Beneficial Effects of Pioglitazone on Hypertensive Cardiovascular Injury Are Enhanced by Combination With Candesartan

Taishi Nakamura, Eiichiro Yamamoto, Keiichiro Kataoka, Takuro Yamashita, Yoshiko Tokutomi, Yi-Fei Dong, Shinji Matsuba, Hisao Ogawa, Shokei Kim-Mitsuyama

Abstract—The effect of pioglitazone, a peroxisome proliferator-activated receptor γ agonist, on hypertensive cardiovascular injury is unknown. We examined the effect of pioglitazone on hypertensive cardiovascular injury and the significance of combination of pioglitazone with angiotensin type 1 receptor blocker. Stroke-prone spontaneously hypertensive rats (SHRSP) were orally given pioglitazone, candesartan, or combined pioglitazone and candesartan for 4 weeks to compare their effects on cardiovascular injury. Pioglitazone, without lowering blood pressure, significantly suppressed cardiac inflammation and fibrosis and reduced vascular endothelial dysfunction, and these beneficial effects were associated with the reduction of superoxide by inhibition of cardiovascular NADPH oxidase. Thus, pioglitazone protects against hypertensive cardiovascular injury, by inhibiting reactive oxygen species (ROS). Combination of pioglitazone and candesartan suppressed cardiac hypertrophy, inflammation, and interstitial fibrosis of SHRSP to a greater extent than either monotherapy, and reduced vascular endothelial dysfunction of SHRSP more than either monotherapy. Furthermore, more beneficial effects of their combination on cardiovascular injury were associated with more reduction of NADPH oxidase–mediated cardiovascular ROS. To elucidate the underlying molecular mechanism, we examined cardiovascular NADPH oxidase subunits. Pioglitazone monotherapy significantly attenuated cardiovascular p22phox and Rac1 in SHRSP, whereas pioglitazone combined with candesartan more attenuated p22phox and significantly reduced Nox1. Thus, additive suppression of cardiovascular NADPH oxidase by the combination was attributed to its additive attenuation of p22phox and Nox1 protein levels. In conclusion, we showed that pioglitazone protected against hypertensive cardiovascular damage, and the combination of pioglitazone and candesartan exerted more beneficial effects on hypertensive cardiovascular injury by more suppressing ROS. (Hypertension. 2008;51:296-301.)

Key Words: cardiac remodeling ■ endothelium ■ hypertension ■ reactive oxygen species ■ inflammation ■ peroxisome proliferator-activated receptor γ ■ AT1 receptor

Hypertension is frequently accompanied by type 2 diabetes in the same patients, and hence, many hypertensive patients are subjected to the combination therapy of antihypertensive drug and antidiabetic drug. Pioglitazone is a peroxisome proliferator-activated receptor γ (PPAR-γ) agonist and is a useful therapeutic drug for type 2 diabetes, by improving insulin resistance. A growing body of experimental and clinical evidence indicates that pioglitazone has beneficial pleiotropic effects on cardiovascular diseases, independently of the improvement of insulin resistance and glycemic control. However, the potential pleiotropic effects of pioglitazone on cardiovascular diseases in hypertension are not clear.

Among antihypertensive drugs, an angiotensin type 1 (AT1) receptor blocker (ARB) has been established to exert favorable pleiotropic effects on various cardiovascular diseases, independently of blood pressure lowering effect. Therefore, it is of great clinical relevance to determine whether the combination of pioglitazone and an ARB exerts more beneficial effects on hypertensive cardiovascular injury than either monotherapy. However, to the best of our knowledge, the significance of the combination of pioglitazone and an ARB for treatment of hypertensive cardiovascular diseases is unknown.

