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Influence of Constitutive Androstane Receptor Activity on the Effects of Pioglitazone on Non-alcoholic Fatty Liver Disease

Constitutive Androstane Receptor의 활성도가 Pioglitazone에 의한 비알콜성지방간 변화에 미치는 영향

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안 화 영

A thesis of the Degree of Doctor of Philosophy

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The Department of Medicine (Molecular and Genomics),

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by
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A thesis submitted to the Department of Medicine in partial fulfillment of the requirements for the Degree of Doctor of Philosophy in Medical Science (Molecular and Genomics) at Seoul National University College of Medicine

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Approved by Thesis Committee:

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Professor _____Vice chairman

Professor _____

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Professor _____

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ABSTRACT

Influence of Constitutive Androstane Receptor Activity on the Effects of Pioglitazone on Non-alcoholic Fatty Liver Disease

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Major in Molecular and Genomics

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INTRODUCTION: We examined whether the effects of pioglitazone on metabolic parameters are different according to the activities of constitutive androstane receptor (CAR) in diet induced obesity mice. Also, we investigated the interaction between pioglitazone/peroxisome proliferator-activated receptor γ (PPAR γ) and CAR.

RESEARCH DESIGN AND METHODS: Three mg/kg of TCPOBOP were injected weekly for CAR activation, and $CAR^{-/-}$ mice were used for CAR depletion. Mice with different CAR activities were subsequently divided into two groups for pioglitazone treatment. To make a diet induced obesity model, high fat diet was supplied to the high fat diet group. We checked body weight changes every week. After 12 weeks, glucose tolerance test was performed and the serum levels of cholesterol and pioglitazone and liver histology were

examined. Fatty acid oxidation rate was measured using [^{14}C]palmitate. Gene expressions related to lipid metabolism were analyzed using real time PCR.

RESULTS: Pioglitazone-treated CAR$^{-/-}$ mice showed less weight gain, comparable improvement of glucose tolerance, and significant improvement of non-alcoholic fatty liver disease (NAFLD) compared with untreated mice. In TCPOBOP treated mice, there was no significant difference by pioglitazone treatment due to the strong effect of TCPOBOP. We identified that activation of CAR by high concentration of pioglitazone was not related to the improvement of NAFLD in CAR$^{-/-}$ mice. Among genes related to lipid metabolism, the expressions of CD36 and SCD-1 were decreased in pioglitazone-treated CAR$^{-/-}$ mice compared with those of pioglitazone-treated CAR$^{+/+}$ mice. These changes might have been regulated by decreased PPARγ2 expression.

CONCLUSIONS: The metabolism of pioglitazone affected by CAR activity was independent of the serum pioglitazone concentration. Decreased PPARγ2 expression by pioglitazone in CAR deleted state might play a role to improve NAFLD possibly through regulating the expressions of CD36 and SCD-1 transiently.

Keywords: non-alcoholic fatty liver disease, thiazolidinedione, drug metabolism, PPARγ

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LIST OF ABBREVIATIONS

CAR	constitutive androstane receptor
CITCO	6-(4-chlorophenyl) imidazo[2,1-b][1,3]thiazole-5-carbaldehyde O-(3,4-dichlorobenzyl) oxime
CPT-1a	Carnitine palmitoyltransferase 1α
CYP	cytochrome P450
DR	direct repeat
ER	everted repeat
FAS	fatty acid synthase
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GRIP-1	Glutamate receptor-interacting protein 1
HDL	High density lipoprotein
LBD	Ligand binding domain
LCAD	Long chain acyl-CoA dehydrogenase
LDL	Low density lipoprotein
MTTP	Microsomal triglyceride transfer protein
NEFA	Non-esterified fatty acid
PGC-1a	Peroxisome proliferator-activated receptor gamma, coactivator 1 alpha
PPARγ	peroxisome proliferator-activated receptor γ
RXR	retinoid X receptor
SCD-1	stearoyl-CoA desaturase
SRC-1	steroid receptor coactivator-1
SREBP-1c	Sterol regulatory element-binding protein-1c
TCPOBOP	1,4-bis[2-(3,5-dichloropyridyloxy)]benzene
TZD	thiazolidinedione
WT	wild type

INTRODUCTION

1. Metabolic regulation by thiazolidinediones

Thiazolidinediones (TZDs) are insulin sensitizing agents that are used to treat patients with type 2 diabetes. Rosiglitazone and pioglitazone are currently available TZDs in clinical use. TZDs act by binding to the peroxisome proliferator-activated receptor gamma (PPARγ), a member of the nuclear receptor superfamily that has a key role in glucose regulation and lipid metabolism (1). Chronic treatment with TZDs improves glucose homeostasis by increasing insulin sensitivity (2, 3). However, diabetic patients treated with TZDs have increased subcutaneous adipose tissue and decreased visceral adipose tissue and hepatic fat content (4, 5). These phenomena were explained by the insulin-sensitizing effects of TZDs. TZDs have been known to induce the differentiation of mesenchymal and preadipocytic precursors in adipose tissue responsible for the shift in lipids from large adipocytes into new small and more insulin-sensitive adipocytes (6). Small adipocytes may be more efficient for storing lipids, and body weight can increase as a result. Therefore, TZDs induce an overall redistribution of fat away from the central compartment to the periphery (7). This process might also reduce the production of harmful adipocytokines such as tumor necrosis factor-α (8) and increase plasma level of beneficial adipocytokines such as adiponectin (9). For lipid profile, TZDs decrease non-esterified fatty acids (NEFA) and

increase HDL cholesterol and LDL cholesterol particle size. Moreover, pioglitazone decrease triglyceride concentration due to its partial agonistic action on PPARα receptors (10).

2. Various responses of individual patients to the pioglitazone treatment

It has been recognized that some patients respond to TZDs (termed responders), whereas others do not (termed non-responders). In previous studies, some clinical parameters which predict the efficacy of TZDs were examined. Kuzuya et al. reported that the glucose lowering effect of troglitazone was related to female gender, BMI, age, and pretreatment fasting plasma glucose level (11). Tajiri et al. reported that age, fasting plasma glucose level and percentage of female gender of pioglitazone responder group was significantly higher than pioglitazone non-responder group (12). However, there has been no study investigating the cause of these differences between responder and non-responder for TZDs.

3. Metabolism of pioglitazone

Pioglitazone is extensively metabolized by hydroxylation and oxidation in the liver to form at least four primary metabolites (designated M-I, M-II, M-IV and M-V) and two secondary metabolites (M-III and M-IV) (13). The

pharmacologically active M-IV and M-III are the main metabolites found in human serum. Therefore, effect of pioglitazone is also variable according to the degree of drug metabolism like other drugs. Pioglitazone are metabolized by multiple cytochrome P450 (CYP) enzymes, mainly by CYP2C8, CYP3A4 and CYP2C9 (13, 14), and they are regulated by xenobiotic receptor CAR and PXR (15).

4. Changes of drug metabolism according to CAR activity

A xenobiotic nuclear receptor, constitutive androstane receptor (CAR) (NR1I3) is abundantly expressed in the liver and small intestine (16). CAR was initially found as a xenoreceptor regulating toxic compounds or drug metabolism by inducing expression of microsomal cytochrome P450 (CYP) (17, 18), UDP-glucuronosyltransferase (19). Like most other nuclear receptors, CAR has an N-terminal DNA binding domain and a C-terminal ligand-binding domain (LBD). CAR is present in the cytoplasm in the liver, and agonists elicit nuclear translocation of CAR, which is essential for the activation of CAR target genes (20). In the nucleus, CAR interacts with its heterodimer partner, retinoid X receptor (RXR), binds to the direct repeat 3 (DR3), DR4, everted repeat 6 (ER6), ER7, or ER8 motifs in the regulatory region of target genes, and activates their transcription (21, 22). Ligand-independent nuclear localization mediated by interaction with the coactivator

GRIP-1 has also been observed (23). Phenobarbital and 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP), 6-(4-chlorophenyl) imidazo[2,1-b][1,3]thiazole-5-carbaldehyde O-(3,4-dichlorobenzyl) oxime (CITCO) promote the transcriptional activity of CAR by further enhancing its interaction with coactivators such as GRIP-1, SRC-1, and PGC-1α (24). Conversely, inverse agonists of CAR, such as androstanol, inhibits the constitutive activity of CAR (25).

Activation of CAR coordinately stimulates the expression of many genes, including CYP2C8, CYP3A4 and CYP2C9, by involved in the metabolism and excretion of xenobiotics from the body. In contrast, loss of CAR function in mice resulted in the complete absence of Cyp2b10 induction, which was representative target gene of CAR, in the liver and decreased drug metabolizing capabilities (16).

The defense against toxicity, by increasing expression of xenobiotic metabolizing enzymes, may occasionally promote drug toxicity if the metabolite produced is more reactive than the parent compound. Indeed, CAR was identified as a key regulator of acetaminophen metabolism and hepatotoxicity (26). High levels of acetaminophen activate CAR, resulting in induction of at least three CYP enzymes involved in generating the more toxic acetaminophen metabolite, N-acetyl parabenzoquinoneimine (NAPQI). In normal mice, activation of CAR by high doses of acetaminophen also increases production of the enzyme primarily responsible for NAPQI conjugation, GSTPi. In the presence of elevated NAPQI levels, GSTPi

induction is deleterious rather than protective because it increases the rate of glutathione depletion. In CAR null animals, the same doses of acetaminophen do not increase production of CYP or GSTPi enzymes and the animals are relatively resistant to acetaminophen toxicity. Consistent with this, inhibition of CAR activity by administration of androstanol blocked hepatotoxicity in wild type but not in CAR null mice. Remarkably, this effect could be observed in animals treated with the inverse agonist one hour after administration of acetaminophen, suggesting that such treatments could be therapeutically useful in some contexts. On the other hand, activation of CAR by PB or TCPOBOP increased the toxicity of acetaminophen (26). Similar to acetaminophen, pioglitazone is also metabolized by CYP (13). Therefore concentration and metabolism of pioglitazone will possibly be different according to CAR deletion and associated CYP activity.

5. Metabolic regulation of CAR

In addition to drug metabolism, CAR also regulates glucose and lipid metabolism. Gain of body weight was significantly inhibited by CAR agonist, TCPOBOP in high fat fed mice (27). Also treatment with TCPOBOP improved glucose tolerance and insulin sensitivity in both the high fat diet induced type 2 diabetes model and the *ob/ob* mice (27, 28).

Activation of CAR suppressed lipid metabolism and lowered serum triglyceride by reducing protein levels of the active form of the lipogenic

transcription factor SREBP-1 (sterol regulatory element-binding protein 1) (29). The inhibitory effect of CAR on lipid metabolism may also be attributed to induction of Insig-1, a protein with anti-lipogenic properties (30). Also CAR activation significantly suppressed the expression of peroxisome proliferator-activated receptors (PPAR) α and its target genes involved in β-oxidation and fatty acid influx, thus reducing incomplete fatty acid oxidation of high fat fed mice (27). For fatty liver, CAR activation dramatically improves fatty liver by inhibition of lipogenesis (27, 28).

6. Pathogenesis of non-alcoholic fatty liver disease (NAFLD) and the role of CAR and TZDs during steatosis

Fatty acids in the liver come from several different sources, derived from dietary fat, released from adipocytes via lipolysis and *de novo* hepatic lipogenesis. An imbalance of any of the pathways involved in triacylglycerol delivery, synthesis, export, or oxidation could contribute to its accumulation in the liver.

As high-fat diets have been shown to produce NAFLD in both animal (31, 32) and human (33) studies and low-fat/high-carbohydrate diets have been shown to increase *de novo* lipogenesis (34), dietary composition can have a major effect by altering the relative sources of liver triacylglycerol.

In addition to the direct effects of diet on hepatic fat accumulation, excess

dietary caloric intake can result in obesity and associated insulin resistance. Insulin resistance may then contribute to the development of NAFLD by impairing the ability of insulin to suppress lipolysis, leading to increased delivery of free fatty acids to the liver (35, 36). This hypothesis is supported by the fact that subjects with NAFLD have been shown to have elevated free fatty acids levels (37, 38) and impaired insulin suppression of lipolysis (37-39).

