Original Article

Beneficial Effects of Pioglitazone on Left Ventricular Hypertrophy in Genetically Hypertensive Rats

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Beneficial effects of thiazolidinediones, peroxisome proliferator–activated receptor γ (PPARγ) agonists, on cardiovascular injuries have been reported. However, the effects of these agonists on left ventricular (LV) hypertrophy have not been clarified. To investigate whether pioglitazone improves LV hypertrophy, we used 32-week-old stroke-prone spontaneously hypertensive rats (SHR-SP) that had been treated or not treated with pioglitazone (10 mg/kg/day) for 8 weeks, and Wistar Kyoto rats (WKY). We evaluated LV geometry by echocardiography; myocyte hypertrophy, tissue fibrosis, and appearance of myofibroblasts by histological examination; mRNA expression by real-time polymerase chain reaction (PCR); protein expression by Western blot; activities of matrix metalloproteinase (MMP) by zymography; and production of reactive oxygen species (ROS) by electron spin resonance spectroscopy or thiobarbituric acid reactive substances (TBARS). SHR-SP showed concentric hypertrophy of the LV, but WKY did not. The myocyte diameter, fraction of tissue fibrosis, and number of myofibroblasts were greater in SHR-SP. mRNA expressions of collagen type I and type III, tissue growth factor (TGF)-β1, and brain natriuretic peptide (BNP); protein expression of connective tissue growth factor (CTGF); activities of MMP2 and MMP9; and ROS were increased in SHR-SP. Pioglitazone did not decrease blood pressure, but partially normalized LV geometry in addition to decreasing myocyte diameter, interstitial fibrosis and number of myofibroblasts; mRNA levels of collagen type I and BNP; MMP2 activity; and protein level of CTGF. However, the mRNA level of collagen type III and TGF-β1, MMP9 activity, and ROS production were not improved. In conclusion, pioglitazone reversed the concentric LV remodeling independently from blood pressure or oxidative stress in chronic hypertension. (*Hypertens Res* 2007; 30: 863–873)

***Key Words*: spontaneously hypertensive rat, left ventricular hypertrophy, peroxisome proliferator–activated receptor γ, thiazolidinedione, myofibroblast**

Introduction

A long-term elevation in systemic blood pressure induces pathogenic cardiac hypertrophy. This process, associated with increased cardiomyocyte size and collagen volume, leads to a deleterious outcome in hypertension by causing heart failure (*1*, *2*). Chronic pressure or volume overload, and various neuron-hormonal stimulations, including stimulation of the renin-angiotensin system, inflammation, and oxidative stress, stimulate the hypertrophy of myocardial cells and advance the proliferation and conversion to myofibroblasts of cardiac fibroblasts, which produce matrix proteins (*3*–*7*).

The peroxisome proliferator–activated receptor γ (PPARγ)

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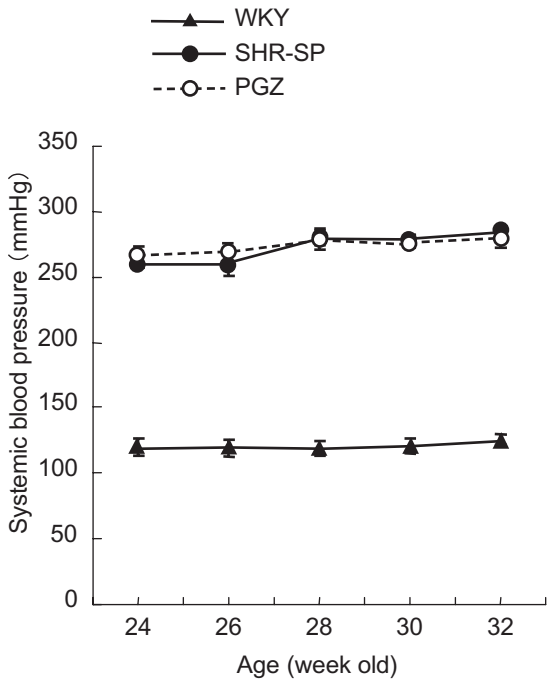

Fig. 1. *Changes in systolic blood pressure (SBP) level in Wistar Kyoto rats (WKY; closed triangles), untreated stroke-prone spontaneously hypertensive rats (SHR-SP; closed circles), and pioglitazone-treated SHR-SP (PGZ; open circles). Pioglitazone was administered from 24 weeks of age for 8 weeks. The SBPs of untreated and pioglitazone-treated SHR-SP were different from those of WKY ($p < 0.0001$). Pioglitazone did not decrease blood pressure.*

agonists known as thiazolidinediones, which include pioglitazone and rosiglitazone, have been developed as anti-diabetic agents that improve insulin sensitivity (*8–12*). Accumulating evidence suggests that thiazolidinediones have anti-inflammatory, anti-oxidant, and anti-fibrotic effects in addition to their anti-diabetic effects (*10*). These actions are considered to come from the stimulation by PPARγ and various mechanisms (*8–12*). Due to these possible beneficial effects, thiazolidinediones are assumed to protect against cardiovascular organ injury, since the evidence has shown that PPARγ is present in the heart and vascular tissues (*11*, *12*). However, the action of thiazolidinediones on the heart has remained controversial. Thiazolidinediones and an intrinsic agonist of PPAR-γ, 15-deoxy-Δ12,14-prostaglandin J2 (15d-PGJ2), suppressed hypertrophy of cardiomyocytes that was induced by various agonists or stretch-stimulation *in vitro* (*13*, *14*). Pioglitazone and troglitazone prevented cardiac hypertrophy in aortic banding model rats (*15*) and N^G-nitro-L-arginine methyl ester (L-NAME)–treated rats (*16*). Thiazolidinediones also inhibited the accumulation of collagen volume in a mouse model of myocardial infarction (*17*) and in deoxycorticosterone acetate (DOCA)–salt rats (*18*). However, in other studies, rosiglitazone (*19*) or pioglitazone (*20*) did not exert