Therefore, in the present study, we examined the effect of pioglitazone monotherapy on hypertensive cardiovascular injury and also the significance of the combination therapy of pioglitazone with candesartan, an ARB, for protection of cardiovascular injury in hypertension. We obtained the evidence that pioglitazone exerted the significant beneficial effects on hypertensive cardiovascular injury, and these effects of pioglitazone were markedly augmented by the combination with candesartan.
Materials and Methods

Animals
Male stroke-prone spontaneously hypertensive rats (SHRSP) and control Wistar-Kyoto rats (WKY) were purchased from Japan SLC (Shizuoka, Japan). They were fed a standard laboratory rat chow (CE2 Clea) and given tap water ad libitum. All procedures were in accordance with institutional guidelines for the care and use of laboratory animals.

Treatment of SHRSP With Pioglitazone, Candesartan, and Their Combination
Eleven-week-old SHRSP were randomly assigned to 5 groups, and were orally given vehicle (0.5% carboxymethyl cellulose), pioglitazone (1 mg/kg per day), pioglitazone (2 mg/kg per day), candesartan (0.3 mg/kg per day), or combined pioglitazone (1 mg/kg per day) and candesartan (0.3 mg/kg per day) by gastric gavage once a day, for 4 weeks. Blood pressure and heart rate were measured before and 1, 2, and 4 weeks after start of drug treatment. After 4 weeks of the treatment, SHRSP and control age-matched WKY were anesthetized with ether, and the blood was collected by cardiac puncture, to measure blood glucose and plasma insulin. After perfusion with phosphate-buffered saline, heart, carotid artery, and aorta were rapidly excised from SHRSP and WKY rats, for measurement of various parameters as described in detail in Online Supplements. The detailed methods are described in online supplements available at http://hyper.ahajournals.org.

Results

Effects on Blood Pressure, Body Weight, Blood Glucose, and Plasma Insulin of SHRSP
As shown in Figure S1 pioglitazone alone at 1 or 2 mg/kg per day or candesartan alone did not lower blood pressure of SHRSP, throughout 4 weeks of the treatment. Moreover, the combination of pioglitazone with candesartan did not reduce blood pressure of SHRSP, throughout 4 weeks of the treatment. Four weeks of treatment with pioglitazone, candesartan, or their combination did not significantly affect body weight, blood glucose, and plasma insulin levels of SHRSP (see Table S2).

Effects on Cardiac Hypertrophy and Remodeling of SHRSP
As shown in Figure 1 and Figure S2, treatment with pioglitazone at 1 mg/kg per day did not significantly decrease the increase in left ventricular (LV) weight of SHRSP, but significantly attenuated the increase in LV brain natriuretic peptide (BNP) mRNA expression (P<0.01), macrophage infiltration (P<0.01), and interstitial fibrosis (P<0.01) of SHRSP. A higher dose of pioglitazone (2 mg/kg per day) and candesartan (0.3 mg/kg per day) exerted comparable effects to pioglitazone (1 mg/kg per day), regarding these parameters. However, the addition of candesartan (0.3 mg/kg per day) to pioglitazone (1 mg/kg per day) conferred the significant prevention of cardiac hypertrophy of SHRSP (P<0.01; Figure 1A), and attenuated LV BNP mRNA, macrophage infiltration, and interstitial fibrosis to a greater extent than candesartan alone or the high dose (2 mg/kg per day) of pioglitazone (Figure 1B through 1D and Figure S2). Furthermore, as shown in Figure S3), pioglitazone or candesartan alone significantly attenuated the increase in LV transforming growth factor (TGF) β1 mRNA expression of SHRSP, and the combination of pioglitazone with candesartan more suppressed this expression than either monotherapy.

Effects on Cardiac NADPH Oxidase Activity and Superoxide of SHRSP
As shown in Figure 2 and Figure S4, pioglitazone (1 mg/kg per day) reduced LV reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity and superoxide levels of SHRSP to a similar degree to candesartan (0.3 mg/kg per day), but a higher dose of pioglitazone (2 mg/kg per day) did not exert more beneficial effects. Whereas, the combination of pioglitazone with candesartan decreased LV NADPH oxidase activity and superoxide of SHRSP more than pioglitazone or candesartan alone.