Increased *de novo* lipogenesis may also contribute to hepatic fat accumulation. Hyperinsulinemia by insulin resistance in subjects with NAFLD increased *de novo* lipogenesis, as insulin stimulates lipogenic enzymes via sterol receptor binding protein-1c (SREBP-1c) even in the insulin resistant state (40). Overexpression of SREBP-1c in transgenic mice leads to increased lipogenesis and the development of NAFLD (41). Inactivation of the SREBP-1c gene in livers of *ob/ob* mice results in an approximate 50% reduction in liver triglyceride (42). Carbohydrates can also stimulate lipogenesis by activating the carbohydrate response element binding protein leading to transcription of genes involved in glycolysis and lipogenesis, thus resulting in the conversion of excess glucose to fatty acids (43).

Increased uptake of fatty acids by hepatocytes also leads to mitochondrial fatty acid oxidation overload, with the consequent accumulation of fatty acids within hepatocytes (44). Increased fatty acid oxidation was associated with prevention of NAFLD in animal study (45).

In the liver, CAR agonist, TCPOBOP, lowered the expression of lipogenic

genes, including SREBP-1c, ACC-1, FAS and SCD-1 (27). In human study, TZDs decreased hepatic fat content and plasma levels of liver aminostransferase , significantly increased hepatic insulin sensitivity (46) and the expressions of PPARγ, adiponectin and lipoprotein lipase mRNA in adipose tissue (9). The expression of PPARγ is lower in the liver than in the adipose tissue. Liver specific PPARγ deficient mice are protected against the development of steatosis suggesting a role for hepatic PPARγ in liver triglyceride accumulation (47).

7. Possible interaction between CAR and PPAR

In addition to the role of xenoreceptor, CAR was also related to the fatty acid metabolism. The effect of CAR on fatty acid metabolism might involve its functional crosstalk with PPARα, a nuclear receptor that plays an important part in energy mobilization during fasting. It has been reported that the PPARα agonist WY14643 induces CAR gene expression in a PPARα dependent manner and also potentiates phenobarbital-induced transcription of CYP2B, a prototypical target gene of CAR (48). A DR1 motif in the CAR gene promoter was identified and shown to be necessary for the CAR induction (48). Consistent with the notion that CAR interacts with PPARα during fasting, CAR has been reported to interfere with fatty acid metabolism by binding to DNA elements overlapping with the PPARα-binding site in the promoter region of the 3-hydroxyacyl-CoA dehydrogenase, an important enzyme of peroxisomal fatty acid oxidation (49). In addition, in wild-type

mice, the CAR agonist, phenobarbital decreases the expression of mitochondrial carnitine palmitoyltransferase 1 (CPT1), the rate limiting enzyme of fatty acid oxidation, an effect not observed in CAR knockout mice (50). However, it has been not known about the interaction between CAR and PPARγ. In one study using microarray analysis, TCPOBOP decreased the expression of PPARγ in the livers of mice with normal chow diet but not with high fat diet (51). From this result, we could speculate on the possibility of interaction between CAR and PPARγ and differences in changes of interaction according to metabolic status.

8. Aim of study

We presumed that effect of pioglitazone on metabolism and NAFLD is different according to the degree of CAR activity. Possible mechanisms for this hypothesis are as follows: (1) CAR activity may affect the metabolism of pioglitazone possibly through a regulation of Cyp2c8 or Cyp3a4 activity. (2) Several genes related with NAFLD may be regulated by an interaction between CAR and PPARγ.

Therefore, we examined whether the effects of pioglitazone on the metabolic parameters are different according to the activities of CAR in diet induced obesity mice. We also investigated whether there exists any interaction between pioglitazone, or PPARγ activities, and CAR activities.

MATERIALS AND METHODS

1. Animals and study design

Homozygous CAR knockout ($CAR^{-/-}$) mice were generated by gene targeting as previously described (16) and were backcrossed to C57BL/6J mice to the tenth generation. $CAR^{+/+}$ (wild type, C57BL/6J) mice were supplied by Orient (Sungnam, Korea). Mice were housed at ambient temperature (23 ± 1℃), with 12:12-h light–dark cycles and free access to water *ad libitum*. Mice were fed regular chow diet or high fat diet (TD.88137, Harlan, 21% (w/w) total lipid (42% calories from fat), 0.2% (w/w) cholesterol).

We used 8-10 week-old male $CAR^{+/+}$ and $CAR^{-/-}$ mice and we divided these mice into vehicle and treatment groups according to administration of pioglitazone (3–4 mice/group) or TCPOBOP. Pioglitazone was given 10 mg/kg/day by oral route mixed with the each diet and 3 mg/kg TCPOBOP was injected intra-peritoneally once a week for 12 weeks. This experiment was repeated 3 times.

To obtain similar serum concentrations of pioglitazone between $CAR^{+/+}$ and $CAR^{-/-}$ mice, we administered different concentrations of pioglitazone to $CAR^{+/+}$ and $CAR^{-/-}$ mice (10 mg/kg, 20 mg/kg and 30 mg/kg) with sonde once daily for 14 days. And then we measured the serum concentration of

pioglitazone in each group. Based on this result, we were able to obtain similar serum concentrations of pioglitazone between $CAR^{+/+}$ and $CAR^{-/-}$ mice by application different concentration of pioglitazone (10 and 20 mg/kg for $CAR^{+/+}$ mice, 1 and 3 mg/kg for $CAR^{-/-}$ mice). We dissolved pioglitazone in solutol HS-15 (9% in phosphate-buffered saline).

To check for acute changes of gene expression according to the duration of pioglitazone treatment, we performed a short time (6 hours) experiment. mice divided into 4 groups in each $CAR^{+/+}$ and $CAR^{-/-}$ mice depending on whether pioglitazone and/or lipid were administered. Three mice were chosen for each group. Pioglitazone (20 mg/kg) and 3 g/kg of 20% intralipid (LIPO MCT injection, Dongkook Pharmaceutical Co., Korea) were administered via intra-peritoneal injection.

All animals were sacrificed after fasting for 6 hours starting from 06:00 a.m. Mice were anesthetized by intraperitoneal injection of zoletil® (Virvac, Carros, France) and blood was drawn by inferior vena cava puncture. Total body fat was measured by small animal body composition analyzer, PIXImus (GE Lunar Co, Winsconsin, USA). The liver was quickly removed, weighed and stored in the ice for measurement of fatty acid oxidation or frozen in liquid nitrogen for RNA extraction. White and brown fat was also removed, weighed and frozen in the liquid nitrogen. The study protocol was approved by the Institutional Animal Care and Use Committee at the Seoul National University Bundang Hospital, Sungnam, Republic of Korea.

2. Materials

Pioglitazone was purchased from Takeda Chemical Industries (Osaka, Japan). Pioglitazone was pulverized and mixed with high fat diet and each treatment mouse was administered pioglitazone 10 mg/kg·day. For measurement of serum concentration of pioglitazone, pioglitazone hydrochloride compound (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) were used for standard preparation. For CAR activation, TCPOBOP (Sigma-Aldrich, St. Louis, MO, USA) and corn-oil (Sigma-Aldrich, St. Louis, MO, USA) were used. For short-term lipid treatment, 20% intralipid was used (LIPO MCT injection, Dongkook Pharmaceutical Co., Korea).

3. Measurement of body weight and glucose tolerance

Body weight was monitored every week. Blood glucose levels were checked with reagent strips read in a glucometer (ACCU-CHEK Active, Roche, Mannheim, Germany). Intraperitoneal glucose tolerance test (IPGTT) carried out after 12 hours of fasting by intraperitoneal injection of 1 g/kg glucose 12 weeks after experiments. Blood glucose levels from tail vein blood were determined using glucometer (ACCU-CHEK Active, Roche, Mannheim, Germany) before and 15, 30, 60, 90, and 120 min after glucose injection.

4. Measurement of lipid profile, liver enzyme and insulin

Total cholesterol, triglyceride, high density lipoprotein cholesterol (HDL-C), low density lipoprotein cholesterol (LDL-C), aspartate aminotransferase (AST) and alanine transferase (ALT) were determined by Beckman Coulter AU480 automatic biochemistry analysis system (Japan) with reagent kits provided by the manufacturer. Insulin was measured by mouse insulin ELISA kit (ALPCO Diagnostics, Windham, NH, USA), following the protocol by the manufacturer.

5. Fatty acid oxidation

The fatty acid oxidation rate was measured as $^{14}CO2$ generation from [^{14}C]palmitate (NEN Life Sciences, Boston, MA, USA). Each liver tissue (20–25mg) were homogenized with buffer (250mM sucrose, 1mM EDTA, 10mM Tris-HCl, pH 7.4) and 50μl homogenates were mixed with reaction buffer containing 0.2 mM palmitate ([^{14}C]palmitate at 0.5 μCi/mL). After incubating for 120 min at 36℃, the reaction was quenched by adding 50 μL of 4 N sulfuric acid. The CO2 produced during the 120-min incubation was trapped with 200μL of 1M sodium hydroxide. The trapped $^{14}CO2$ and ^{14}C-

labeled acid soluble products were determined by liquid scintillation counting. The measured fatty acid oxidation rate was corrected according to the protein content of the tissues.

6. Histology

The left lobes of the livers were removed and fixed in 10% formaldehyde-PBS solution, embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin and eosin.

7. Measurement of pioglitazone concentration

The concentrations of pioglitazone were analyzed using a high-performance liquid chromatography (HPLC, Agilent 1200 series, Aglient Technologies, Santa Clara CA, USA). Pioglitazone hydrochloride was diluted in 50% acetonitrile to obtain 100ug/mL as working solution. This working solution was diluted with blank plasma to make standard solution with desired concentration level (5, 10, 50, 100, 500, 1000 and 2000 ng/mL). This standard solution (0.2ml) was mixed with 10ul of 1ug/ml formoterol and 600 μL of acetonitrile and centrifuged for 5 minutes at 13000 rpm. And then, 100 μg/mL supernant mixed with 500 μL f 5 mM ammonium formate: acetonitrile (20:80, 0.1% TFA). This mixture (0.1 μL) was injected to LC/MS/MS and the graphs

were analyzed.

8. RNA isolation and quantitative real time PCR

Total RNA was isolated from frozen liver and cells using Trizol® Reagents (Invitrogen, Carlsbad, CA) according the manufacturer's instruction. First-strand cDNA was synthesized from 1ug of RNA using Omniscript RT kit (Qiagen, Santa Clarita, CA, USA). Quantitative RT-PCR was performed using SsoFast EvaGreen Supermix (Bio-Rad Laboratories Inc., Hercules, CA, USA) and CFX96^{TM} real-time PCR system (Bio-Rad Laboratories Inc., Hercules, CA, USA). The used primer sequences are shown in Table 1.

