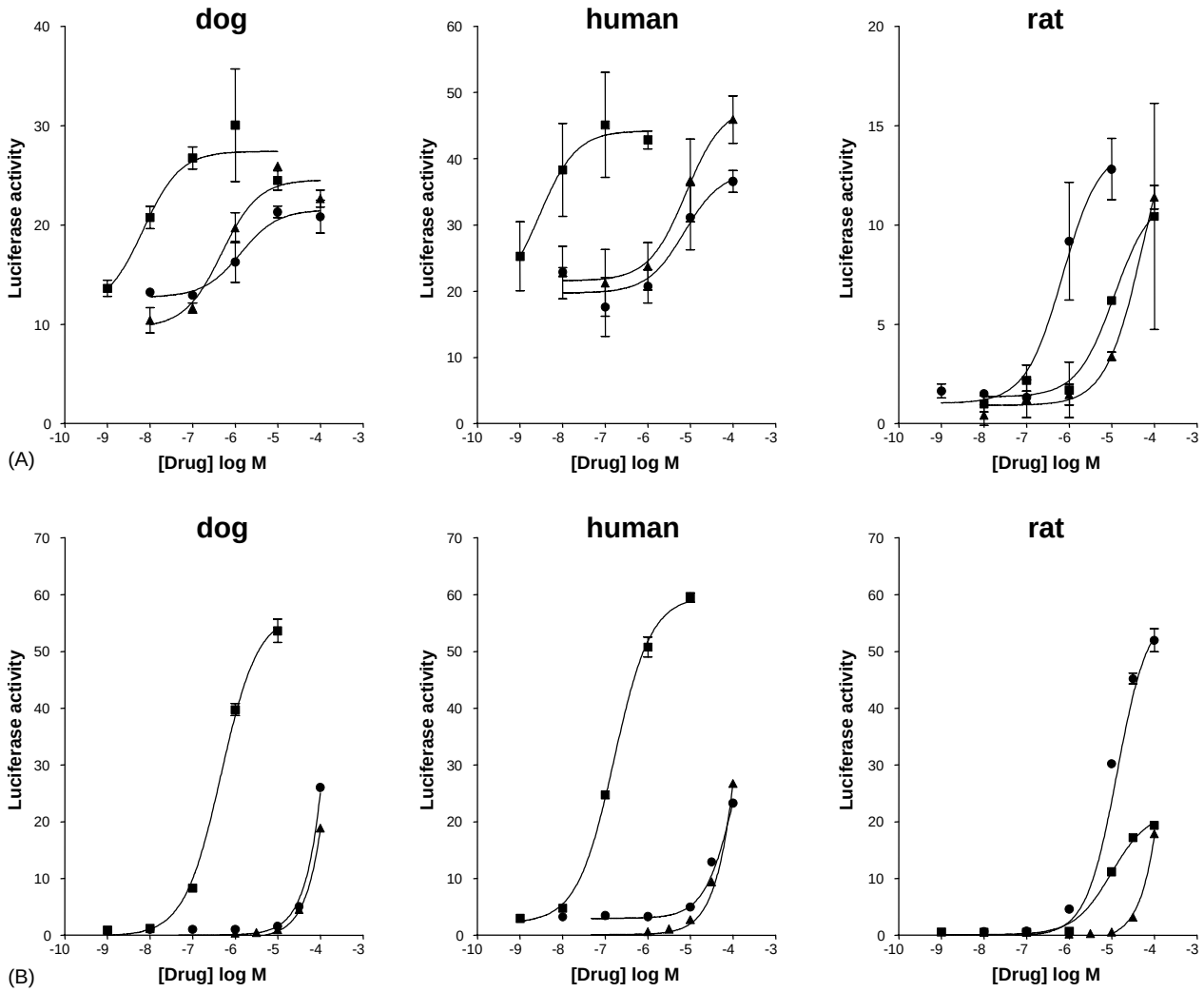

Fig. 5. Transactivation assay using full length or LBD of PPARα in dogs, rats, and humans. CHO-K1 cells were transfected with expression plasmids of the full length of PPARα and reporter plasmids containing PPRE of rat AOX (A) or expression plasmids of GAL4-chimeric PPARα LBDs and its reporter (B). Transfected cells were treated with various concentrations of KCL (■), Wy-14,643 (●), and fenofibric acid (▲) for 24 h. Data are presented as the means ± S.E.M. of three independent experiments.

