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Histopathological Evaluation of Heart Toxicity of a Novel Selective PPAR-γ Agonists CKD-501 in db/db Mice

Hyun-Il Yang[1], Woo Sik Kim[2], Dal-Hyun Kim[2] and Jin Seok Kang[1,*]

[1]Department of Biomedical Laboratory Science, Namseoul University, Cheonan 331-707,
[2]Divison of Pharmacology & Toxicology, CKD Research Institute, Chong Kun Dang, Yongin 446-527, Republic of Korea

Abstract

High risk of cardiovascular diseases caused by existing PPAR-γ agonists such as rosiglitazone and pioglitazone has been recently reported. CKD-501 is a novel selective PPAR-γ agonist as a potential target to reduce cardiovascular risk in non-insulin dependent diabetes mellitus (NIDDM). In this study, We investigated potential cardiotoxicity of CKD-501 and compared its toxicity with that of rosiglitazone or pioglitazone using db/db mice. After 12-week repeated administration of CKD-501 at doses of 3, 10 and 30 mg/kg/day or rosiglitazone at doses of 10 and 30 mg/kg/day or pioglitazone at doses of 200 and 540 mg/kg/day, animals were sacrificed for investigation of potential toxicities. Diameters of left ventricles and areas of cardiomyocytes were measured. And lipid accumulation and apoptosis in heart muscle were examined by oil red O staining and TUNEL staining, respectively. Diameters of left ventricles were significantly increased in high dose treatment group of pioglitazone compared to control (p<0.05), while other groups showed a tendency for an increase. All test articles induced significantly the increase of area of cardiomyocytes in heart compared to control (p<0.01), in regular order as pioglitazone > CKD-501 ≥ rosiglitazone. However, lipid accumulation and apoptotic changes in heart were not observed in all dosing groups. Taken together, the myocardial cell hypertrophy of CKD-501 are relatively lower than that of pioglitazone and similar to rosiglitazone. And it is suggested that the myocardial cell hypertrophy of CKD-501 are less adverse in clinical use for the management of the NIDDM.

Key Words: PPAR-γ agonist, Cadiotoxicity, CKD-501, Rosiglitazone, Pioglitazone

INTRODUCTION

Non-insulin dependent diabetes mellitus (NIDDM) has become an epidemic and serious worldwide public health issue, characterized by insulin resistance, hyperglycemia and often accompanied with dyslipidemia and obesity (Chen *et al.*, 2009). As the prevalence of this health ailment is increasing dramatically, various therapeutic compounds have been developed to treat this disease, mainly based on targeting for peroxisome proliferator-activated receptors (PPAR).

New drugs based on thiazolidinediones (TZDs) structural motif have been developed. TZDs is a PPAR-γ agonist, which is found in insulin-dependent glucose-requiring tissues such as adipose tissue, skeletal muscle, and liver tissue (Lehmann *et al.*, 1995; Spiegelman, 1998; Young *et al.*, 1998). However, PPAR-γ agonists are known to be at extraordinarily high risk for cardiovascular disease, while they have no or only a slight significant effect on triglycerides (TG), high density lipoprotein (HDL), and low density lipoprotein (LDL) levels (van Wijk *et al.*, 2003). Rosiglitazone and piolgitazone are well known PPAR-γ agonists (Lee, 2008). But it has been reported that use of rosiglitazone was associated with increased the odds ratio for myocardial infarction as 1.43 and for death from cardiovascular causes as 1.64. Therefore, rosiglitazone has recently been withdrawn from the European market and given status of restricted usage in USA (Momose *et al.*, 1991; Cantello *et al.*, 1994). A recent outcomes study of pioglitazone showed a trend toward reduction in vascular events but the increased incidence of congestive heart failure (Nesto *et al.*, 2003). Efforts for developing new mechanism drugs have been continued to reduce these side effect as much as possible, and it is necessary to develop effective therapies for treating NIDDM.

CKD-501 is a novel selective PPAR-γ agonist containing the TZDs group used for the management NIDDM. Generally, a selective affinity to PPAR-γ was associated with better efficacy and pharmacokinetic properties in NIDDM animal model. Based on the previous experiments that compounds which belong to the class of potent selective PPAR-γ agonist have

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***Corresponding Author**
E-mail: kang@nsu.ac.kr
Tel: +82-41-580-2721, Fax: +82-41-580-2932

relatively lower effective concentration 50% than that of pioglitazone and rosiglitazone, CKD-501 has been developed to be a better compound for the treatment of NIDDM compared to rosiglitazone and pioglitazone. However, the cardiotoxicty of CKD-501 was not examined yet. In this study, we investigated the potential cadiotoxicity of CKD-501 compared with rosiglitazone and pioglitaszone in db/db mice.