9. Statistical analysis

Statistical analysis was performed by non-parametric analysis by using the Mann-Whitney tests. Statistical significance was assumed at $P < 0.05$

Table 1. Primer sequences using Real-time PCR in this study

Gene name	Forward	Reverse
PPARα	aatcctgtgccaaccagaag	atcgccactaaggtgtcagg
PPARγ1	tgcagctcaagctgaatcac	acgtgctctgtgacgatctg
PPARγ2	tgcagctcaagctgaatcac	cacgtgctctgtgacgatct
PGC-1α	aagagcgccgtgtgatttac	tgcattcctcaatttcacca
CD36	aaaccagtgctctcccttga	ctgcaccaataacagctcca
SREBP-1c	tgacccggctattccgtga	ctgggctgagcaatacagttc
FAS	cccttgatgaagagggatca	caaggcgttagggttgacat
SCD-1	aggtgcctcttagccactga	ccaggagtttcttgggttga
CPT-1α	acagtgggacattccaggag	aaggaatgcaggtccacatc
LCAD	tcaccacacagaatgggaga	tttctctgcgatgttgatgc
MTTP	gtattcccacctcagccaga	gtcaggcacgtcaaagcata
GAPDH	tgtgtccgtcgtggatctga	cctgcttcaccaccttcttga
CAR	catatggcaccgaggatacc	atctcaggaactgccaggaa
Cyp2b10	ctgtgaatggcactggagaa	gggaggaaaagggaagtgac
Cyp2c8	cagccaaagtccaggaagag	tctcatgcaacacagcatca
Cyp3a11	agggaagcattgaggaggat	cccatatcggtagaggagca
Cyp4a14	gctcctgttgaatgggaaga	tccgccatgattttgacata

PPAR α , Peroxisome proliferator-activated receptor α; PPARγ, Peroxisome proliferator-activated receptor γ1; PGC-1α, Peroxisome proliferator-activated receptor gamma, coactivator 1 alpha; SREBP-1c, Sterol regulatory element-binding protein-1c; FAS, fatty acid synthase; SCD-1, stearoyl-CoA desaturase CPT-1α, Carnitine palmitoyltransferase 1α; LCAD, Long chain acyl-CoA dehydrogenase; MTTP, Microsomal triglyceride transfer protein, GAPDH, Glyceraldehyde-3-phosphate dehydrogenase;

RESULTS

1. Changes of body weight, organ weights and glucose tolerance by pioglitazone treatment in diet induced obesity mice and different CAR activity

Pioglitazone increases insulin sensitivity in adipose tissue, liver and muscle and lowered blood glucose level. However, in addition to improved glycemic control, it causes weight gain, edema and redistribution of body fat in individuals with type 2 diabetes (7).

First of all, pioglitazone induced weight gain in $CAR^{+/+}$ (wild type) mice. In contrast, pioglitazone did not induce weight gain in $CAR^{-/-}$ mice. Moreover, pioglitazone-treated $CAR^{-/-}$ mice weighed less than vehicle-treated $CAR^{-/-}$ mice. In TCPOBOP-treated mice, there was no significant weight difference between vehicle and pioglitazone treatment. When the weight of fat and liver were measured individually, the weight of epididymal white fat which was included in visceral fat was significantly lower in pioglitazone-treated $CAR^{-/-}$ mice than in vehicle-treated $CAR^{+/+}$ mice (Figure 1B).

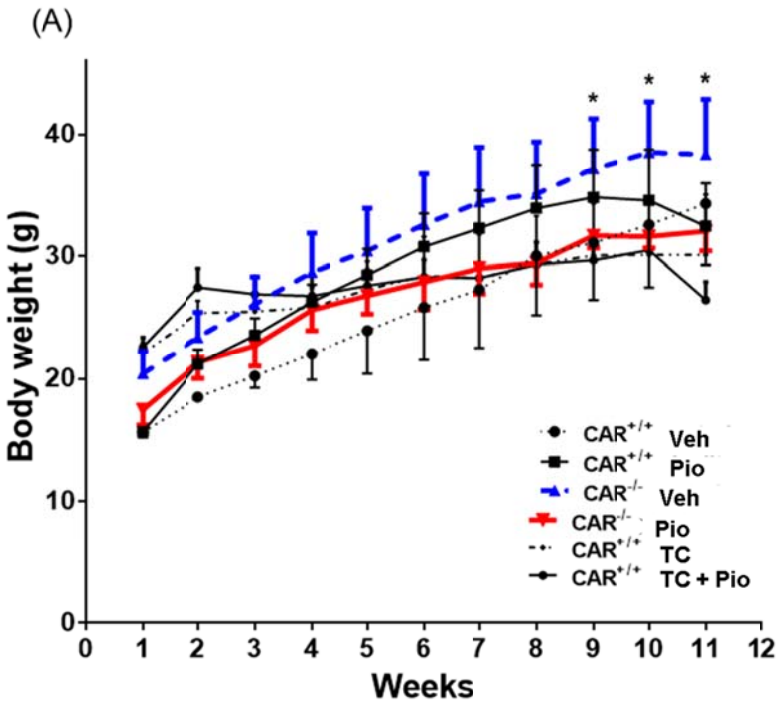
The weight of liver in $CAR^{+/+}$ mice was significantly higher in TCPOBOP-treated group compared with untreated mice irrespective of co-treatment with pioglitazone (Figure 1B). This result was the same in previous studies (52, 53),

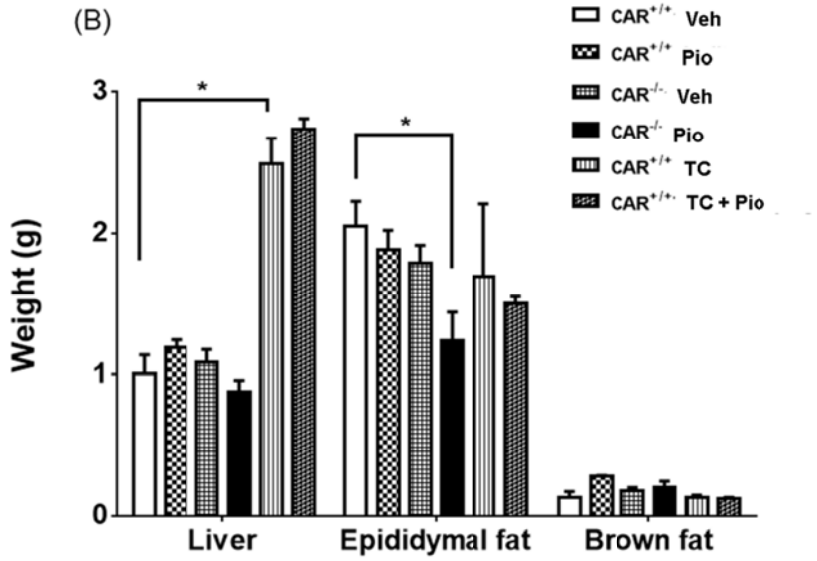
which reported that TCPOBOP induced hepatocyte hyperplasia and hepatomegaly.

When we performed 1 g/kg IPGTT in each group, pioglitazone treatment lowered glucose level in both $CAR^{+/+}$ and $CAR^{-/-}$ mice (Figure 1C). However, the difference of glucose level at each time point was significantly higher in $CAR^{-/-}$ mice. TCPOBOP-treated mice showed lower glucose level than that of $CAR^{+/+}$ mice, but the difference was not significant according to pioglitazone treatment. Fasting insulin level showed no significant difference between according to pioglitazone treatment (Figure 1D).

2. Measurements of serum lipids and liver enzymes in $CAR^{+/+}$ and $CAR^{-/-}$ mice

We measured the lipid profile and liver enzyme levels in $CAR^{+/+}$ and $CAR^{-/-}$ mice on high fat diet. The lipid profile and liver enzyme levels was not significantly changed by pioglitazone treatment (Figure 2). Serum total and HDL cholesterol level was significantly higher in $CAR^{-/-}$ mice than in $CAR^{+/+}$ mice (Figure 2A). This result coincide with the previous study which reported $CAR^{-/-}$ mice displayed modestly elevated total cholesterol , HDL cholesterol and apoA-I levels compared with $CAR^{+/+}$ mice (54).


(A)
Body weight (g)
Weeks
CAR+/+ Veh
CAR+/+ Pio
CAR-/- Veh
CAR-/- Pio
CAR+/+ TC
CAR+/+ TC + Pio


(B)
Weight (g)
CAR+/+ Veh
CAR+/+ Pio
CAR-/- Veh
CAR-/- Pio
CAR+/+ TC
CAR+/+ TC + Pio
Liver
Epididymal fat
Brown fat

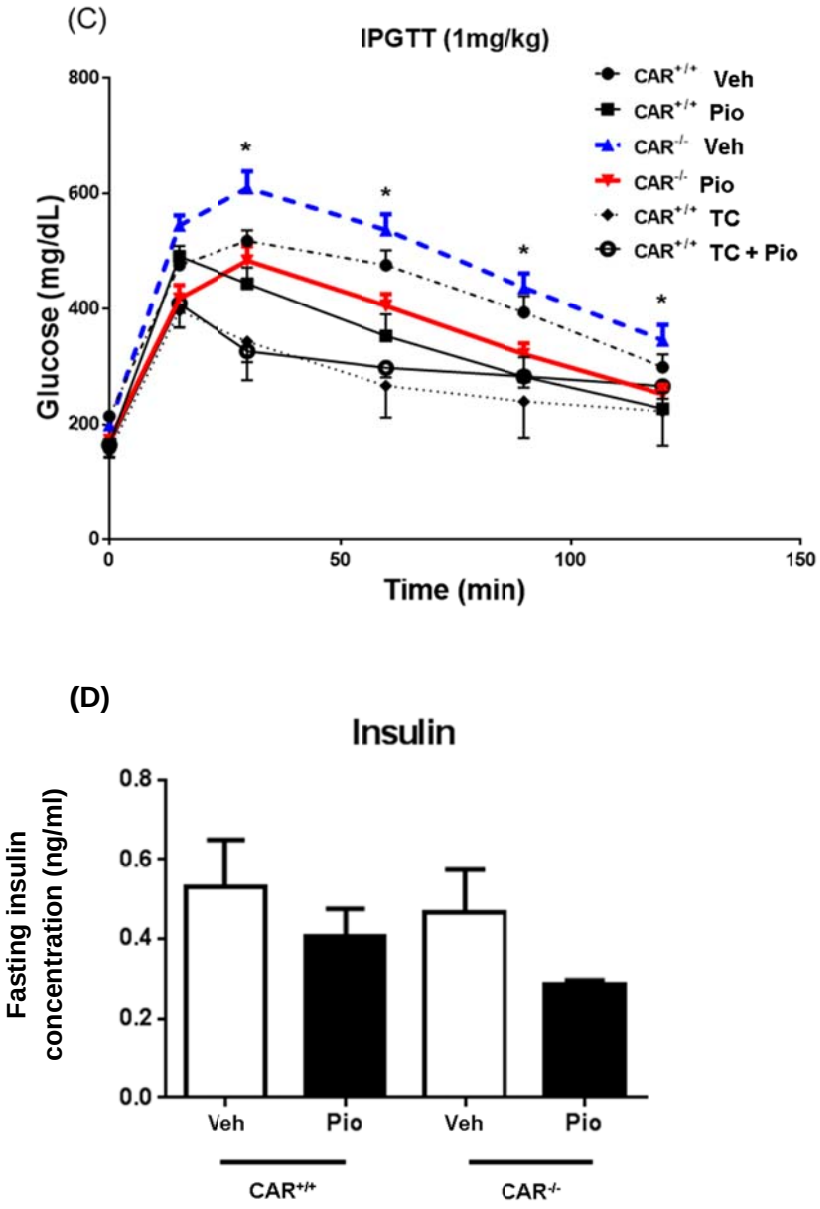


Figure 1. Metabolic changes according to CAR activity. (A) Body weight changes by pioglitazone in $CAR^{+/+}$, TCPOBOP-treated $CAR^{+/+}$ or $CAR^{-/-}$ mice. (B) Liver and fat tissue weight after 12 weeks treatment. (C) Intraperioneal glucose tolerance test in each group. (D) Fasting insulin level in $CAR^{+/+}$ and $CAR^{-/-}$ mice. Veh, vehicle; Pio, pioglitazone, * $P < 0.05$

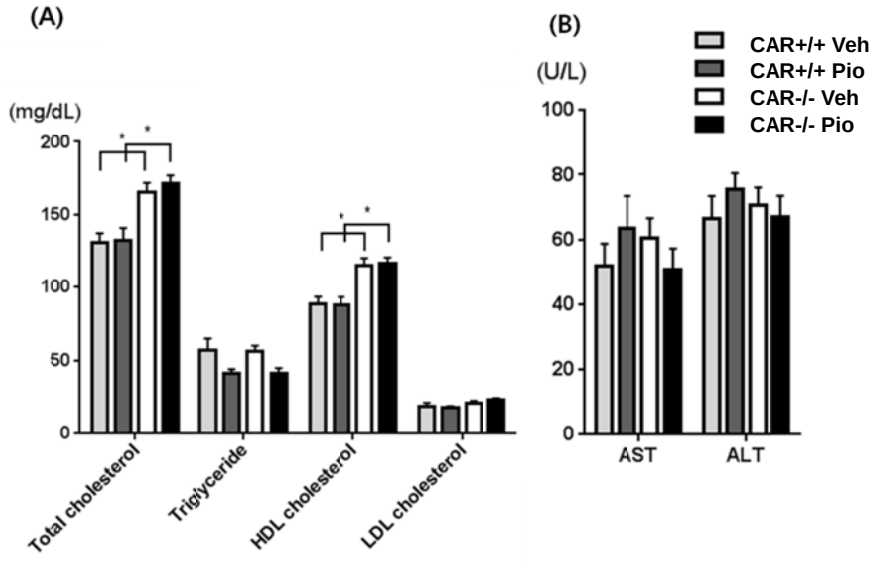


Figure 2. Lipid profile and liver enzyme levels in $CAR^{+/+}$ and $CAR^{-/-}$ mice.