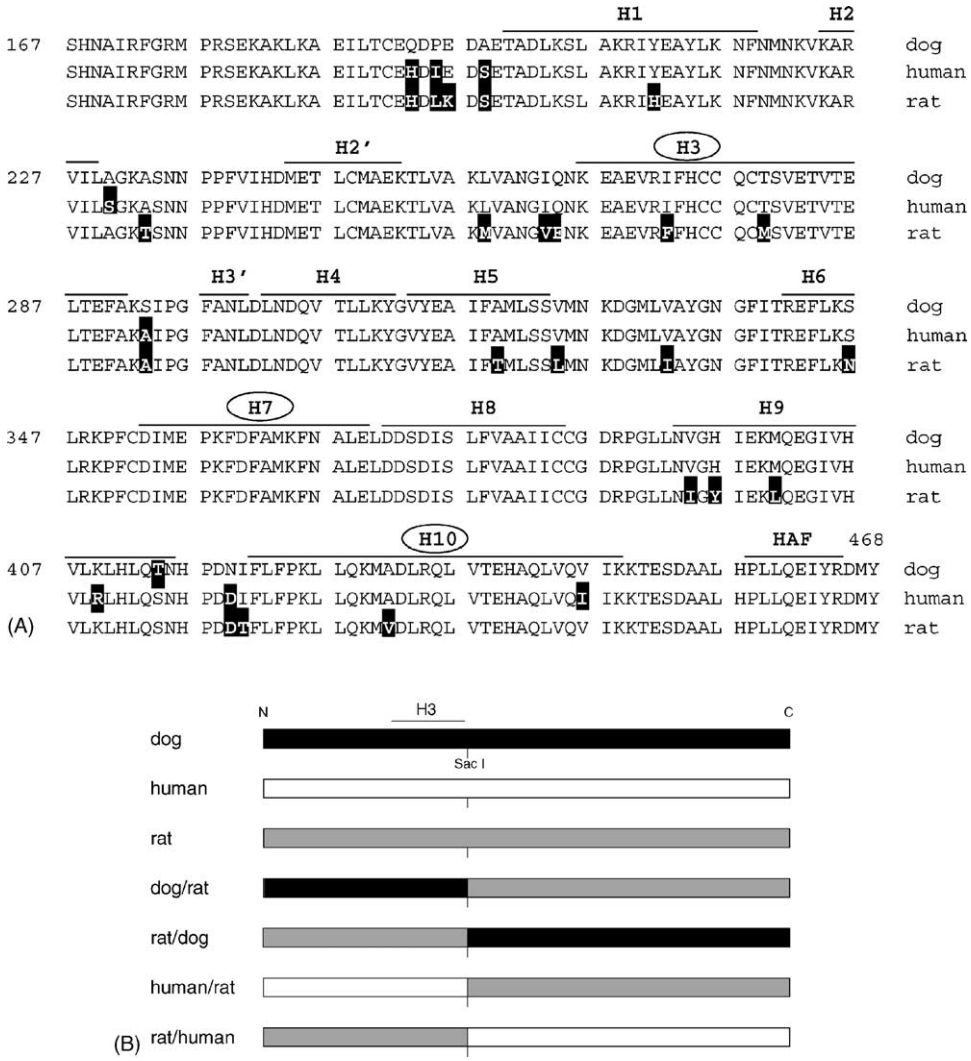

Fig. 6. (A) Alignment of deduced amino acid sequences of PPARα LBD among dogs, humans, and rats. Different amino acids among the three species are shown in boxes. Putative amino acids for the α-helices are underlined. (B) Schematic diagram of chimeric PPARα LBD. Helix 3 is underlined, and *Sac*I recognition sites are indicated.

3.4. KCL induces PPARα-regulated gene expression in dog hepatocytes

Mitochondrial HMG-CoA synthase is one of the PPARα-regulated genes used as a marker for evaluation of PPARα-dependent gene expression [33]. We next investigated the effects of KCL and WY-14,643 on mRNA expression levels of mitochondrial HMG-CoA synthase in dog and rat hepatocytes. Dog and rat primary hepatocytes were treated with KCL, Wy-14,643, and fenofibric acid, and mRNA levels of mitochondrial HMG-CoA synthase were measured by Northern blotting. In dog primary hepatocytes, KCL most potently increased the levels of HMG-CoA synthase mRNA among the tested agonists (Fig. 9A). Wy-14,643 and fenofibric acid slightly increased HMG-CoA synthase mRNA in dog primary hepatocytes only at 10^{-4} M. Unlike dog primary hepatocytes, Wy-14,643 was more potent than KCL and fenofibric acid in increasing levels of HMG-CoA synthase mRNA in rat primary hepatocytes. Similar results were obtained from the study on AOX as another PPARα-regulated gene (Fig. 9B).