MATERIALS AND METHODS

Chemicals

CKD-501 was provided by the CKD Research Institute of Chong Kun Dang. Rosiglitazone and pioglitazone were purchased from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO, USA) and 10% solutol (Solutol HS 15, BASF Company Ltd., Seoul, Korea), which is non-ionic solubilizer for use in injections, was selected as a vehicle control.

Animals and treatment

Mice (C57BLKS/J-db/db) were used for this study. Forty male mice at 6 weeks of age were provided by Central Lab. Animal Inc. (Seoul, Korea). Throughout the study period, the animals were housed in a room that was maintained at a temperature of 23 ± 0.5°C and a relative humidity of 20-60%. Animals were housed in each solid bottom polycarbonate cage (W 200 × D 260 × H 130 mm) with sterilized bedding and were offered pellet food for lab animal purchased from Samtako Bio Korea Co., Ltd. (Osan, Korea), and tap water was given via water bottle, *ad libitum*. CKD-501 at doses of 3, 10 and 30 mg/kg/day or rosiglitazone at doses of 10 and 30 mg/kg/day or pioglitazone at doses of 200 and 540 mg/kg/day was daily treated for a 12 weeks by oral gavage. Oral administration was selected according to the intended clinical administration route of above compounds. All compounds were suspended in 10% solutol at dosing volume of 10 ml/kg on the basis of the body weight measured most recently in the repeated administration period.

This study was performed in accordance with the Animal Experimentation Policy of CKD Research Institute of Chong Kun Dang Inc.

Histopathological examination of hearts and livers

Hearts and livers were removed and fixed in 10% neutral buffered formalin (NBF). Formalin-fixed tissues were processed for paraffin sections, embedded in paraffin, sectioned at 4-μm thickness, stained with hematoxylin and eosin (H&E), and examined microscopically.

Measurement of diameters of left ventricles in heart

For histological analysis, the specimens were microscopically examined using 4-fold magnification and were analyzed by image analyzer program (Motic software, Hong Kong).

Measurement of areas of cardiomyocytes of left ventricles in heart

For histological analysis, the specimens were microscopically examined using 40-fold magnification and were analyzed by image analyzer program (Motic software).

Oil red O staining

For oil red O staining, the hearts were embedded in optimal cutting temperature compound (OCT) compound, sectioned at 8-μm thickness. These tissues fixed with ice cold 10% formaldehyde for 5-10 min, distilled water, dried again, sections were incubated in oil red O solution (0.5% oil red O dissolved in 100% propylene glycol) for 8-10 min at 60°C, washed with 85% propylene glycol solution for 2-5 min and counterstained with hematoxylin and distilled water.

Terminal deoxynucleotidyl-mediated dUTP nick labeling (TUNEL) assay

The avidin-biotin complex method was used to detect apoptosis in 5-μm sections of heart tissue that had been dewaxed with xylene and hydrated using a graded ethanol series. Sections were treated sequentially with proteinase K and 3% hydrogen peroxide, and then treated with equilibration buffer, TdT enzyme and anti-digoxigenin peroxidase conjugate according to the manufacturer's instructions (ApopTag® Peroxidase In Situ Apoptosis Detection Kit; Chemicon International, CA). Immune complexes were visualized using DAB (Sigma-Aldrich) as the chromogen. Phosphate buffered saline, instead of TdT enzyme, was used as a negative control. Sections were counterstained with hematoxylin to facilitate their examination under a light microscope.