* $P < 0.05$

3. The improvement of NAFLD in pioglitazone-treated $CAR^{-/-}$ mice

In histologic examination, NAFLD in $CAR^{+/+}$ mice was slightly aggravated by pioglitazone treatment. TCPOBOP, a CAR agonist, treated mice showed no fatty change in the liver by histology regardless of pioglitazone treatment. In $CAR^{-/-}$ mice, pioglitazone treatment showed definite improvement of NAFLD (Figure 3F). Therefore, we focused our study on the effect of pioglitazone on the improvement of NAFLD in $CAR^{-/-}$ mice.

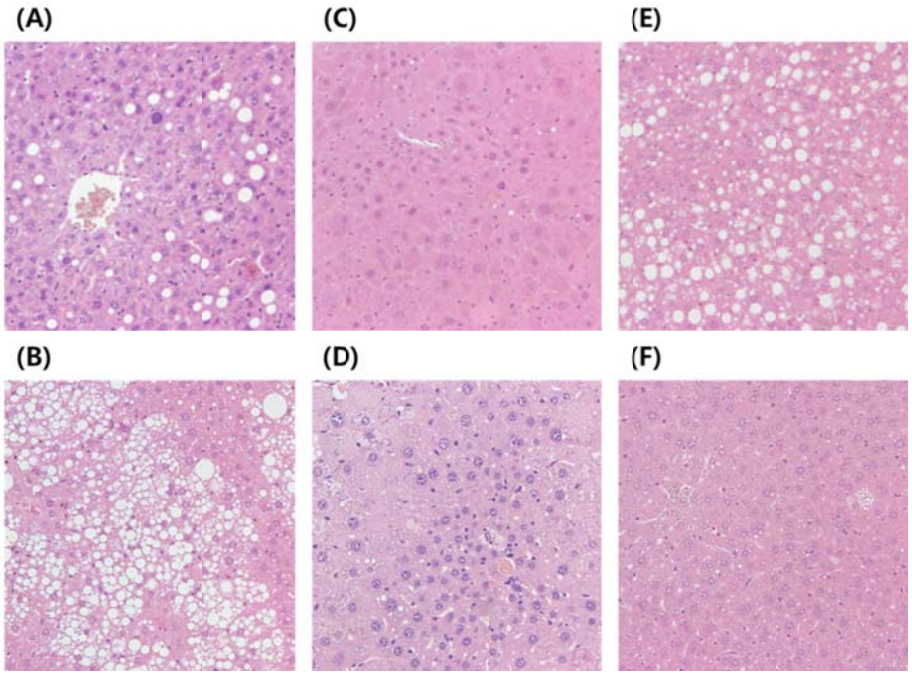


Figure 3. Liver histology after treatment with pioglitazone and/or TCPOBOP in high fat diet fed $CAR^{+/+}$ and $CAR^{-/-}$ mice after 12 weeks.

(A) vehicle-treated $CAR^{+/+}$, (B) $CAR^{+/+}$ with 10 mg/kg/day pioglitazone, (C) $CAR^{+/+}$ with 3 mg/kg/week TCPOBOP, (D) $CAR^{+/+}$ with 3 mg/kg/week TCPOBOP and 10 mg/kg/day pioglitazone, (E) vehicle-treated $CAR^{-/-}$, (F) $CAR^{-/-}$ with 10 mg/kg/day pioglitazone

4. Differences in the expression of Cytochrome P450s and the concentration of serum pioglitazone between $CAR^{-/-}$ and $CAR^{+/+}$ mice

Subsequently, we investigated whether the improvement of NAFLD by pioglitazone in $CAR^{-/-}$ mice was affected by pioglitazone concentration or CYP activity.

Expression of CAR target gene, cyp2b10, was increased by pioglitazone in only $CAR^{+/+}$ mice (Figure 4) regardless of diet. However, increased CAR activity was not related with the improvement of NAFLD. Instead, pioglitazone-treated $CAR^{+/+}$ mice showed severe fatty liver in histology as shown figure 3. The expression of cyp2c8 was not affected by pioglitazone in $CAR^{+/+}$ and $CAR^{-/-}$ mice. In contrast, the expression of cyp3a11 was significantly decreased in pioglitazone-treated $CAR^{-/-}$ mice compared with pioglitazone-treated $CAR^{+/+}$ mice in high fat diet group (Figure 4). Therefore, in high fat condition, we presumed that the concentration of pioglitazone might be higher in $CAR^{-/-}$ mice than $CAR^{+/+}$ mice. To confirm this, we measured serum concentration of pioglitazone in the high fat group.

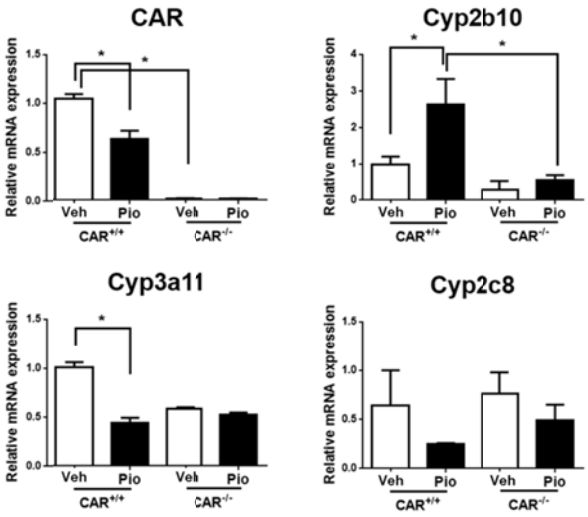
Serum concentration of pioglitazone was higher in $CAR^{-/-}$ mice than $CAR^{+/+}$ mice as was expected (Figure 5A). To confirm that the improvement of NAFLD was due to high serum concentration of pioglitazone, we measured serum concentration of pioglitazone after administration of different

concentrations of pioglitazone (10, 20 and 30mg/kg) for 2 weeks with high fat diet (Figure 5B). $CAR^{-/-}$ showed approximately 3 to 10 times higher concentrations of pioglitazone compared with $CAR^{+/+}$ mice under the same dose of pioglitazone administration. Therefore, we administered 10 and 20mg/kg of pioglitazone in $CAR^{+/+}$ mice and 1 and 3mg/kg of pioglitazone in $CAR^{-/-}$ mice for 2 weeks in conjunction with high fat diet. Consequently, we were able to obtain comparable serum concentrations of pioglitazone between $CAR^{+/+}$ and $CAR^{-/-}$ mice (Figure 5C).

5. Concentration independent improvement of NAFLD in pioglitazone-treated $CAR^{-/-}$ mice

After adjusting for serum concentration of pioglitazone, we compared liver histology in the last experimental group. Although the serum concentration of pioglitazone was similar between pioglitazone-treated $CAR^{+/+}$ and $CAR^{-/-}$ mice, the improvement of NAFLD remained significantly greater in the $CAR^{-/-}$ mice (Figure 6).

(A) Chow diet group



(B) High fat diet group

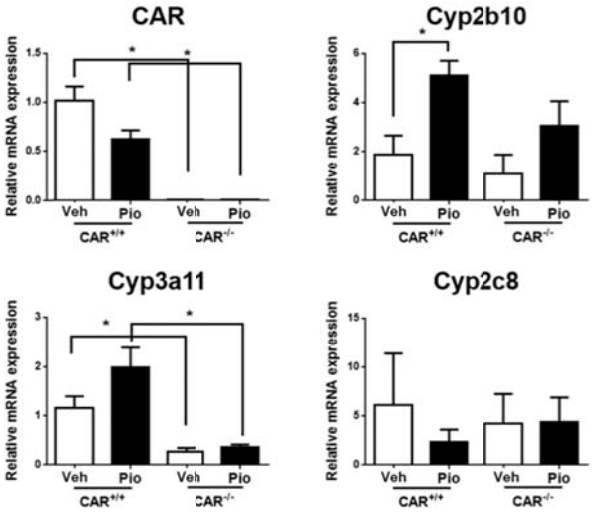


Figure 4. Expressions of CAR and its target genes by pioglitazone in $CAR^{+/+}$ and $CAR^{-/-}$ mice. Veh, vehicle; Pio, pioglitazone, * $P < 0.05$

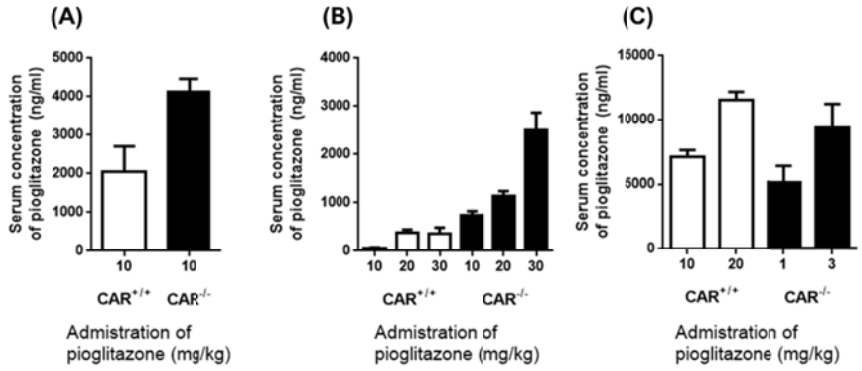


Figure 5. Serum concentrations of pioglitazone in pioglitazone-treated $CAR^{+/+}$ and $CAR^{-/-}$ mice according to the different concentration of pioglitazone. (A) 12 weeks of pioglitazone treatment with high fat diet, (B) and (C) 2 weeks of pioglitazone treatment with high fat diet

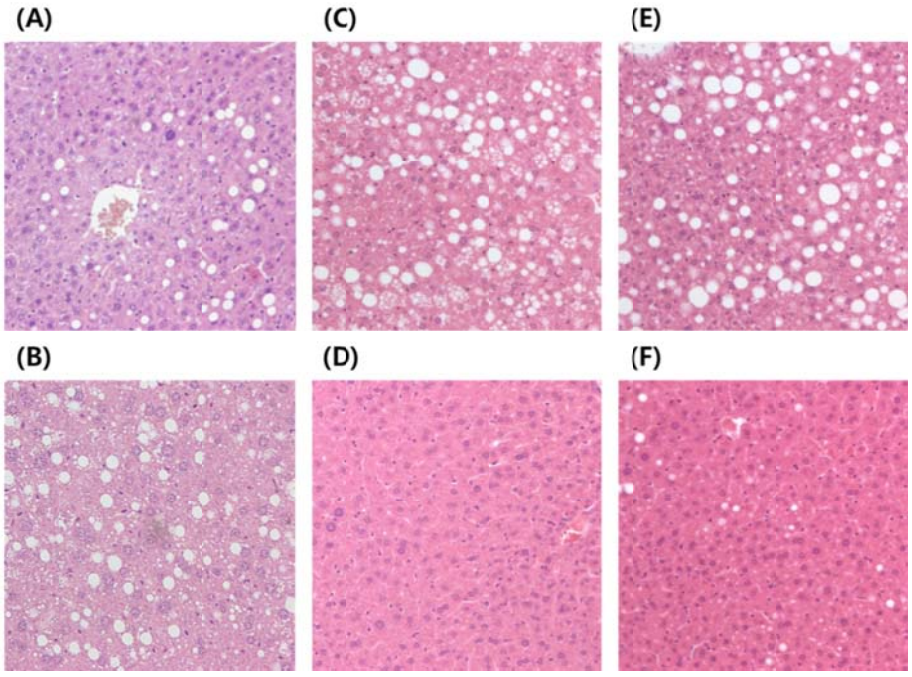


Figure 6. Liver histology when serum concentration of pioglitazone was similar between $CAR^{+/+}$ and $CAR^{-/-}$ mice.