Table 1. Body Weight and Heart Weight of Rat at 32 Weeks of Age

	Parameter		
	Body weight (g)	Heart weight (g)	(Heart weight/body weight)×100
WKY	448±6	1.39±0.04	0.31±0.01
SHR-SP	353±11*	1.96±0.05*	0.56±0.02*
PGZ	346±8*	1.79±0.04*,†	0.52±0.01*

WKY, Wistar Kyoto rats; SHR-SP, stroke-prone spontaneously hypertensive rats; PGZ, pioglitazon-treated SHR-SP. Values are mean±SEM. $n=12$ per group. *$p<0.001$ *vs*. WKY; †$p<0.05$ *vs*. SHR-SP.

such beneficial actions on myocardial infarction. In addition, evidence of the effects of thiazolidinediones on left ventricular (LV) hypertrophy in chronic hypertension is limited; for example, there has been only one study examining these effects in genetically hypertensive rats (*21*). Since LV hypertrophy in patients with essential hypertension develops gradually, use of chronic hypertension model animals is valuable. In the present study, we investigated whether pioglitazone administration from 24 to 32 weeks of age could improve hypertensive cardiac hypertrophy in stroke-prone spontaneously hypertensive rats (SHR-SP).

Methods

Animals

This study was approved by the Animal Care and Use Committee, University of the Ryukyus, and was conducted in accordance with its recommendations. Male SHR-SP/IZM ($n=24$) and Wistar Kyoto (WKY)/IZM ($n=12$) rats (Disease Model Cooperative Research Association, Kyoto, Japan) were fed a standard rat chow (CE-2 containing 0.5% NaCl; Japan Clea, Tokyo, Japan) and had free access to tap water. At 24 weeks of age, SHR-SP were assigned to either a control group or a pioglitazone group; pioglitazone (10 mg/kg/day) was mixed with chow and administered for 8 weeks. The amount of pioglitazone mixed with chow was calculated from the weight of chow eaten in the preceding day. Systolic blood pressure (SBP) was measured by the tail-cuff method every 2 weeks from 24 to 32 weeks of age. At 32 weeks, rats were used for the experiments.

Rats were deeply anesthetized with diethyl ether and then sacrificed. The hearts were immediately excised and weighed. The left ventricle (LV) was separated from the atrium and right ventricle, and was frozen in liquid nitrogen and stored at −80°C until use. Plasma was collected and stored at −80°C for future measurement of glucose and insulin. Insulin and glucose were measured by enzyme assay and enzyme immunoassay, respectively. The other rats were killed with a sodium pentobarbital overdose and were perfu-

Table 2. Echocardiographic Parameters for the Left Ventricle

	Parameter						
	IVS (cm)	LVPW (cm)	LVDd (cm)	LVDs (cm)	RWT	EF	%FS
WKY	0.24±0.01	0.25±0.01	0.64±0.01	0.30±0.01	0.43±0.01	78.1±1.4	53.4±1.4
SHR-SP	0.34±0.03**	0.33±0.02**	0.54±0.02*	0.27±0.02	0.55±0.01**	75.7±2.2	51.3±2.3
PGZ	0.28±0.01†	0.27±0.01†††	0.63±0.03††	0.30±0.03	0.47±0.01**,††	76.1±2.6	52.2±3.0

WKY, Wistar Kyoto rats; SHR-SP, stroke-prone spontaneously hypertensive rats; PGZ, pioglitazon-treated SHR-SP; IVS, thickness of interventricular septum; LVPW, thickness of left ventricular posterior wall; LVDd, end-diastolic left ventricular dimension; LVDs, end-systolic left ventricular dimension; RWT, relative wall thickness; EF, ejection fraction; %FS, %fractional shortening. Values are mean±SEM. $n=12$ per group. *$p<0.005$, **$p<0.001$ *vs*. WKY; $^{\dagger}p<0.05$, $^{\dagger\dagger}p<0.01$, $^{\dagger\dagger\dagger}p<0.001$ *vs*. SHR-SP.

sion-fixed *via* the apex for 5 min at 120 mmHg in WKY, and at 200 mmHg in SHR-SP and pioglitazone-treated SHR-SP with 4% paraformaldehyde buffered with 0.1 mol/L NaH_2PO_4. The heart was then excised and processed in paraffin using routine techniques.

Echocardiography

Rats were anesthetized with an intraperitoneal injection of sodium pentobarbital (50 mg/kg). Echocardiographic studies were performed with a 7.5-MHz linear-array transducer (Sonolayer, Toshiba, Tokyo, Japan) by one of the authors (T.S.). The two-dimensional short-axis view of the LV and M-mode tracings at the papillary muscle level were recorded. The end-diastolic left ventricular dimension (LVDd), end-systolic left ventricular dimension (LVDs), diastolic interventricular septal thickness (IVS), and diastolic left ventricular posterior wall thickness (LVPW) were measured. The relative wall thickness (RWT), LV fractional shortening (%FS), and LV ejection fraction (EF) were calculated as a percentage from the LVDd and LVDs.

Histological Examination

Three micrometer–thick sections of LV, embedded in paraffin, were used for the staining with the hematoxylin-eosin for evaluation of cardiomyocyte hypertrophy and that with the Masson's trichrome for evaluation of tissue fibrosis. In addition, other transverse sections were stained with mouse anti–α-smooth muscle actin monoclonal antibody (1:5 dilution; Nichirei, Tokyo, Japan) for evaluation of the myofibroblast appearance by a standard immunostaining technique.

To determine the cardiomyocyte size, the shortest transverse diameter was measured in 30 nucleated transverse sections of the myocytes per heart at ×400 magnification. To determine the percent area of myocardial interstitial fibrosis, 30 fields of the heart were recorded at ×200 magnification. To evaluate the perivascular fibrosis, short-axis images of the 8–16 round vessels of the heart were studied at ×100 to ×200 magnification. The perivascular fibrosis was determined as the ratio of the area of fibrosis surrounding the vessel wall to the total vessel area.