Effects on Vascular Endothelial Function, NADPH Oxidase Activity, Superoxide, and eNOS Activity of SHRSP
As shown in Figure 3, pioglitazone (1 mg/kg per day) significantly prevented the impairment of vascular endothelial-dependent relaxation in SHRSP to a similar degree to candesartan. In contrast to no more beneficial effects of the high dose of pioglitazone (2 mg/kg per day), the low dose of...
pioglitazone combined with candesartan reduced vascular endothelial dysfunction more than pioglitazone or candesartan monotherapy. Vascular endothelium-independent relaxation, obtained by the vascular relaxation with sodium nitroprusside, was not different between WKY and SHRSP, and was not affected by any drug treatments (Figure S5).

Pioglitazone or candesartan monotherapy significantly attenuated the enhancement of vascular NADPH oxidase activity and superoxide of SHRSP, to a comparable degree (Figure 4A and 4B and Figure S6). The combination of pioglitazone with candesartan exerted more attenuation of LV NADPH oxidase activity and superoxide of SHRSP than either monotherapy.

As shown in Figure S7, pioglitazone treatment did not affect the decrease in vascular endothelial nitric oxide synthase (eNOS) activity of SHRSP, whereas candesartan significantly normalized vascular eNOS activity of SHRSP. Combined pioglitazone and candesartan exerted no more improvement of vascular eNOS activity, compared with candesartan monotherapy.

Effects on Cardiac and Vascular NADPH Oxidase Subunits of SHRSP
As shown in Figure 5 and Figure S8, p22phox, Nox1, and Rac1 levels in membrane fraction of cardiac and vascular tissues of SHRSP were significantly higher than those of WKY. p22phox levels in both cardiac and vascular tissues were significantly reduced by pioglitazone or candesartan, to a similar extent. Moreover, the combination of pioglitazone and candesartan reduced cardiac and vascular p22phox levels in SHRSP more than either monotherapy (Figure 5). Pioglitazone did not alter cardiac and vascular Nox1 levels of SHRSP, whereas candesartan significantly attenuated cardiac and vascular Nox1 levels of SHRSP. Cardiac and vascular Rac1 levels were significantly reduced by pioglitazone, candesartan, and their combination, to a similar degree.

As shown in Figure S9, cardiac and vascular copper-zinc superoxide dismutase (Cu/Zn SOD) levels in SHRSP were larger than WKY, but were not altered by pioglitazone, candesartan, or their combination.

**Discussion**

Our present work provided 2 major findings. One was that pioglitazone, independently of blood pressure and blood glucose, significantly inhibited cardiac inflammation and fibrosis, and reduced vascular endothelial dysfunction in
hypertensive rats, by suppressing reactive oxygen species (ROS). The other was that the combination of pioglitazone with candesartan exerted more beneficial effects on cardiovascular injury of hypertensive rats than either monotherapy, by attenuating NADPH oxidase-mediated ROS more.

In the present study, to rule out the effects of blood pressure lowering on cardiovascular injury in SHRSP, we used pioglitazone at the dose of 1 to 2 mg/kg per day and candesartan at a very low dose (0.3 mg/kg per day), because in our preliminary experiments, we noticed that such doses of pioglitazone and candesartan did not affect blood pressure of SHRSP. Previously, Sugiyama et al. who examined the effect of various doses of pioglitazone on Wistar fatty rats which are the useful model of type 2 diabetes and obesity, show that 1 mg/kg per day of pioglitazone significantly improves insulin resistance, lowers blood glucose, and increases body weight in Wistar fatty rats, confirming that 1 mg/kg per day of pioglitazone in the rat is the sufficient dose to activate tissue PPARγ in vivo. Furthermore, the administration of 1 mg/kg per day of pioglitazone in the rat is shown to produce comparable plasma pioglitazone concentrations to those in the patients receiving clinical dose of pioglitazone. Therefore, the dose of pioglitazone used in our study was optimal and clinically relevant. Accumulating experimental and clinical evidence have established that AT1 receptor blockade by an ARB, independently of blood pressure lowering, significantly suppresses cardiac hypertrophy and remodeling, and significantly reduces vascular endothelial dysfunction. In this study, we examined the effect of the low dose of candesartan (0.3 mg/kg per day) without blood pressure lowering in SHRSP. As expected, such a low dose of candesartan significantly suppressed cardiac inflammation and fibrosis, and markedly attenuated vascular endothelial dysfunction in hypertensive rats, being consistent with the previous findings. Of note, despite no reduction of blood pressure, pioglitazone monotherapy significantly reduced cardiac BNP mRNA expression, macrophage infiltration, and interstitial fibrosis and attenuated vascular endothelial dysfunction in SHRSP to a similar extent to the low dose of candesartan. Thus, pioglitazone, a PPARγ agonist, appears to provide similar benefit on hypertensive cardiovascular injury to an ARB. Furthermore, cardiac TGFβ1 mRNA expression in SHRSP was significantly suppressed by pioglitazone as well as candesartan. Taken together with the fact that TGFβ1 is the major growth factor responsible for cardiac fibrosis, our results suggest that the inhibition of cardiac fibrosis by pioglitazone might be partially mediated by the inhibition of TGFβ1 expression.