(A) vehicle-treated $CAR^{+/+}$, (B) vehicle-treated $CAR^{-/-}$, (C) $CAR^{+/+}$ with 10 mg/kg pioglitazone, (D) $CAR^{-/-}$ with 1 mg/kg pioglitazone, (E) $CAR^{+/+}$ with 20 mg/kg pioglitazone, (F) $CAR^{-/-}$ with 3 mg/kg pioglitazone

6. Comparable rates of fatty acid oxidation and gene expressions related to fatty acid oxidation between pioglitazone-treated $CAR^{+/+}$ and $CAR^{-/-}$ mice

Next, we investigated whether the increased fatty acid oxidation or decreased *de novo* lipogenesis or lipid transport that were previously observed play a role in the improvement of NAFLD by pioglitazone in CAR-deleted DIO mice.

In order to investigate the mechanisms of the improvement of NAFLD in $CAR^{-/-}$ mice with pioglitazone treatment, we measured fatty acid oxidation in 12-week treated mice. However, that rates of fatty acid oxidation in the liver were not different between $CAR^{+/+}$ and $CAR^{-/-}$ mice regardless of diet (Figure 7).

Subsequently, we analyzed the gene expression associated with fatty acid oxidation, lipid transport and lipogenesis in the liver. In order to exclude the secondary effects of high fat diet, only the mice fed with chow diet were included for analysis. Also, we analyzed and compared the hepatic gene expression of 2-week and 6-hour treatment group to see the changes of gene expression according to the duration of pioglitazone treatment.

In the analysis of genes related to fatty acid oxidation, the expression of PPARα was decreased by 12 weeks of pioglitazone treatment in $CAR^{+/+}$ mice fed with high fat diet. Also, vehicle-treated $CAR^{-/-}$ mice showed lower

expressions of PPARα compared vehicle-treated $CAR^{+/+}$ mice in the high fat fed group (Figure 8) However, there was no significant difference between pioglitazone-treated $CAR^{+/+}$ and $CAR^{-/-}$ mice in all treatment condition.

The expression of CPT1α showed no significant difference between pioglitazone-treated $CAR^{+/+}$ and $CAR^{-/-}$ mice (Figure 9).

On the other hand, pioglitazone-treated $CAR^{-/-}$ mice showed lower expression of long chain acyl-CoA dehydrogenase (LCAD), a mitochondrial enzyme responsible for the fatty acid oxidation (55), compared with that of pioglitazone-treated $CAR^{+/+}$ mice in 2-week high fat diet group (Figure 10). This change, however, was not shown in other treatment condition.

The expression of PPARα marker gene, Cyp4a14, was increased in pioglitazone-treated $CAR^{-/-}$ mice compared with $CAR^{+/+}$ mice in 12-week chow diet group (Figure 11).

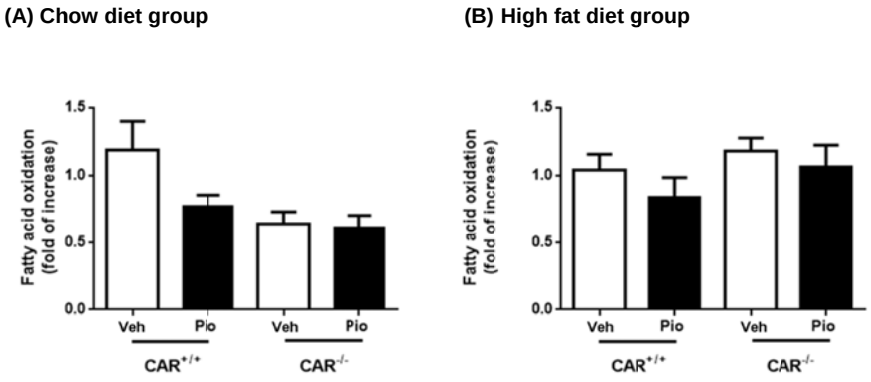


Figure 7. Fatty acid oxidation rate in 12-week treatment group

Veh, vehicle; Pio, pioglitazone

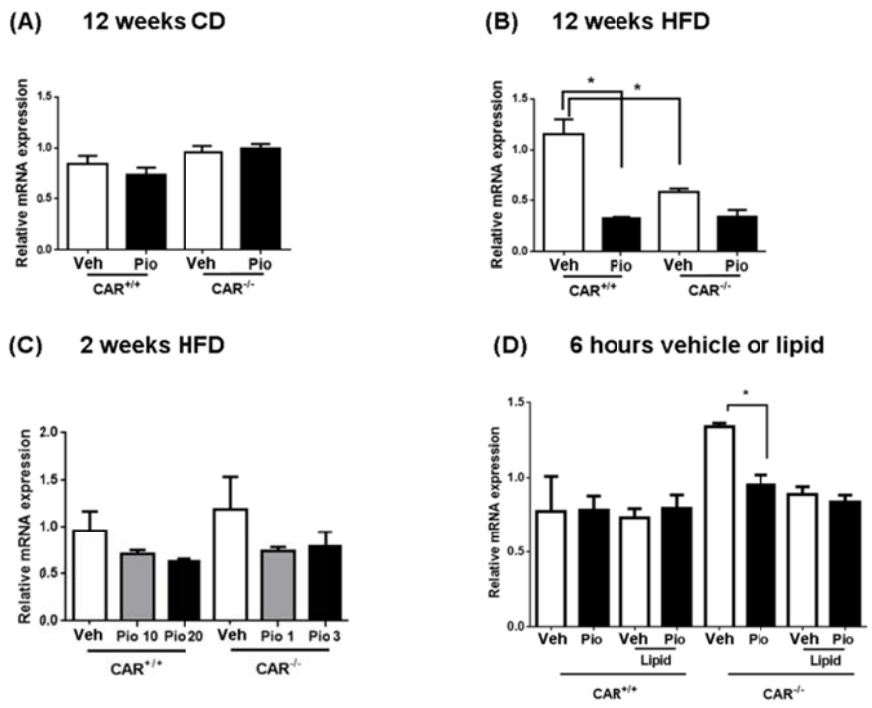


Figure 8. Expression of PPARα according to treatment groups with or without pioglitazone in $CAR^{+/+}$ and $CAR^{-/-}$ mice. Veh, vehicle; Pio, pioglitazone; CD, chow diet; HFD, high fat diet, * P <0.05

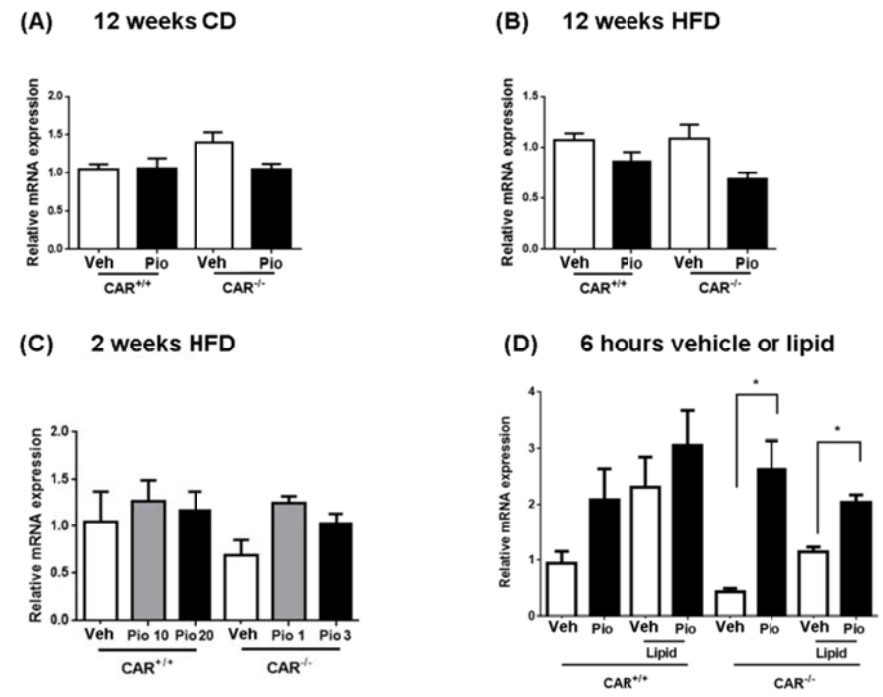


Figure 9. Expression of CPT-1α according to treatment groups with or without pioglitazone in $CAR^{+/+}$ and $CAR^{-/-}$ mice. Veh, vehicle; Pio, pioglitazone, * P <0.05

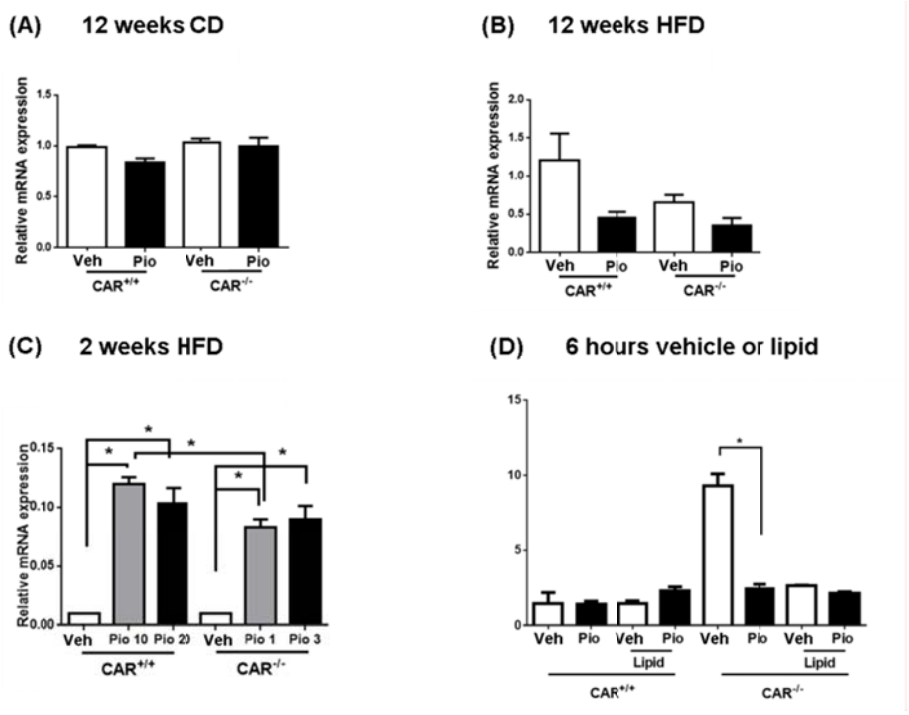


Figure 10. Expression of long-chain acyl-CoA dehydrogenase (LCAD) according to treatment groups with or without pioglitazone in $CAR^{+/+}$ and $CAR^{-/-}$ mice. * P <0.05