Real-Time Reverse Transcription–Polymerase Chain Reaction

The mRNA expression levels of collagen type I, collagen type III, transforming growth factor-β1 (TGF-β1), brain natriuretic peptide (BNP), PPARγ, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were analyzed by real-time polymerase chain reaction (PCR). Total RNA was isolated from homogenized LV myocardium tissue using ISOGEN (Nippon Gene Co., Tokyo, Japan). The reverse transcription (RT) reaction was performed with a TaKaRa High Fidelity RNA PCR Kit (Takara Bio Co., Tokyo, Japan). PCR was carried out with an LC Fast Start DNA Master SYBR Green kit (Roche Applied Science, Mannheim, Germany) using 2 μL of cDNA, corresponding to 50 ng of total RNA in a 20 μL final volume, 3 mmol/L $MgCl_2$ and 0.5 μmol/L of each primer (final concentration). Briefly, quantitative PCR was performed using a LightCycler (Roche Applied Science). Amplification specificity was checked using a melting curve following the manufacturer's instructions. mRNA expression values were normalized to those of GAPDH expression.

Gelatin Zymography

Tissues were homogenized in RIPA buffer (in mmol/L: 150 NaCl, 10 Tris-HCl, pH 7.40, 1 EDTA, 10 NaF, 0.2 Na_3VO_4, 1% NP-40, 0.1% sodium deoxycholate, 0.1% sodium dodecylsulfate [SDS], 1 phenylmethylsulfonyl fluoride [PMSF], 1 μg/mL protease inhibitor) with a Polytron homogenizer for 30 s, and debris was removed by centrifugation at 600 × *g* for 10 min. The activities of matrix metalloproteinase (MMP)-2 and MMP-9 were measured by gelatin zymography. Samples (50 μg protein) were separated on a 10% SDS-polyacrylamide gel impregnated with 1 mg/mL gelatin. After the electrophoresis, the gel was washed at room temperature for 30 min in washing buffer (2.5% Triton X-100), then incubated overnight at 37°C with shaking in the development buffer (50 mmol/L Tris-HCl, pH 8.8, 5 mmol/L $CaCl_2$, 0.02% NaN_3). The gel was stained with a solution of 0.25% Coomassie brilliant blue R-250. Clear zones against the blue background indicated the presence of gelatinase activity. The band intensity was estimated with the use of NIH Image software.