Table 1. Body and heart weights in PPAR-γ agonists-treated mice

Group	Treatment and dose	Number of mice	Initial body weight (g)	Final body weight (g)	Absolute heart weight (g)	Relative heart weight (%)
1	Vehicle control	5	39.5 ± 1.2[a]	59.5 ± 7.3	0.11 ± 0.01	0.19 ± 0.03
2	Rosiglitazone 10 mg/kg	5	40.7 ± 1.5	68.7 ± 3.1	0.15 ± 0.03	0.22 ± 0.02
3	Rosiglitazone 30 mg/kg	5	41.3 ± 3.1	71.0 ± 5.7	0.17 ± 0.04	0.24 ± 0.07
4	Pioglitazone 200 mg/kg	5	40.7 ± 1.0	75.3 ± 4.4**	0.17 ± 0.03	0.22 ± 0.03
5	Pioglitazone 540 mg/kg	5	40.6 ± 2.4	70.1 ± 8.3	0.18 ± 0.07	0.27 ± 0.14
6	CKD-501 3 mg/kg	5	39.8 ± 2.1	72.7 ± 5.0*	0.16 ± 0.03	0.22 ± 0.04
7	CKD-501 10 mg/kg	5	42.1 ± 3.0	75.1 ± 6.9**	0.17 ± 0.03	0.22 ± 0.03
8	CKD-501 30 mg/kg	5	40.2 ± 1.6	73.3 ± 5.7*	0.16 ± 0.02	0.22 ± 0.03

[a]Data represent mean ± SD.

*, **Significantly defferent from vehicle control at $p<0.05$ or $p<0.01$, respectively.

Statistical analysis

All results are expressed as mean ± SD. For multiple comparisons, the statistical analysis was performed by Tukey-Kramer methods using a JMP program (SAS Institute, Cary, NC). $p<0.05$ was considered to be statistically significant.

RESULTS

Body and heart weights

Initial body weights of treatment groups were not different compared to vehicle control group. However, final body weight was significantly increased in the pioglitazone 200 mg/kg dose group and CKD-501 3, 10 and 30 mg/kg dose groups compared to the vehicle control group ($p<0.01$, $p<0.05$, $p<0.01$ or $p<0.05$, respectively). On the while, absolute heart and relative heart weights of treatment groups were not different compared to vehicle control group (Table 1).

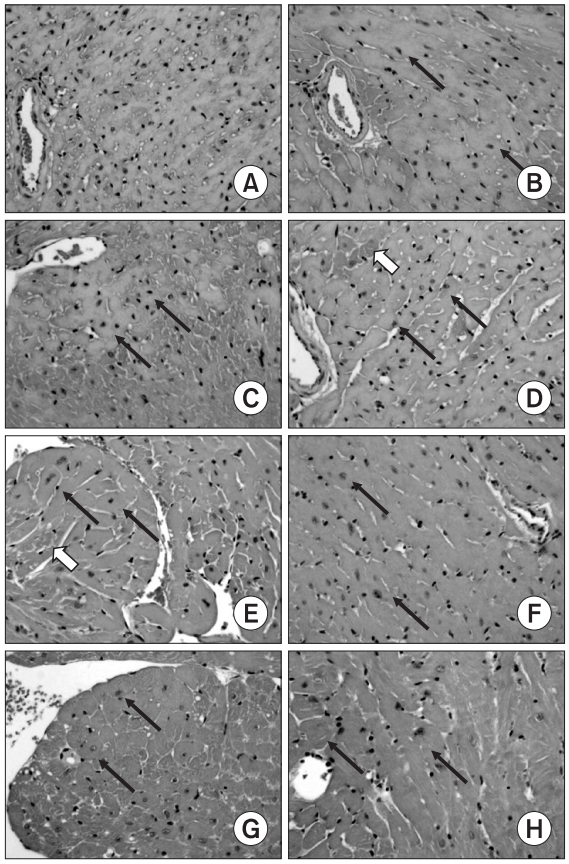

Fig. 1. Histopathological features of heart. (A) G1 as Vehicle; (B) G2 as Rosiglitazone 10 mg/kg; (C) G3 as Rosiglitazone 30 mg/kg; (D) G4 as Pioglitaszone 30 mg/kg; (E) G5 as Pioglitaszone 540 mg/kg; (F) G6 as CKD-501 3 mg/kg; (G) G7 as CKD-501 10 mg/kg; (H) G8 as CKD-501 30 mg/kg. Note the hypertrophy of some cardiomyocytes in hearts treated with test chemicals (arrow) and degeneration of cardiomyocytes (white arrow). Hematoxylin & Eosin (H&E) staining of paraffin embedded sections from the heart from animals, ×400.

Histopathological examination of heart

Histopathological lesions were not observed in the vehicle control groups, but the myocardial cell hypertrophy was observed to varying degrees in all drug-treated groups (Fig. 1). An increasing tendency was observed in the extent and area of myocardial cell hypertrophy depending on the dose of rosiglitazone and pioglitazone. The myocardial cell hypertrophy was also observed in all dose groups of CKD-501.