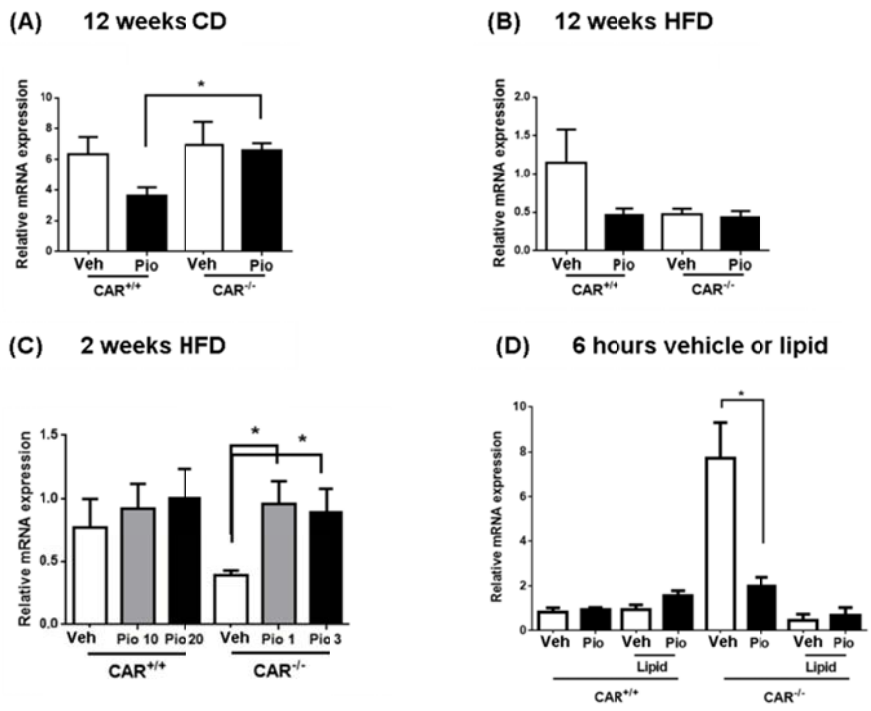


Figure 11. Expression of Cyp4a14 according to treatment groups with or without pioglitazone in $CAR^{+/+}$ and $CAR^{-/-}$ mice. Veh, vehicle; Pio, pioglitazone, * P <0.05

7. Different expressions of genes associated with lipid transport between pioglitazone-treated $CAR^{+/+}$ and $CAR^{-/-}$ mice

CD36, also known as FAT (fatty acid translocase) is a key protein involved in regulation of fatty acid uptake. In 12-week and 2-week high fat diet group (Figure 12B, 12C), pioglitazone-treated $CAR^{-/-}$ mice showed lower CD36 expression than that of pioglitazone-treated $CAR^{+/+}$ mice. This result suggested that pioglitazone reduced fatty acid uptake in high fat condition through decreasing CD36. In contrast, the expression of MTTP (microsomal triglyceride transfer protein) showed no difference between pioglitazone-treated $CAR^{+/+}$ and $CAR^{-/-}$ mice in all treatment condition (Figure 13). Since MTTP is required for the assembly and secretion of very low density lipoproteins by the liver, it was conceivable that excretion of triglyceride in pioglitazone-treated $CAR^{-/-}$ mice might not be different from that of pioglitazone-treated $CAR^{+/+}$ mice.

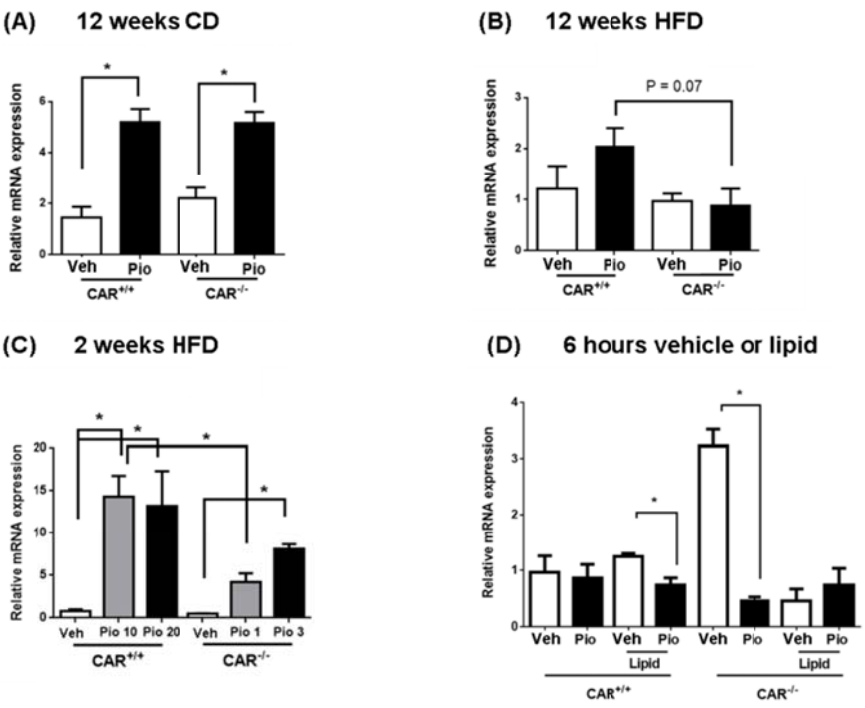


Figure 12. Expression of CD36 according to treatment groups with or without pioglitazone in $CAR^{+/+}$ and $CAR^{-/-}$ mice. Veh, vehicle; Pio, pioglitazone, * P <0.05

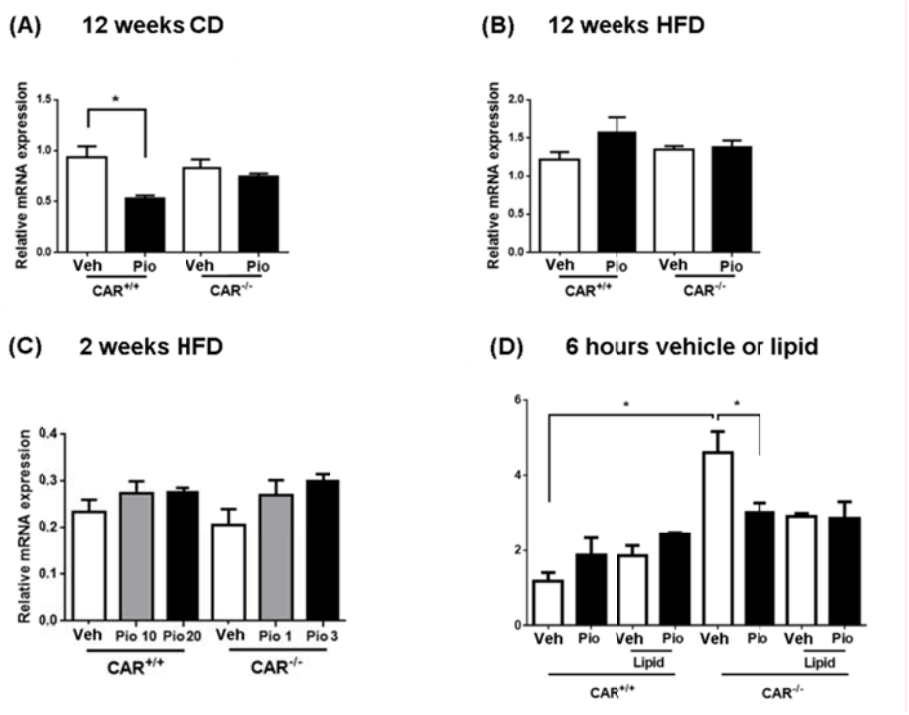


Figure 13. Expression of MTTP according to treatment groups with or without pioglitazone in $CAR^{+/+}$ and $CAR^{-/-}$ mice. Veh, vehicle; Pio, pioglitazone, * P <0.05

8. Different expression of genes related to lipogenesis between pioglitazone-treated $CAR^{+/+}$ and $CAR^{-/-}$ mice

In $CAR^{-/-}$ mice, pioglitazone treatment reduced the expression of sterol regulatory element-binding protein-1c (SREBP-1c) compared with vehicle-treated in groups in mice with 12 weeks of dietary intervention with chow diet, high fat diet, or6 hours of intralipid treatment group (Figure 14A, 14B and 14D). However, there was no significant difference between pioglitazone-treated $CAR^{+/+}$ and $CAR^{-/-}$ mice in all treatment condition.

The expression of FAS (fatty acid synthase) showed no difference in pioglitazone-treated $CAR^{+/+}$ and $CAR^{-/-}$ mice in all treatment condition (Figure 15).

In contrast, the expression of SCD-1 (stearoyl-CoA desaturase) which is a rate-limiting enzyme responsible for converting palmitic and stearic acid to palmitoleci and oleic acids and associated with lipogenesis and obesity (56), was decreased in greater extent in pioglitazone-treated $CAR^{-/-}$ mice compared with pioglitazone-treated $CAR^{+/+}$ mice in 12 weeks and 2 weeks of dietary intervention with high fat diet (Figure 16B and 16C).

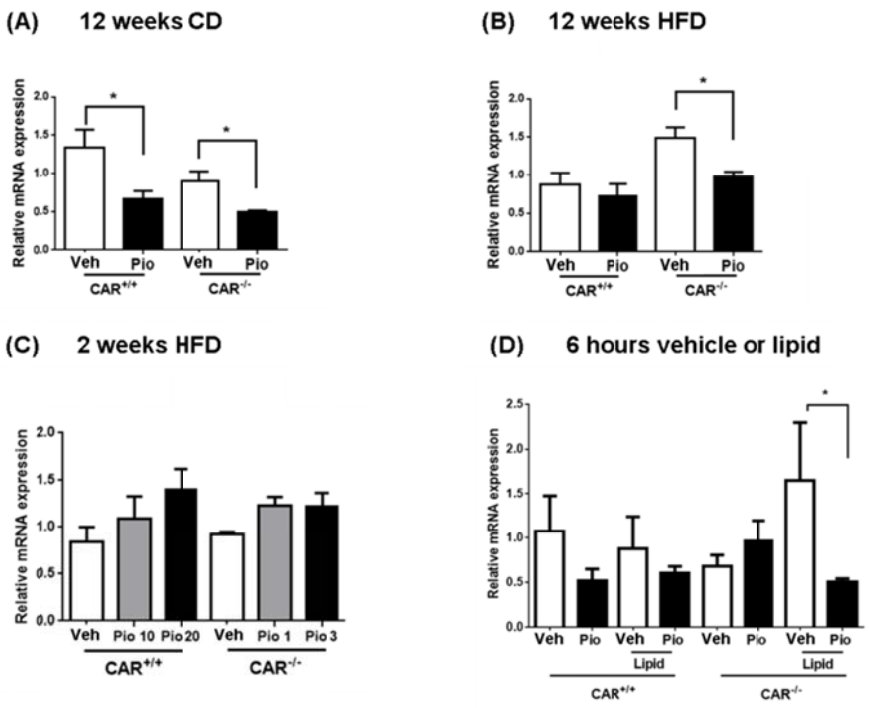


Figure 14. Expression of SREBP-1c according to treatment groups with or without pioglitazone in $CAR^{+/+}$ and $CAR^{-/-}$ mice. Veh, vehicle; Pio, pioglitazone, * P <0.05

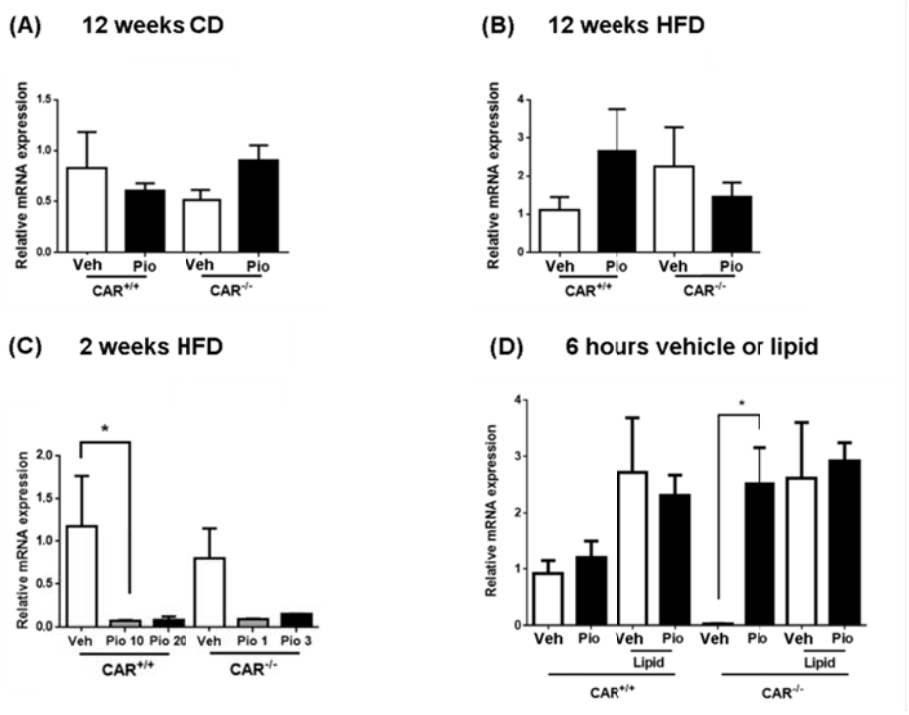


Figure 15. Expression of FAS according to treatment groups with or without pioglitazone in $CAR^{+/+}$ and $CAR^{-/-}$ mice. Veh, vehicle; Pio, pioglitazone, * P <0.05