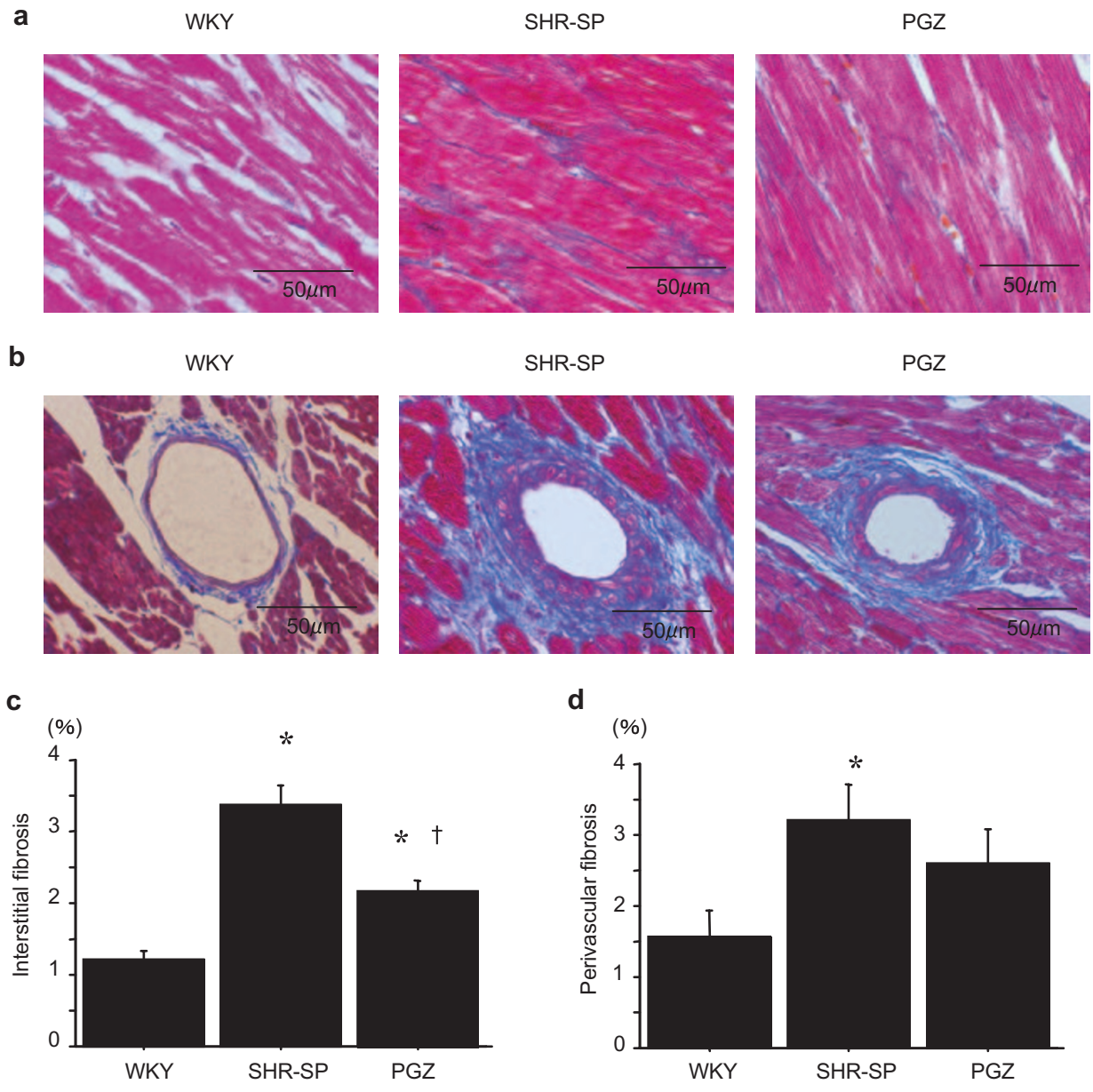

Fig. 2. *Interstitial fibrosis (a) and perivascular fibrosis (b) of the left ventricle (LV) from WKY, untreated SHR-SP, and pioglitazone-treated SHR-SP (PGZ). Quantitative analysis of interstitial fibrosis (c) and perivascular fibrosis (d) in the LV. The fibrosis was determined by a light microscope after Masson's trichrome staining. Results are the mean±SEM (n = 3 per group). *p < 0.001 vs. WKY, †p < 0.0001 vs. SHR-SP.*

Western Blot Analysis

Connective tissue growth factor (CTGF) protein expression was determined by Western blotting analysis. Samples (50 μg protein) were separated on a 12.5% SDS-polyacrylamide gel and were transferred onto PVDF membranes (Bio-Rad). The membranes were blocked with 5% nonfat dry milk and thereafter probed with primary antibody against either CTGF (1:5,000 dilution) (Abcam, Cambridge, UK) or α-tubulin (TU-02) (1:1000 dilution) (Santa Cruz Biotechnology, Santa Cruz, USA). Horseradish peroxidase–linked antibodies (Amersham Biosciences, Piscataway, USA) were then used as secondary antibodies. Blotted membranes were visualized with ECL-plus reagent (Amersham Biosciences). The band intensity was estimated with the use of NIH Image software. The expression level of CTGF protein was normalized to that of α-tubulin protein.

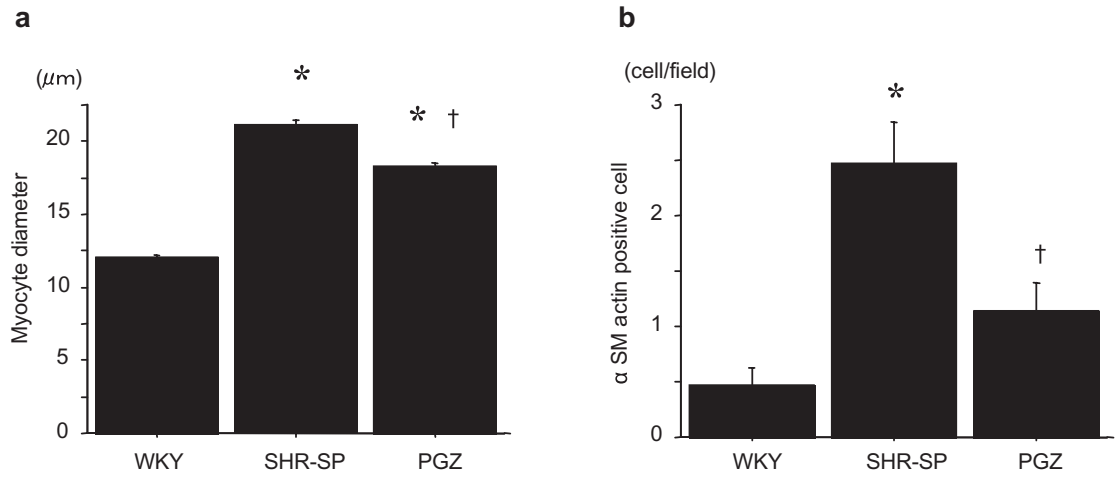

Fig. 3. *a: Transverse diameter of cardiomyocytes in the LV of WKY, untreated SHR-SP, and pioglitazone-treated SHR-SP (PGZ). Results are the mean±SEM (n=3 per group). b: Number of myofibroblasts in the LV of WKY, untreated SHR-SP, and pioglitazone-treated SHR-SP (PGZ). Results are the mean±SEM (n=3 per group). *$p<0.001$ vs. the WKY, †$p<0.0001$ vs. SHR-SP.*

Evaluation of Oxidative Stress

Reactive oxygen species (ROS) production was quantified in the LV tissue by electron spin resonance (ESR) spectroscopy with 4-hydroxy-2,2,6,6-tetramethyl-piperidinyloxy (hydroxy-TEMPO) (SIGMA ALDRICH Inc., Steinheim, Germany) (*22*). Measurements were performed at room temperature using an X-band (9.45 GHz) ESR spectrometer (JES-RE-1X; JEOL, Tokyo, Japan). LV myocardial samples were homogenized in 50 mmol/L sodium phosphate buffer (pH 7.4) containing protease inhibitors. The homogenate was immediately reacted with hydroxy-TEMPO (0.1 mmol/L), and its ESR spectra were recorded for up to 10 min at intervals of 1 to 3 min. The rate of signal decay reveals the presence of ROS.

The thiobarbituric acid reactive substance (TBARS) levels of the LV were determined by a spectrophotometric method using thiobarbituric acid (*23*). The homogenate (0.1 mL) was mixed with 0.2 mL of 8.1% SDS, 1.5 mL of 20% acetic acid (pH 3.5), 1.5 mL of 0.8% thiobarbituric acid, and 0.05 mL of butylated hydroxytoluene (BHT). The mixture was incubated at 100°C for 60 min and then cooled. One milliliter of distilled water and 5 mL of an *n*-butanol:pyridine (15:1 v/v) mixture was added to the mixture and centrifuged at $700 \times g$ for 10 min. TBRAS was extracted with a mixture of butanol: pyridine (15: 1 v/v), and the absorbance was read at 532 nm.

Statistical Analyses

All data are presented as the mean±SEM. Statistically significant differences in mean values were tested by analysis of variance. Fisher's least significant difference test was used for comparisons between the groups. The differences were considered statistically significant at a value of $p<0.05$.