Measurement of diameters of left ventricles in heart

The diameters of left ventricles were increased significantly in the pioglitazone 540 mg/kg dose group compared to the vehicle control group ($p<0.05$). However, there were no significantly difference between the vehicle control group and rosi-

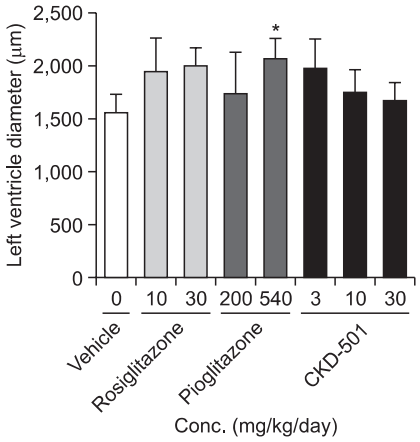

Fig. 2. Diameter of left ventricle in heart. Note the significant difference of diameter of left ventricle in hearts between animals treated with vehicle and Pioglitaszone 540 mg/kg. X axis represents test groups and Y axis represents left ventricle diameter (μm); All results are expressed as mean ± SD. *$p<0.05$ vs vehicle-treated animals.

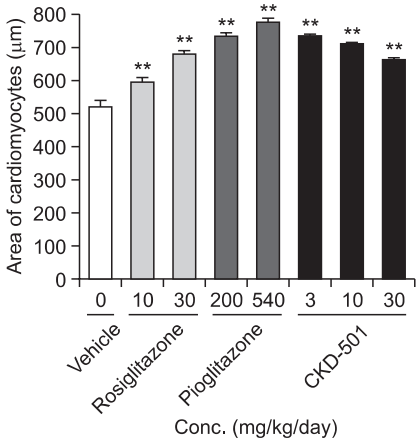

Fig. 3. Area of cardiomyocytes in heart. Note also the significant difference of area of cardiomyocytes in hearts between animals treated with CKD-501 30 mg/kg and rosiglitazone 10 mg/kg, pioglitaszone 30 mg/kg and 540 mg/kg and CKD-501 3 mg/kg and 10 mg/kg. X axis represents test groups and Y axis represents area of cardiomyocytes (μm); All results are expressed as mean ± SD. **$p<0.01$ vs. vehicle-treated animals.

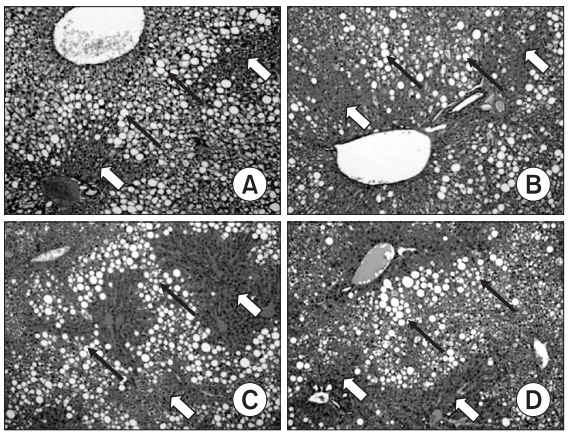

Fig. 4. Histopathological features of liver. (A) Vehicle; (B) Rosiglitazone 30 mg/kg; (C) Pioglitaszone 540 mg/kg; (D) CKD-501 30 mg/kg. Note numerous large and clear fat vacuoles within hepatocytes and displacement of nucleus to the periphery of cell (black arrow) and remaining parenchyma cells (white arrow). However, there are no differences of intensity of fatty change in drug-treated-animals and vehicle control. Hematoxylin & Eosin (H&E) staining of paraffin embedded sections from the liver from animals, ×100.

glitazone dose groups, pioglitazone 200 mg/kg dose group or CKD-501 dose groups (Fig. 2).

Measurement of areas of cardiomyocytes of left ventricles in heart

Treatment of rosiglitazone, pioglitazone or CKD-501 induced a significant increase of areas of cardiomycytes of left ventricles in hearts compared to the vehicle control group (p<0.01) (Fig. 3). However, an increasing degree of the area of myocardial cells was observed in the order as rosiglitazone, CKD-501 and pioglitazone.

Oil red O staining and TUNEL assay

Frozen tissue of abdominal fat was dyed in the positive control group and fat was detected as a result. But the accumulation of fat was not observed in the heart in the vehicle control group and all test substance-treated groups (data not shown).

Apoptosis was not observed in the heart in the vehicle control group and all test articles-treated groups in the present study (data not shown).