3.5. KCL is a potent hypolipidemic agent in dogs

Because we have found that KCL is the potent agonist in induction of dog PPARα activation and PPARα-dependent gene expression in dog hepatocytes, we attempted to determine whether KCL is a potent lipid-lowering agent in dogs. When administered orally to beagle dogs, rats and mice once daily for 7 days, KCL lowered plasma triglyceride levels in those animals in a dose-dependent manner (Fig. 10). Oral administration of KCL to dogs (0.03 mg/kg per day), rats (3 mg/kg per day), and mice (3 mg/kg per day) decreased plasma triglyceride levels by 40–60%. As expected from results on transactivation assays, the effective dose of KCL in dogs was at least 100-fold more potent than those in rodents (Fig. 10).

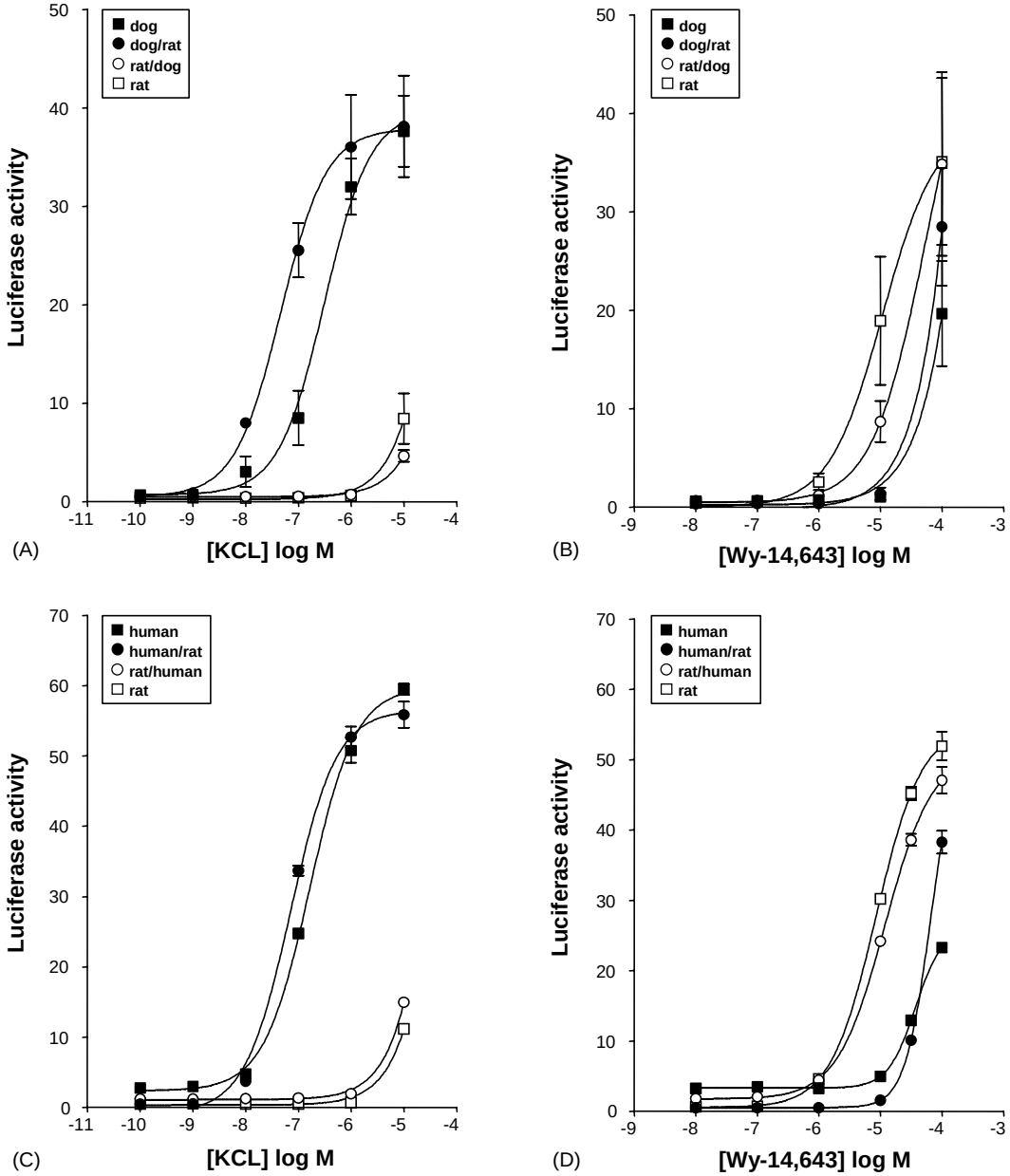

Fig. 7. Transactivation assay using chimeric PPARα LBD. (A and B) CHO-K1 cells were transfected with expression plasmids of chimeric PPARα LBD of dog (■), dog/rat (●), rat/dog (○), and rat (□). (C and D) Cells were transfected with expression plasmids of chimeric PPARα LBD of human (■), human/rat (●), rat/human (○), and rat (□). Data are presented as the means ± S.E.M. of three independent experiments.

4. Discussion

We initially performed cDNA cloning of dog PPARα and found that dog PPARα shares 94.7 and 91.0% amino acid sequence identity with human and rat PPARα, respectively, and that, especially in the LBD, dog PPARα is 97.0 and 92.4% identical in human and rat PPARα, respectively. These data indicate that dog PPARα is more similar to human PPARα than rodent PPARα.

In a transactivation assay, we showed that KCL potently activates dog and human PPARα, but not rat PPARα. Wy-14,643 more potently activated rat PPARα than dog and human PPARα. These results indicate that KCL is a human- and dog-specific PPARα agonist, while Wy-14,643 is a rat-specific PPARα agonist. Binding ability of KCL to human PPARα LBD was tested by a binding assay using recombinant human PPARα LBD proteins and radiolabeled synthetic PPARα agonist, KRP-297. In addition, we showed that KCL more potently induces typical PPARα-upregulated genes such as HMG-CoA synthase and AOX in dog hepatocytes, compared to Wy-14,643 and fenofibric acid. These findings suggest that KCL acts as a potent PPARα agonist in dogs.

A point-mutation study showed that the agonistic activities of KCL and Wy-14,643 are primarily affected by the 272- and 279-amino acids, respectively, in helix 3 of