Results

Blood Pressure, Heart Weight, and Plasma Levels of Insulin and Glucose

Changes in SBP from 24 to 32 weeks of age are shown in Fig. 1. Untreated SHR-SP and pioglitazone-treated SHR-SP had comparable SBP levels, which were significantly greater than those of WKY. Body weight and heart weight at 32 weeks of age are presented in Table 1. Untreated SHR-SP had a greater heart weight and heart weight/body weight ratio compared with age-matched WKY. Pioglitazone administration to SHR-SP decreased the heart weight, but not the heart weight/body weight ratio.

Plasma glucose levels did not significantly differ among WKY, untreated SHR-SP, and pioglitazone-treated SHR-SP (140.5±9.3 mg/dL, 164.3±2.3 mg/dL, 149.5±10.0 mg/dL, respectively; $n=6$ in each group). The plasma levels of insulin in WKY and untreated SHR-SP were comparable, and were lower in pioglitazone-treated SHR-SP than in WKY (WKY 1.00±0.15 ng/mL, SHR-SP 0.68±0.28 ng/dL, pioglitazone-treated SHR-SP 0.42±0.09 ng/dL, $p<0.05$ for both SHR-SP groups *vs*. WKY; $n=6$ in each group).

Echocardiographic Results

The structure and function of the LV were evaluated by echocardiography (Table 2). The IVS, LVPW, and RWT were increased, and LVDd was decreased in untreated SHR-SP compared with WKY. Pioglitazone administration decreased IVS, LVPW, and RWT, and increased LVDd.

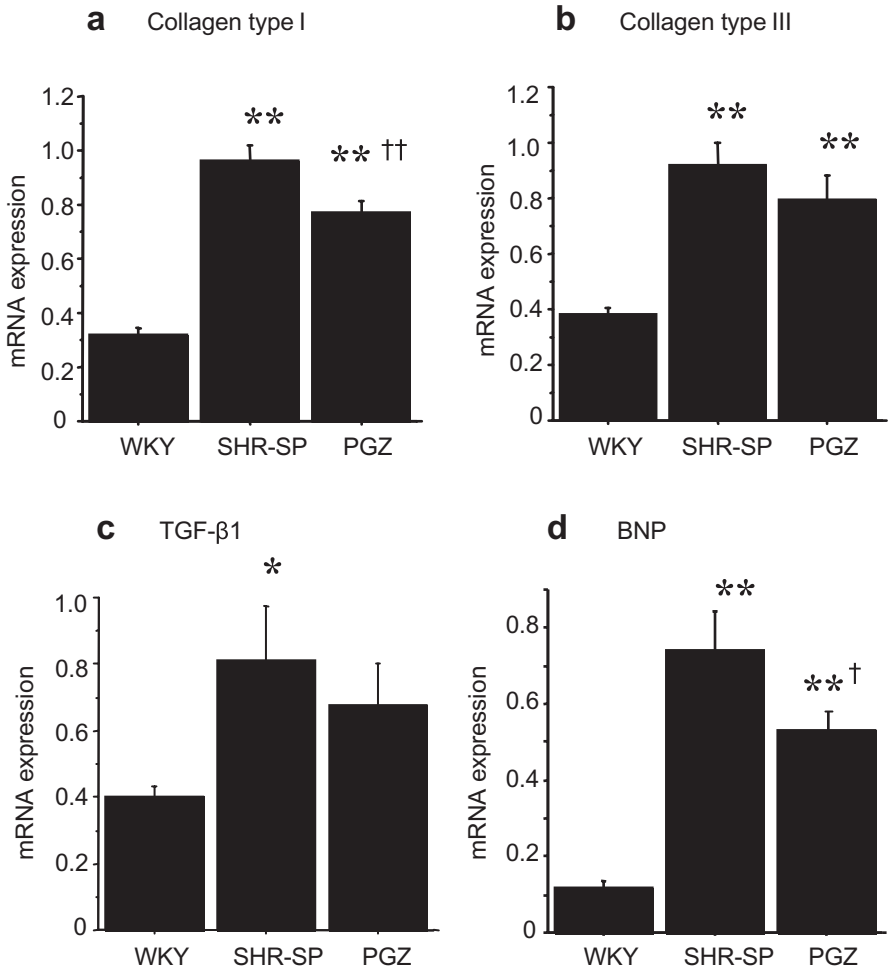

Fig. 4. *Expressions of mRNA in the LV of collagen type I (a), collagen type III (b), transforming growth factor (TGF)-β1 (c), and brain natriuretic peptide (BNP: d) from WKY, untreated SHR-SP, and pioglitazone-treated SHR-SP (PGZ). The expression was normalized to that of GAPDH, a housekeeping gene. Results are the mean±SEM. n=6 to n=10 per group. *p<0.05, **p<0.0005 vs. WKY; †p<0.05, ††p<0.005 vs. SHR-SP.*

There was no significant difference among the three groups in the EF and %FS of the LV.

Histological Analysis

The interstitial and perivascular collagen volume fraction were significantly higher in untreated SHR-SP than in WKY (Fig. 2). Pioglitazone administration significantly decreased the interstitial fibrosis. The decrease in the perivascular fibrosis by pioglitazone was small.

The diameter of cardiomyocytes was significantly greater in the untreated SHR-SP than in the WKY (Fig. 3a). Pioglitazone administration decreased the myocyte size. In untreated SHR-SP, the number of fibroblasts expressing α-smooth muscle actin, a marker for differentiation to the myofibroblast phenotype, was greater than in WKY. This increase was also significantly reduced by pioglitazone (Fig. 3b).

mRNA Expressions of Collagen Type I, Collagen Type III, and TGF-β1, and BNP

In untreated SHR-SP, mRNA levels of collagen type I, collagen type III, TGF-β1, and BNP were increased compared with those in WKY (Fig. 4). Pioglitazone administration decreased the mRNA levels of collagen type I and BNP. However, those of collagen type III and TGF-β1 were comparable between untreated and pioglitazone-treated SHR-SP.