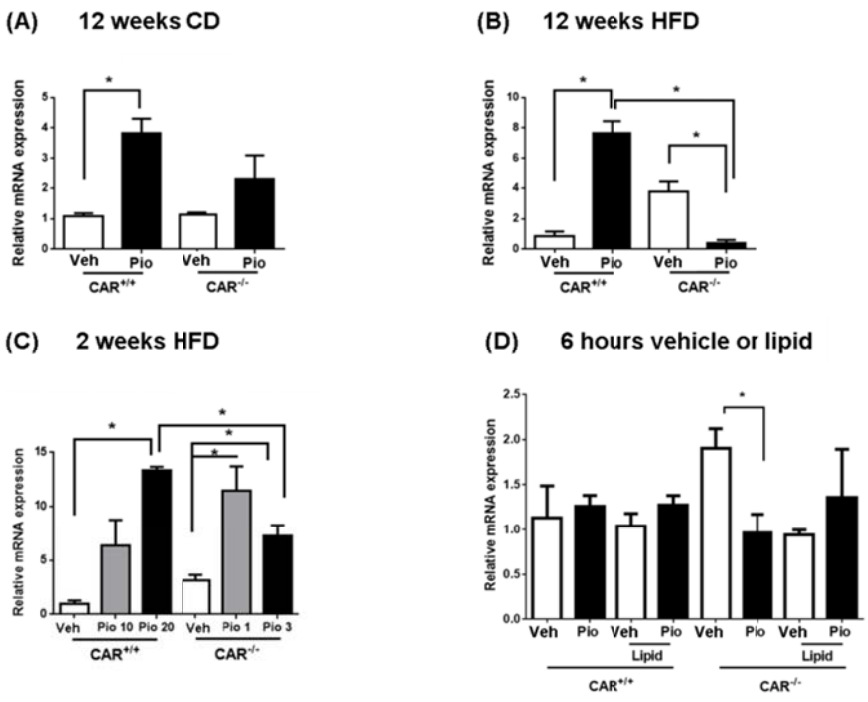


Figure 16. Expression of SCD-1 FAS according to treatment groups with or without pioglitazone in $CAR^{+/+}$ and $CAR^{-/-}$ mice. Veh, vehicle; Pio, pioglitazone, * P <0.05

9. Different expressions of PPARγ and PGC-1α between pioglitazone-treated $CAR^{+/+}$ and $CAR^{-/-}$ mice

As shown figure 17, the expression of PPAR γ1 was decreased by pioglitazone treatment in both $CAR^{+/+}$ and $CAR^{-/-}$ mice in groups with 2 weeks or 6 hours of treatment. However, the expression of PPARγ1 showed no significant change according to the presence of CAR. On the other hand, the expression of PPARγ2 was decreased consistently in pioglitazone-treated $CAR^{-/-}$ mice compared with pioglitazone-treated $CAR^{+/+}$ mice in all high fat conditions regardless of treatment periods (Figure 18B, 18C and 18D).

PGC-1α (peroxisome proliferator-activated receptor gamma coactivator 1-alpha) interacts with the nuclear receptor PPARγ, which permits the interaction with multiple transcription factors. The expression of PGC-1α was also decreased in pioglitazone-treated $CAR^{-/-}$ mice than pioglitazone-treated $CAR^{+/+}$ mice in high fat diet condition (Figure 19B, 19C and 19D).

We summarized the comparison of gene expression between pioglitazone-treated $CAR^{-/-}$ mice and $CAR^{+/+}$ mice in Table 2.

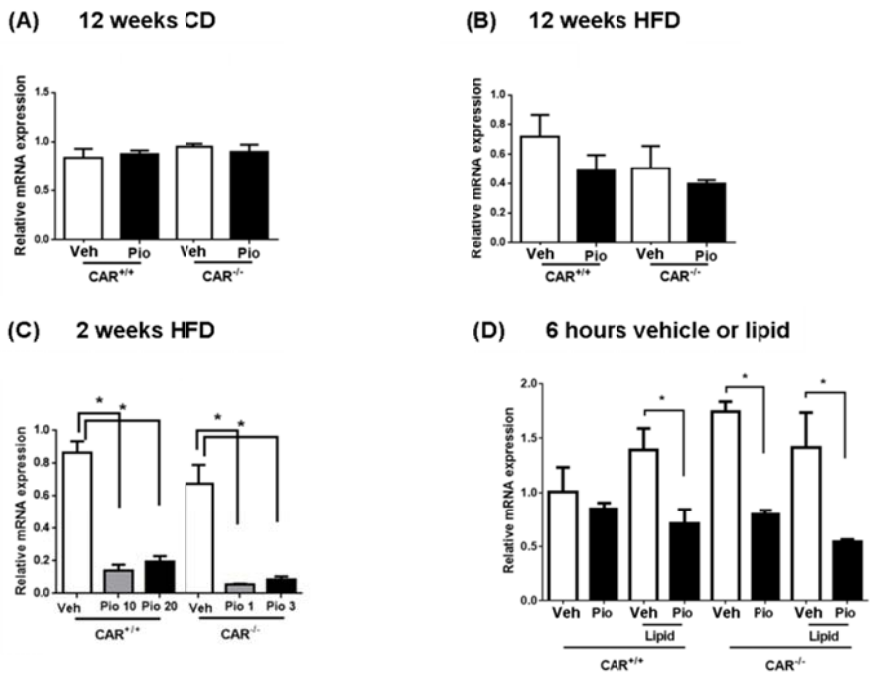


Figure 17. Expression of PPARγ1 according to treatment groups with or without pioglitazone in $CAR^{+/+}$ and $CAR^{-/-}$ mice. Veh, vehicle; Pio, pioglitazone, * P <0.05

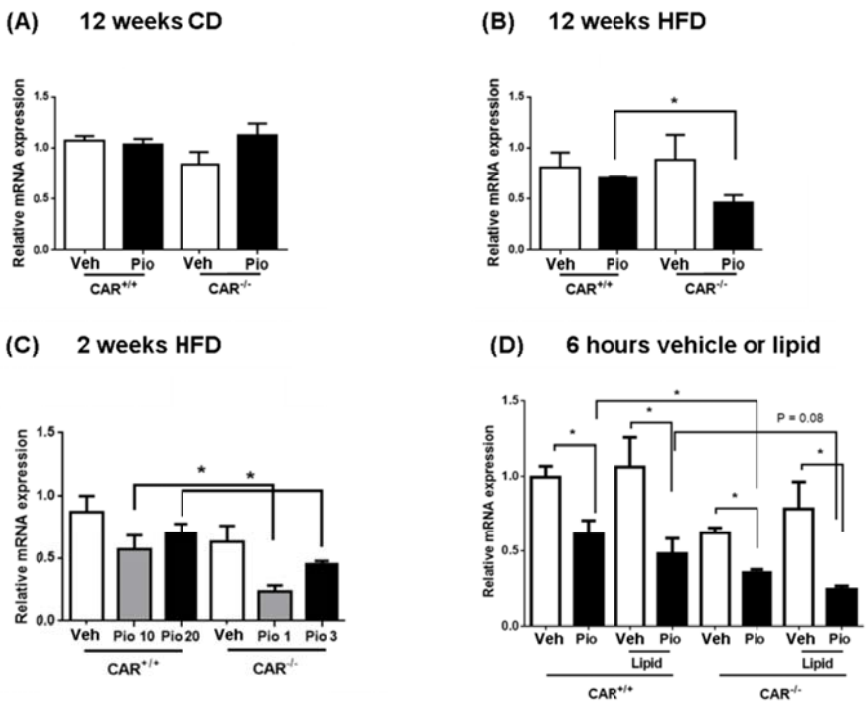


Figure 18. Expression of PPARγ2 according to treatment groups with or without pioglitazone in $CAR^{+/+}$ and $CAR^{-/-}$ mice. Veh, vehicle; Pio, pioglitazone, * P <0.05

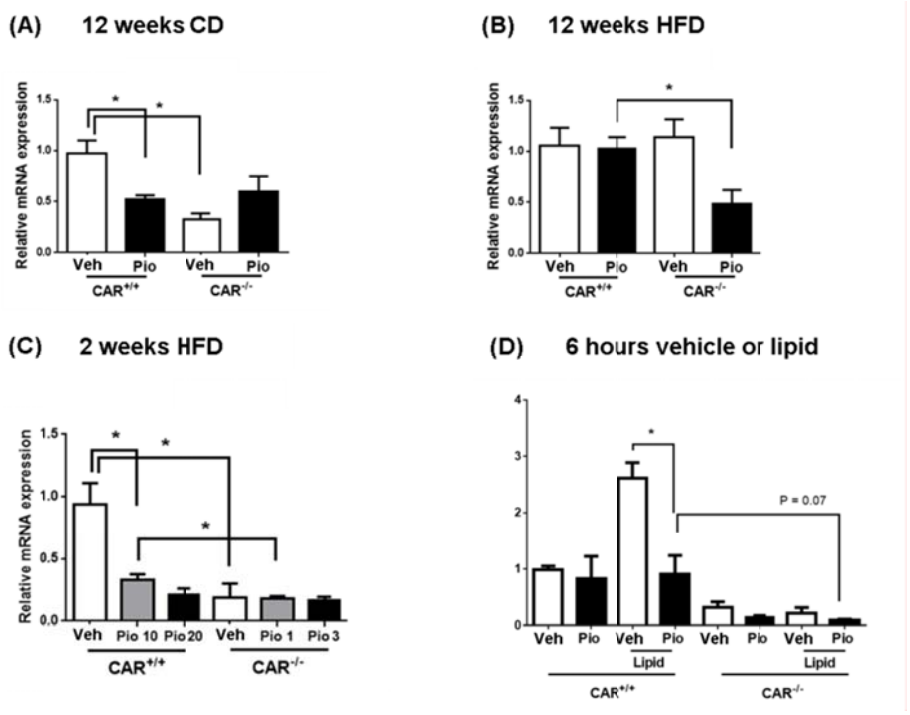


Figure 19. Expression of PGC-1α according to treatment groups with or without pioglitazone in $CAR^{+/+}$ and $CAR^{-/-}$ mice. Veh, vehicle; Pio, pioglitazone, * P <0.05

Table 2. Comparison of changes in gene expression between pioglitazone-treated $CAR^{-/-}$ and $CAR^{+/+}$ mice according to treatment period and diet

		6hours HF	2weeks HF	12weeks HF	12weeks CD
	Gene	$CAR^{-/-}$ pio Vs. $CAR^{+/+}$ pio	$CAR^{-/-}$ pio Vs. $CAR^{+/+}$ pio	$CAR^{-/-}$ pio Vs. $CAR^{+/+}$ pio	$CAR^{-/-}$ pio Vs. $CAR^{+/+}$ pio
Fatty acid oxidation	PPARα	↔	↔	↔	↔
	CPT1α	↔	↔	↔	↔
	LCAD	↔	↓	↔	↔
	Cyp4a14	↔	↔	↔	↑
Lipid transport	CD36	↔	↓	↓	↔
	MTTP	↔	↔	↔	↔
Lipogenesis	SREBP1c	↔	↔	↔	↔
	FAS	↔	↔	↔	↔
	SCD-1	↔	↓	↓	↔
PPAR and PGC1α	PPARγ1	↔	↔	↔	↔
	PPARγ2	↓	↓	↓	↔
	PGC1α	↓	↓	↓	↔

PPAR α , Peroxisome proliferator-activated receptor α; PPARγ, Peroxisome proliferator-activated receptor γ1; PGC-1α, Peroxisome proliferator-activated receptor gamma, coactivator 1 alpha; SREBP-1c, Sterol regulatory element-binding protein-1c; FAS, fatty acid synthase; SCD-1, stearoyl-CoA desaturase CPT-1α, Carnitine palmitoyltransferase 1α; LCAD, Long chain acyl-CoA dehydrogenase; MTTP, Microsomal triglyceride transfer protein, GAPDH, Glyceraldehyde-3-phosphate dehydrogenase;

DISCUSSION

In this study, pioglitzone treatment resulted in more favorable responses in glucose tolerance and the improvement of NAFLD in $CAR^{-/-}$ mice compared with $CAR^{+/+}$ mice. Unexpectedly, pioglitazone-treated $CAR^{-/-}$ mice gained less weight than vehicle-treated $CAR^{-/-}$ mice, whereas pioglitazone-treated $CAR^{+/+}$ mice gained more body weight than vehicle-treated $CAR^{+/+}$ mice (Figure 1A). Also the weight of epididymal white fat, which constitutes visceral fat, was decreased (Figure 1B) and NAFLD was significantly improved in pioglitazone-treated $CAR^{-/-}$ mice (Figure 3). These results suggest the possibility of negative lipid balance induced by pioglitazone treatment in $CAR^{-/-}$ mice. Since CAR exists mainly in the liver and is not expressed in the adipose tissue, the improvement of NAFLD might be a primary effect in this change.