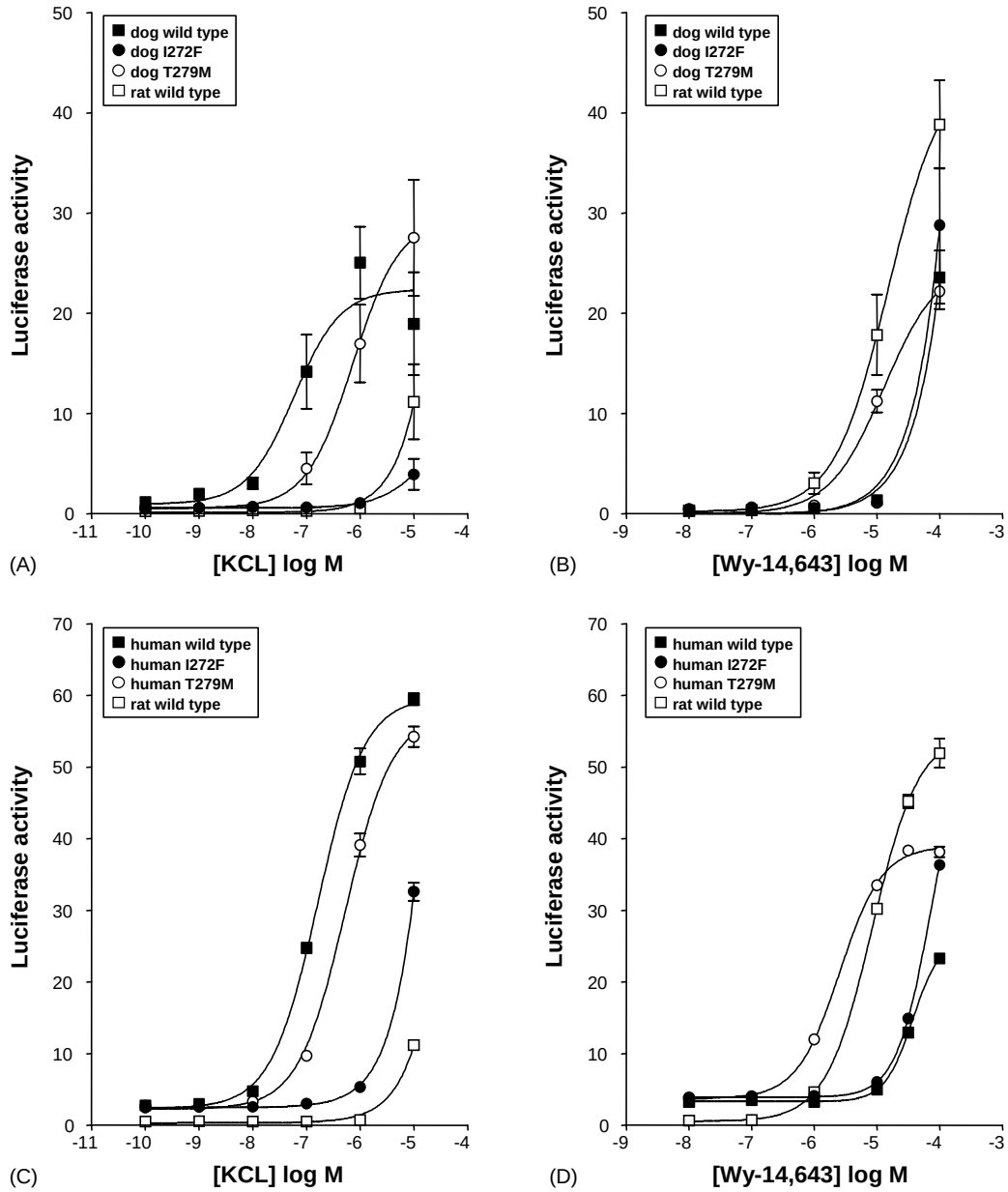

Fig. 8. Transactivation assay using point mutants of PPARα LBD. (A and B) CHO-K1 cells were transfected with expression plasmids of dog PPARα LBD wild type (■), I272F (●), T279M (○), and rat PPARα LBD wild type (□). (C and D) CHO-K1 cells were transfected with expression plasmids of human PPARα LBD wild type (■), I272F (●), T279M (○), and rat PPARα LBD wild type (□). Data are presented as the means ± S.E.M. of three independent experiments.

PPARα LBD. It has been reported that agonists interact with regions of helices 3, 7, and 10 in the LBDs of PPARγ and PPARδ based on X-ray crystal structure analysis [36,37]. X-ray crystal structures of PPARα have revealed that the agonist-binding site is located in the central core of PPARαLBD, flanked by helices 3, 5, 7, and 12 [38]. We showed that fenofibrate had no species difference in its PPARα response, suggesting that the interaction mode of weaker agonists such as fenofibrate with PPARα may be different from those of the potent agonists KCL and Wy-14,643. The interaction with helix 3 of PPARα may be needed to produce high-affinity agonists.

Finally, we found that KCL reduces plasma triglyceride levels >100-fold more potently in dogs than in rats, which is consistent with the observations from transactivation assays. Our data showed the high expression of dog PPARα in lipid-catabolic tissues such as skeletal muscle, kidney, liver, and heart (Fig. 2A), as previously described in humans [6,39] and rodents [5]. Hepatic activation is known to be primarily responsible for the triglyceride-lowering effects of PPARα agonists [9–14]. However, the relative expression levels of PPARα in dog tissues were found to be the highest in skeletal muscle, unlike rodents exhibit the highest expression of PPARα in liver [5]. The