MMP Activities

Myocardial gelatinase activities at 72 kD (MMP-2) and genolytic activities at 92 kD (MMP-9) were evaluated by gelatin zymography. Both MMP-2 and MMP-9 activities were increased in untreated SHR-SP compared with WKY. Pioglitazone administration significantly decreased MMP-2 activ-

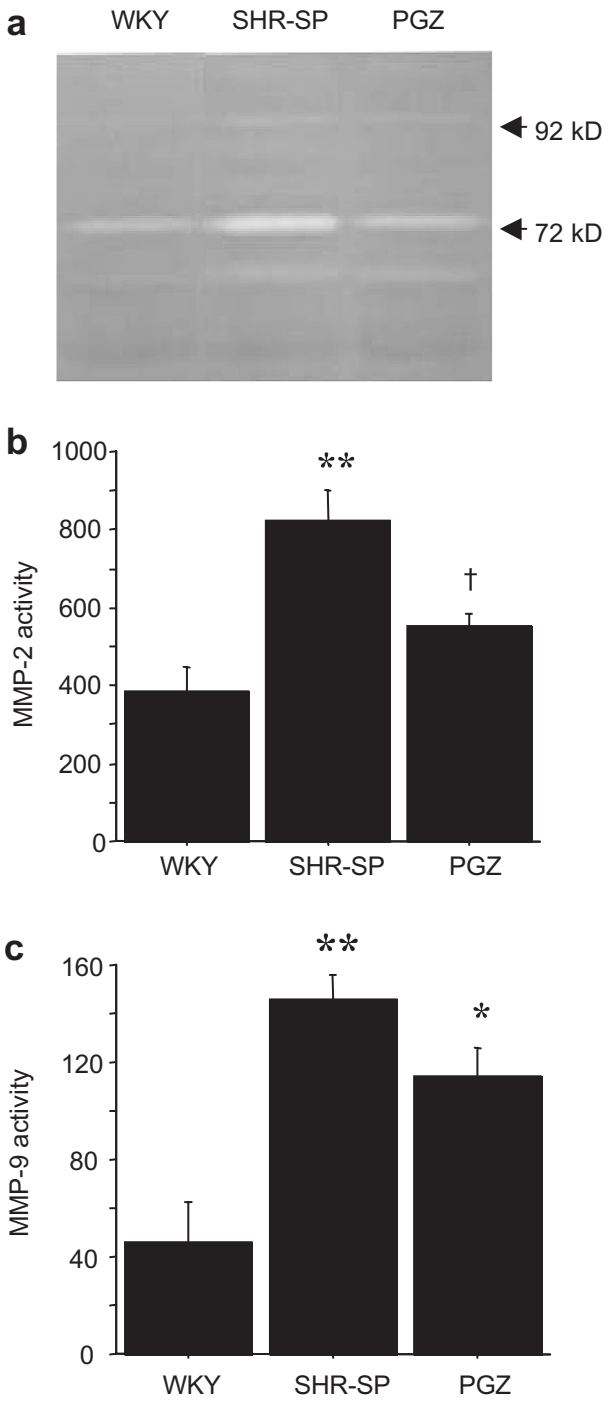

Fig. 5. *a: Representative data from zymography comparing the myocardial matrix metalloproteinases (MMP)-2 and MMP-9 in WKY, untreated SHR-SP, and pioglitazone-treated SHR-SP (PGZ). b: Densitometric analysis of MMP-2 activity. c: Densitometric analysis of MMP-9 activity. Results are the mean±SEM (n=4). *p<0.005, **p<0.001 vs. WKY; †p<0.05 vs. SHR-SP.*

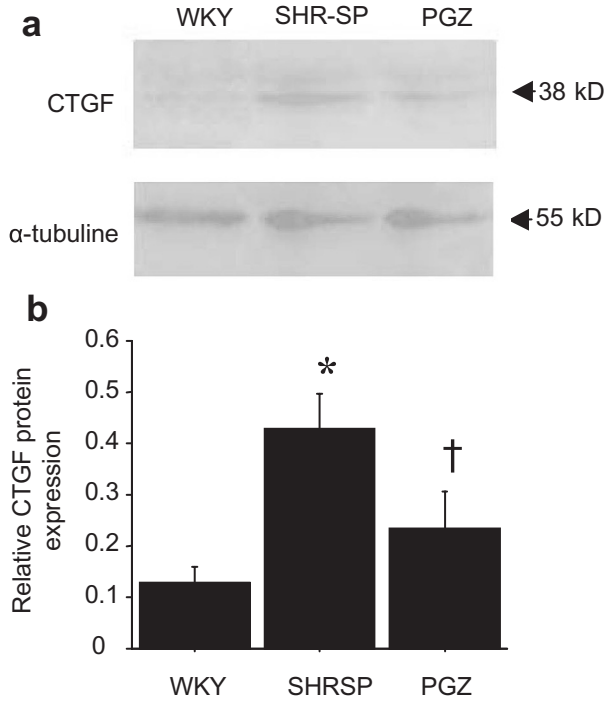

Fig. 6. *a: Representative blots comparing myocardial connective tissue growth factor (CTGF) protein expression and α-tubulin protein expression by Western blot analysis in WKY, untreated SHR-SP, and pioglitazone-treated SHR-SP (PGZ). b: Densitometric analysis of the immunoreactive bands of myocardial CTGF relative to α-tubulin. Results are the mean±SEM (n=3). *p<0.05 vs. WKY, †p<0.05 vs. SHR-SP.*

ity, and tended to decrease MMP-9 activity (Fig. 5).

CTGF Protein Expression

CTGF protein expression was greater in untreated SHR-SP than in WKY (Fig. 6). Pioglitazone administration significantly reduced this up-regulation of CTGF protein.