Histopathological examination of liver

Numerous large and clear fat vacuoles in the hepatocytes was observed in the vehicle control group and all test substance-treated groups. But, there was no difference in degree of severity between compounds (Fig. 4).

DISCUSSION

PPAR-γ agonists were empirically discovered by their abilities to improve insulin sensitivity in rodent models (Reifel-Miller *et al.*, 2005). However, there have been a lot of reports related to the side effects caused by PPAR-γ agonist such as not only weight gain, edema and increased risk of fracture and also hepatic failure, heart failure, increased heart weight (Breider *et al.*, 1999; Malinowski and Bolesta, 2000; Funk *et al.*, 2001; Kahn *et al.*, 2008; Singh and Loke, 2008). So, new compounds have been studied to reduce these side effects as much as possible. CKD-501 was developed as a novel selective PPAR-γ agonist containing the TZD group used for the management of NIDDM.

Based on the result of the histopathological evaluation of the heart in db/db mice treated by rosiglitazone and pioglitazone, all test chemical induced the myocardial cell hypertrophy to varying degrees. And dose-dependent increasing tendency for myocardial cell hypertrophy was observed in rosiglitazone and pioglitazone treated mice. However, this pattern was not appeared in CKD-501 treatment groups.

In measurement of the left ventricular thickness, high dose treatment of pioglitazone induced increase of diameters of left ventricles. Other treatment also showed increased pattern, even though there were no the statistical significance. In measurement of area of cardiomyocytes in hearts, all test articles induced a significant increase of it, which represented increasing rates in order as pioglitazone > CKD-501 ≥ rosiglitazone. Treatment of pioglitazone and rosiglitazone showed a dose-dependent increasing pattern, however, treatment of CKD-501 did not showed this pattern. Further studies will be warranted to elucidate the mechanism of this pattern.

Myocardial cell hypertrophy was known to be caused from increased hemodynamic loading and subsequent abnormal systolic and diastolic stress in the myocardial fibers. It showed two pathological patterns: 1) concentric hypertrophy induced by the blood pressure overload and 2) eccentric hypertrophy induced by the blood volume overload (Grant *et al.*, 1965; Grossman *et al.*, 1975). It seemed that it was difficult to distinguish these two hypertrophy patterns by the observation of myocardial cells with an optical microscope from a cross-section of the hearts in this study.

For the measurement of the diameter by selecting only the myocardial cells located in the left ventricular wall some variations were appeared depending on the location and there were somewhat differences in diameter of myocardial cell depending on the anatomical location in the same dose group. As a cross-sectional shape of myocardial cells was variously observed, it is difficult to identify of groups quantified by measuring the diameter of myocardial cells in the current research. To reduce the variation as much as possible, additional molecular imaging technologies such as pre-clinical magnetic resonance imaging and computed tomography will be required in further studies.

In additions, a number of studies have shown that PPAR partial agonists including selective PPAR modulators have improved side effect profiles compared with full agonists (Reifel-Miller *et al.*, 2005). CKD-501 is developed as a selective PPAR-γ agonist and therefore, it is assumed that the side effects of CKD-501 in heart are relatively lower than those of rosiglitazone and pioglitazone.

It was necessary to investigate the lipid accumulation or apoptosis in the heart because myocardial lipid accumulation might be the mechanisms underlying the detrimental effects during the development of pathological cardiac hypertrophy (He *et al.*, 2012) and myocardial lipid accumulation was associated with the increased cardiomyocyte apoptosis in response to the PPAR-γ expression in the heart (Son *et al.*, 2007; Son *et al.*, 2010). However, the lipid accumulation or apoptosis in the heart was not observed in all test article-treated groups. So, it seemed that test articles used in this study did not affect

lipotoxicity in the heart. It may be associated with the relatively short dosing period or lower doses than other studies reporting the lipotoxicity in rodents (Golfman *et al.*, 2005; Vikramadithyan *et al.*, 2005; Wang and Unger, 2005).

Fatty degeneration in the liver was observed in all test article-treated mice. It was considered as the characteristics of db/db mice, showing obesity and severe NIDDM (Lee *et al.*, 2010; Yamaguchi *et al.*, 2007). And there were no alteration of it associated with test articles in this study.

In conclusion, the myocardial cell hypertrophy of CKD-501 are relatively lower than that of pioglitazone and similar to rosiglitazone. And it is suggested that the myocardial cell hypertrophy of CKD-501 are less adverse than when compared to reference compounds in clinical use for the management of the NIDDM.

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