CAR activator, TCPOBOP reduced body weight and improved glucose tolerance and NAFLD (Figure 1A, 1C and 3). However, we were not able to examine any difference according to treatment groups with or without pioglitazone in TCPOBOP-treated mice due to the strong effect of TCPOBOP itself. Furthermore, the changes caused by TCPOBOP were similar to the results shown in previous studies (27, 28). For these reasons, we focused on the effect of pioglitazone on the improvement of NAFLD in $CAR^{-/-}$ mice.

Firstly, we investigated whether increased CYP activity or high concentration of pioglitazone affect the improvement of NAFLD in $CAR^{-/-}$ mice.

Since there is no currently known method for the direct measurement of CAR activity, we checked the expression of Cyp2b10, which was a prototypical target of CAR (48). As shown figure 4, the expression of Cyp2b10 was increased by pioglitazone in both chow and high fat diet condition. However, despite the increased CAR activity, pioglitazone-treated $CAR^{+/+}$ mice showed severe fatty liver (Figure 3). Therefore, increased CAR activity per se was not associated with the improvement of NAFLD.

The expression of Cyp3a11, the murine homolog of human CYP3A4, was decreased in $CAR^{-/-}$ mice in high fat condition (Figure 4B) and concentration of pioglitazone was higher in high fat diet $CAR^{-/-}$ mice (Figure 5A). Therefore, we assumed that the high concentration of pioglitazone resulted by low Cyp3a11 might have been affected by the improvement of NAFLD. To confirm this hypothesis, we adjusted the concentration of pioglitazone in $CAR^{+/+}$ and $CAR^{-/-}$ mice (Figure 5C). Contrary to our expectation, however, fatty liver was still improved in $CAR^{-/-}$ mice (Figure 6).

From these results, we confirmed that the improvement of NAFLD in $CAR^{-/-}$ mice was not associated with pioglitazone concentration or the activity of CYP enzyme. These results compelled us to search for a different mechanism associated with the improvement of NAFLD by pioglitazone in $CAR^{-/-}$ mice.

To investigate the mechanism, we compared the expression of several genes related to lipid metabolism between pioglitazone-treated $CAR^{-/-}$ and $CAR^{+/+}$ mice. Among several genes, CD36, a key protein involved in the regulation of fatty acid uptake, and SCD-1, an enzyme associated with lipogenesis, were decreased in greater amount in pioglitazone-treated $CAR^{-/-}$ mice than in pioglitazone-treated $CAR^{+/+}$ mice in high fat condition. These results suggested that decreased lipid uptake and lipogenesis in pioglitazone-treated $CAR^{-/-}$ mice had contributed to the improvement of NAFLD.

Previous studies reported that specific induction of CD36/FAT transporters in the liver leads to hepatomegaly and fatty liver, which suggests a role in fatty acid uptake (57, 58). In particular, recent findings suggest increased hepatic CD36 activity may be critical for the development of steatosis under pathologic conditions such as high fat diet, obesity and diabetes (59, 60). Conversely, when CD36 was deleted, liver was protected from the development of NAFLD (61). In human, hepatic CD36 gene expression was increased and correlated with liver fat content in patients with NAFLD (62).

SCD1 has recently become a target of interest for the reversal of NAFLD and insulin resistance. Mice with a global knockout of SCD1 fed a high carbohydrate/high fat diet are lean, insulin sensitive and have decreased liver fat content due to both decreased lipogenic activity and increased fatty acid oxidation (63-65), suggesting that whole-body SCD1 deficiency protects from obesity-associated insulin resistance and steatosis.

Both CD36 and SCD-1 genes were regulated by PPARγ (56, 66) and they had

PPARγ response element on their promoter (67, 68). Therefore, decreased expression of PPARγ might affect the expression of CD36 and SCD-1.

Several murine models of obesity and diabetes, including *ob/ob*, A-ZIP and KKAy, develop fatty livers that express enhanced levels of PPARγ (47, 69-71). PPARγ has two major isoforms, γ1 and γ2, generated from the same gene by alternative splicing (72). PPARγ2 is highly expressed in adipose tissue and is upregulated in steatotic livers of *ob/ob* mice, whereas PPARγ1 is found at low levels in many tissues (73). A number of studies have demonstrated enhanced expression of lipogenic genes and increased expression of PPARγ in animal models of steatotic liver (47, 71, 74). Moreover, a role for PPARγ has been established in the maintenance of a steatotic phenotype in the liver (47). In previous study using a hepatic stable cell line expressing PPARγ2, the expression of PPARγ2 induced lipid accumulation in the hepatocytes by upregulating adipogenic and lipogenic gene expression (75). Culturing of PPARγ2-expressing hepatocytes in the absence of serum (exogenous lipids) resulted in lipid accumulation, suggesting that *de novo* lipid synthesis may be an important mechanism contributing to steatosis in the hepatocytes (75).

In our study, the expression of PPARγ2 was decreased in pioglitazone-treated $CAR^{-/-}$ mice in high fat diet condition. This change occurred in early period of pioglitazone treatment and was maintained throughout pioglitazone treatment (Figure 17B, C and D). Therefore, we could speculate that decreased expression of PPARγ2 in pioglitazone-treated $CAR^{-/-}$ mice might have caused the improvement of NAFLD by decreasing expression of gene related to lipid

uptake (CD36) and lipogenesis (SCD-1). These results were summarized at figure 20.

The limitation to our study is that we were not able to find the exact molecular mechanism for the interaction between pioglitazone/PPARγ2 and CAR. Further study will be needed to uncover the underlying mechanisms.

However, our study results may be useful in establishing a novel treatment approach for patients with NAFLD by suggesting the benefits of combination treatment with pioglitazone and CAR antagonist, or drugs that modulate CAR activity.

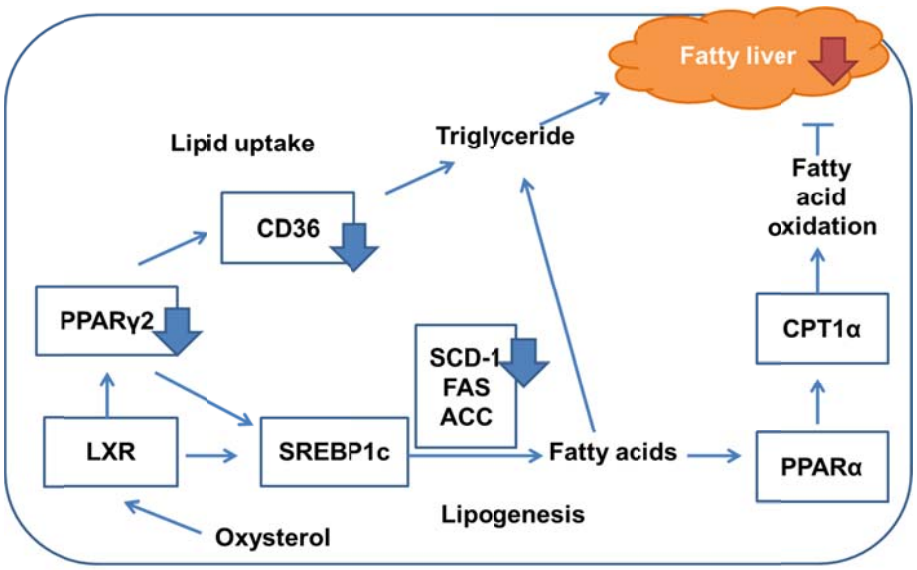


Figure 20. Possible changes of genes related to lipid metabolism in the hepatocytes of pioglitazone-treated $CAR^{-/-}$ mice compared with $CAR^{+/+}$ mice. PPAR α , Peroxisome proliferator-activated receptor α; PPARγ, Peroxisome proliferator-activated receptor γ1; PGC-1α, Peroxisome proliferator-activated receptor gamma, coactivator 1 alpha; SREBP-1c, Sterol regulatory element-binding protein-1c; ACC, acetyl-CoA carboxylase; FAS, fatty acid synthase; SCD-1, stearoyl-CoA desaturase CPT-1α, Carnitine palmitoyltransferase 1α; LXR, liver X receptor

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국문 초록

Constitutive Androstane Receptor의 활성도가 pioglitazone에 의한 비알콜성지방간 변화에 미치는 영향

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안 화 영

서론 본 연구는 CAR의 활성도에 따라 비만을 유도한 쥐에서 피오글리타존이 대사 조절에 미치는 영향이 어떻게 달라지는지를 알아보고자 하였고, 또한 피오글리타존/PPARγ 와 CAR 사이에 어떤 상호작용이 존재하는지 알아보고자 하였다.

방법 CAR의 활성화를 위해서는 3mg/kg 의 TCPOBOP를 매주 주사하였고, CAR의 활성도를 없애기 위해서는 $CAR^{-/-}$ 쥐를 이용하였다. 비만을 유도하기 위해 고지방식을 주었고, 각각의 다른 CAR 의 활성도를 갖는 마우스들은 피오글리타존 투여 여부에 따라 두군으로 나누었다. 12주 동안 매주 체중을 측정하였고, 이후 당부하검사를 하였으며, 혈중 콜레스테롤 및 피오글리타존 농도를 측정하고 지

방간 변화를 현미경으로 관찰하였다. 지방산 산화율을 측정하였고, 지방 대사 관련 유전자의 변화는 real time PCR 방법을 이용하였다.

결과 피오글리타존을 투여한 $CAR^{-/-}$ 마우스는 피오글리타존을 준 정상 마우스에 비해 체중이 덜 증가하는 소견을 보였고, 당부하에서는 비슷한 호전을 보였으며, 지방간은 매우 호전되는 소견을 보였다. TCPOBOP 로 CAR 를 활성화시킨 경우에는 TCPOBOP 자체의 효과가 너무 강하여 피오글리타존 투여로 인한 변화를 관찰할 수 없었다. 피오글리타존에 의해 CAR 의 활성도가 증가하는 것과 피오글리타존 자체의 혈중 농도는 지방간의 호전과는 관련이 없었다. 피오글리타존을 준 $CAR^{-/-}$ 마우스의 간조직에서 유전자 변화를 살펴본 결과 CD36 과 SCD-1 의 발현이 피오글리타존을 준 정상 마우스에 비해 감소하였고, 이 유전자들은 PPARγ2 의 발현 감소에 의해 조절되는 것으로 보였다.

결론 피오글리타존에 의한 대사에 CAR 의 활성도가 미치는 영향은 CAR의 농도와는 무관하였으며, CAR 유전자가 없는 상태에서 피오글리타존의 투여는 PPARγ2 의 발현을 감소시켜, 이를 통해 CD36 과 SCD-1 의 발현을 감소시킴으로써 지방간의 호전에 기여하는 것으로 나타났다.