ROS Production

The rate of electron spin resonance signal decay, an index of the amount of ROS, was significantly greater in untreated SHR-SP than in WKY (Fig. 7). Pioglitazone administration did not significantly decrease the rate of ESR signal decay. Myocardial TBARS levels were also comparable between pioglitazone-treated (10.5±0.7 nmol/g; $n=5$) and untreated SHR-SP (10.4±0.6 nmol/g; $n=5$).

PPARγ Levels

Expression levels of PPARγ in the LV were examined by real-time PCR. The mRNA levels of PPARγ were comparable

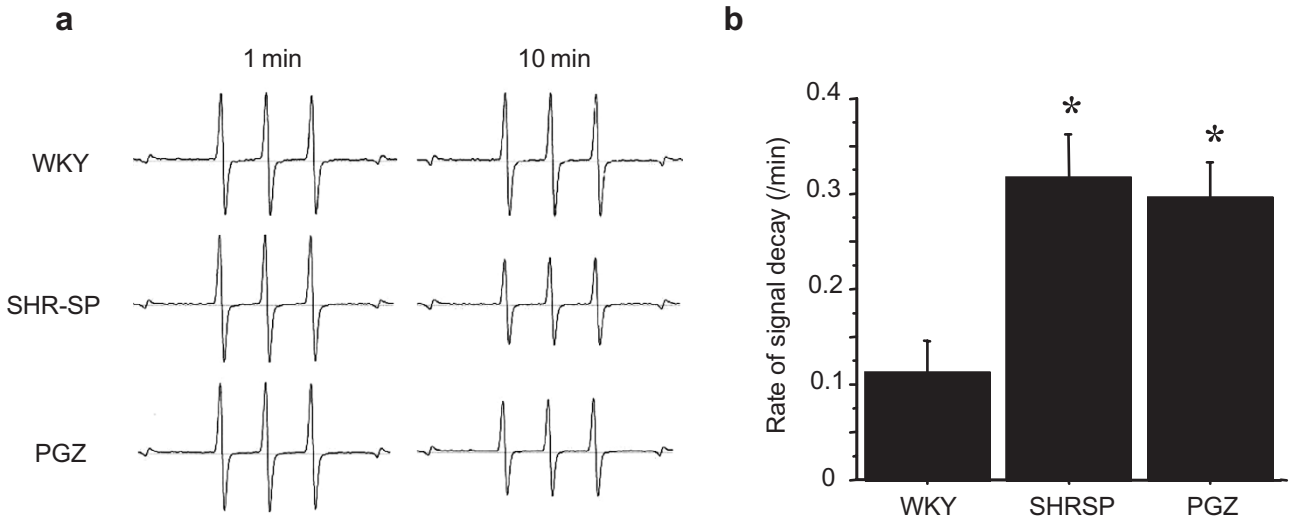

Fig. 7. *Electron spin resonance (ESR) analysis with hydroxy-TEMPO in myocardial tissues from WKY, untreated SHR-SP, and pioglitazone-treated SHR-SP (PGZ). a: Sequential sample of ESR spectra of hydroxy-TEMPO. b: Summary data for the rate of hydroxy-TEMPO signal decay for LV tissues from WKY, SHR-SP, and PGZ. Results are the mean±SEM (n=7). The decay rates of the three groups are comparable. *p<0.005 vs. WKY.*

among WKY, untreated SHR-SP, and pioglitazone-treated SHR-SP, although pioglitazone tended to increase the level (WKY 0.86±0.08; untreated SHR-SP 1.05±0.05; pioglitazone-treated SHR-SP 1.22±0.11; $n=6$ in all groups, values are relative to the GAPDH level).

Discussion

Several studies have shown that thiazolidinediones or PPARγ ligands prevent pathogenic cardiac hypertrophy and/or fibrosis in animal models of hypertension (*13–17*). In previous studies, the action of thiazolidinediones was evaluated primarily in models with acute pressure overload (*13*, *15*) or those with angiotensin II-administration (*24*). In addition, the administration of thiazolidinediones preceded the development of hypertension or cardiac hypertrophy in these studies (*13–17*). In contrast, we have shown that pioglitazone ameliorated the cardiac hypertrophy and fibrosis that had already developed.

In the present study, pioglitazone did not alter the SBP level, glucose level, or insulin level in SHR-SP. These results indicate that the changes observed in the LV were independent of the anti-depressor action or anti-diabetic action of this drug. However, we have not examined the insulin-sensitivity in these rats. Also, although the difference was not statistically significant, pioglitazone treatment tended to decrease the glucose level and insulin level in SHR-SP. Tokudome *et al*. reported that high glucose and high insulin had direct effects on protein synthesis in cardiac myocytes and on DNA and collagen synthesis in cardiac fibroblasts (*25*). The insulin resistance may unfavorably effect cardiac structure and function in hypertensive patients (*26*). The possibility that improvement of insulin sensitivity would result in beneficial effects should be further tested.

Diep *et al*. reported that pioglitazone administration from 6 weeks of age for 20 weeks only slightly attenuated LV hypertrophy or fibrosis, whereas this treatment improved cardiac inflammatory parameters (*21*). We observed that the same dose of pioglitazone, when administered from 24 weeks of age for 8 weeks, recovered the LV hypertrophy. The reason for this discrepancy remains unknown; it is possible, however, that the long duration (20 weeks) of drug administration may have masked the beneficial effects of pioglitazone in their study, since the severe hypertension persisted during the protocol.