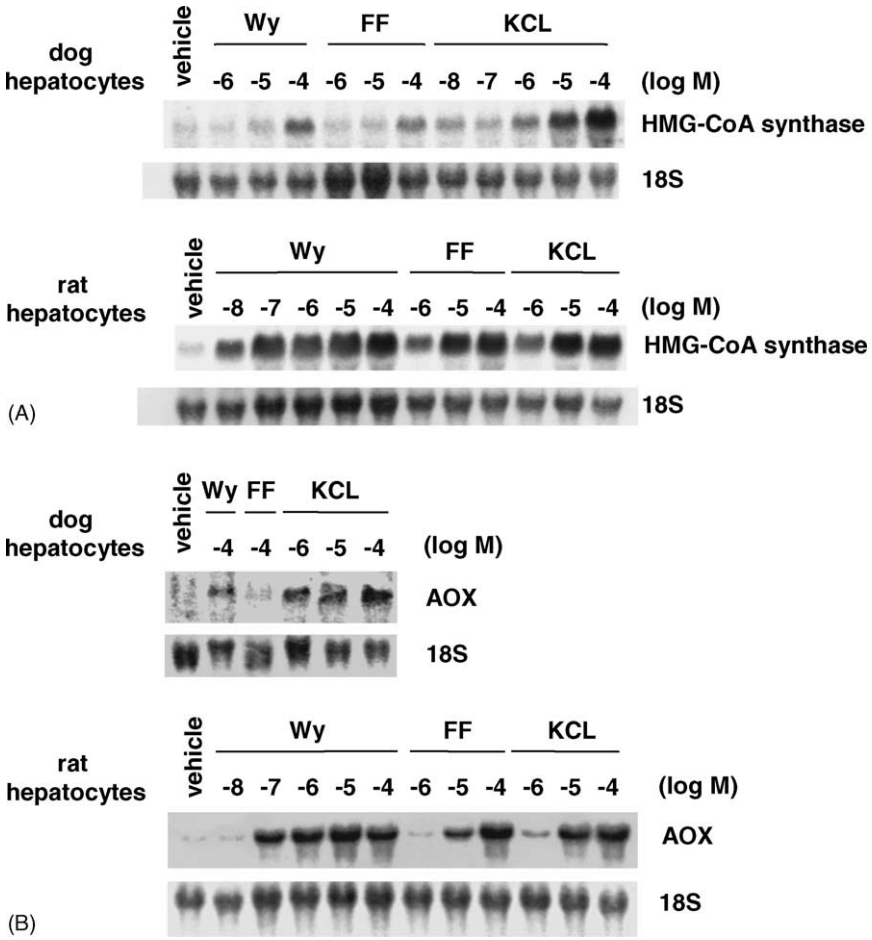

Fig. 9. Induction of HMG-CoA synthase mRNA and AOX mRNA by PPARα agonists in primary hepatocytes. Dog and rat hepatocytes were treated with indicated concentrations of WY-14,643 (Wy), fenofibric acid (FF), or KCL for 24 h. HMG-CoA synthase mRNA (A) and AOX mRNA (B) were measured by Northern blotting.

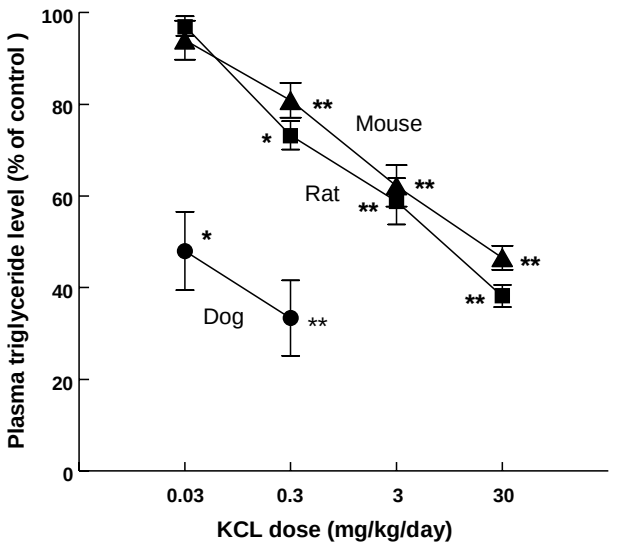

Fig. 10. Lowering action of KCL on plasma triglyceride levels in dogs, rats, and mice. KCL was administered orally once a day to dogs ($n = 3$), rats ($n = 6$), or mice ($n = 6$). Data are presented as the means ± S.E.M. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. vehicle-treated control group. Pretreatment levels of triglyceride in dogs, rats, and mice were 42 ± 4 mg/dl, 81 ± 2 mg/dl, and 61 ± 1 mg/dl, respectively.

expression pattern in dogs was similar to that in humans [6,39]. Moreover, PPARα mRNA level in dog liver was markedly lower than that in rodent liver (Fig. 2B). Taken together, there is the possibility that PPARα activation in other tissues such as skeletal muscles than in liver may responsible for triglyceride-lowering effect of KCL in dogs.

In conclusion, apparent species differences were observed between human/dog and rat PPARα in a trans-activation assay, gene expression in hepatocytes, and decreases in plasma triglyceride levels in response to a novel agonist, KCL. We here show that KCL is a potent human- and dog-PPARα agonist that has lipid-lowering ability in vivo. Our results suggest that rodents are unsuitable models for the assessment of potent agonists for human PPARα. We found, however, that dog PPARα is conserved to the human type in key amino acids involved in species differences, and that the pharmacological responses of dog PPARα are similar to those of human PPARα. These data suggest that the dog might be a useful animal for bettering our understanding of the benefits and risks in the human treatment of PPARα agonists.

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