Evidence has accumulated that cardiac fibroblasts play an important role in the development of cardiac hypertrophy and pathogenesis of cardiac failure *via* the production of matrix proteins in the extracellular spaces (*3–7*). In the present study, the cardiac collagen volume, the number of myofibroblasts, the mRNA levels of collagen type I, collagen type III, and TGF-β, and the protein levels of CTGF were increased in untreated SHR-SP more than in WKY. Pioglitazone decreased the number of myofibroblasts, decreased the levels of collagen type I mRNA, and improved the interstitial fibrosis of the LV. The proliferation of fibroblasts and their transition to myofibroblasts are important regulatory mechanisms for the generation of collagen (*4*). It is thus likely that pioglitazone improved the LV fibrosis at least in part by affecting fibroblasts or myofibroblasts. In contrast, pioglitazone did not enhance, but rather inhibited the MMP activities. Thus, the change in MMP activities may not be a mechanism for the

antifibrotic action of pioglitazone. Conversely, the decrease in MMP activities may confer protection against cardiac failure, since the increase in MMP activities has been reported as one of the mechanisms for the deterioration of LV structure and function (*27*).

Fibroblast proliferation and myofibroblast differentiation are regulated by various mechanism, including calcineurin-nuclear factor of the activated T cells (NFAT) pathway (*28*) and the TGF-β/CTGF pathway (*29*). In the present study, pioglitazone decreased the expression of CTGF, but not that of TGF-β. CTGF has been shown to stimulate proliferation and differentiation of fibroblasts, and regulates extracellular matrix protein synthesis in association with TGF-β (*30*, *31*). However, recent studies have shown that CTGF gene expression is also regulated by the calcineurin-NFAT pathway (*32*) and the Smad pathway (*33*), independently from TGF-β.

The inflammatory response stimulates TGF-β activation *via* proinflammatory transcriptional factors such as nuclear factor-κB (NF-κB), activating protein-1(AP-1), and the signal transducer and activator of transcription (STAT) (*34*). It is possible that thiazolidinediones suppress NF-κB activation, and modify cytokine production, including that of tumor necrosis factor-α (TNFα) and TGF-β, as reported in previous papers (*17*, *21*, *35*). Diep *et al*. reported that pioglitazone administration for 20 weeks inhibits cardiac inflammatory responses, including NF-κB activation, AP-1 binding, and expression of TNFα in the LV of SHR-SP (*21*). However, a recent study showed that pioglitazone caused an anti-proliferative effect and reduced extracellular matrix production through mechanisms independent of TGF-β in human renal fibroblast cells (*36*). In addition, in L-NAME–treated rats, which developed cardiac hypertrophy, pioglitazone did not reduce TGF-β levels, but improved LV fibrosis (*16*). Although we have not examined in detail the change in cardiac inflammatory responses, mRNA levels of TGF-β were not altered by pioglitazone in the LV from adult SHR-SP. Thus, the mechanism that explains the anti-fibrotic effects of thiazolidinediones may differ among various disease models and various organs.

In the present study, the myocardial cell hypertrophy was also improved by pioglitazone. The present results are in good accordance with the previous studies indicating that thiazolidinediones or stimulation of PPARγ prevented the hypertrophy of myocardial cells or LV hypertrophy (*13*, *14*). Asakawa *et al*. reported that angiotensin II–induced myocardial hypertrophy was inhibited by treatment with PPARγ ligands (*13*). They also showed that heterozygous PPARγ-deficient mice display an exaggerated hypertrophic response to aortic banding, and that pioglitazone significantly blunts myocardial hypertrophy in banded wild-type mice. Yamamoto *et al*. also reported that both troglitazone and 15d-PGJ2 prevented hypertrophy of cultured cardiomyocytes (*14*). We have shown, using adult SHR-SP, that pioglitazone, a PPARγ agonist, improves the hypertrophy of myocardial cells in chronic hypertension.

Ciglitazone, another thiazolidinedione, and 15d-PGJ2 decreased the expression of nicotinamide adenine dinucleotide phosphate (NAD(P)H) oxidase subunits (Nox1, Nox2, and Nox4), and decreased ROS production in endothelial cells *in vitro* (*37*, *38*). Pioglitazone also decreased the expression of the NAD(P)H oxidase subunits p47phox and Nox2, and suppressed superoxide production in the renal cortices of obese rats treated with a high-fat diet (*39*). In cultured rat fibroblasts, pioglitazone inhibited angiotensin II–induced production of ROS, resulting in a reduction of collagen type I synthesis (*40*). However, in the present study, the ROS production, which was estimated by the ESR method and TBARS levels, was not decreased in the LV by pioglitazone. We also observed that the mRNA expression of Nox family members (Nox1, Nox2, and Nox4) and p22phox were not altered by pioglitazone (data not shown). Thus, it is unlikely that the ROS played a major role in the beneficial action of pioglitazone on the LV hypertrophy of the present study. However, in our recent unpublished observations, pioglitazone administration decreased the ROS level of the LV in Dahl salt-sensitive rats fed a high salt diet. Thus, the action of pioglitazone on oxidative stress may be different among various models.

Pioglitazone reversed the concentric remodeling of the LV in the present study. This action was accompanied by an increase in the diastolic diameter. One may speculate that this change in the LV was due to a deterioration of LV function. This mechanism is unlikely, however, because the mRNA levels of BNP from the LV actually decreased with pioglitazone treatment, and because the EF and %FS of the LV were not decreased.

One known side effect of pioglitazone in humans is an increase in circulation volume. This effect could induce an exacerbation of congestive heart failure. However, in the present study, pioglitazone did not significantly increase the body weight, the mRNA level of BNP, or the cardiac function. It is thus unlikely that this fluid retention explains the changes in the LV.

In summary, we have shown in the present study that pioglitazone improves LV hypertrophy without a deterioration of LV function in genetically hypertensive rats. We administered pioglitazone from 24 weeks of age, when hypertension and LV hypertrophy had already been established. This timing of drug administration may have clinical importance, since patients with hypertension take the medicine after they are diagnosed as hypertensive or after hypertensive organ damage has appeared. The present observation may point to a possible clinical application of thiazolidinediones in the treatment of LV hypertrophy in patients with hypertension and normal systolic function. This possibility may be tested in future clinical studies, although caution should be taken because thiazolidinediones have been reported to cause fluid retention and worsened congestive heart failure in clinical use (*41*).

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