Accumulating evidence indicate that ROS plays a crucial role in cardiac hypertrophy and remodeling and vascular endothelial impairment. NADPH oxidase is well known to be the major enzyme generating superoxide in cardiovascular tissues and plays a critical role in the mechanism of cardiac remodeling and vascular endothelial dysfunction. Therefore, to elucidate the underlying mechanism of cardiovascular protection by pioglitazone, we examined the effect of pioglitazone on cardiac and vascular NADPH oxidase and ROS of SHRSP. We found that pioglitazone markedly reduced cardiac and vascular superoxide in SHRSP, which was associated with the inhibition of NADPH oxidase activity. We also examined the effect of pioglitazone on cardiovascular Cu/Zn SOD, one of the major ROS scavenging enzymes, and found no effect of pioglitazone on this enzyme. Therefore, the inhibition of cardiac and vascular NADPH oxidase-mediated ROS appears to be responsible for the protective effects of pioglitazone against cardiovascular injury of SHRSP. However, in contrast to the significant normalization of vascular eNOS activity in SHRSP by candesartan, pioglitazone failed to normalize vascular eNOS.
activity, which may explain a trend toward more improvement of vascular endothelial function by candesartan compared with pioglitazone. Hence, differing from the case of ARB, eNOS appears to play a minor role in the attenuation of vascular endothelial dysfunction in SHRSP by pioglitazone.

Insulin resistance is supposed to play a causative role in either hypertension or type 2 diabetes, and hypertensive patients are frequently accompanied by type 2 diabetes. Therefore, many hypertensive patients are receiving the combination therapy of antihypertensive drugs, such as an ARB, and antidiabetic drugs, such as pioglitazone. However, to the best of our knowledge, the significance of combination therapy of an ARB and pioglitazone for treatment of cardiovascular complications is unknown. Accordingly, we compared the effect of pioglitazone (1 mg/kg per day) combined with candesartan, with a higher dose of pioglitazone (2 mg/kg per day) monotherapy, regarding the effects on cardiovascular injury. In this work, we found that the combination of pioglitazone with candesartan did not alter blood pressure, body weight, blood glucose, or plasma insulin in SHRSP, which permitted us to examine the potential pleiotropic effect of their combination on cardiovascular injury, beyond blood pressure or blood glucose. Of note are the observations that their combination significantly suppressed cardiac hypertrophy of SHRSP, differing from either monotherapy. Furthermore, the combination of pioglitazone with candesartan suppressed cardiac inflammation, TGFβ1 expression, and fibrosis and prevented vascular endothelial impairment in SHRSP to a greater extent than their monotherapy, demonstrating the benefits of their combination on hypertensive cardiovascular injury. To examine whether these beneficial effects of their combination in SHRSP were attributed to ROS or not, we investigated the effects of their combination on cardiac and vascular NADPH oxidase and superoxide. Notably, cardiac and vascular NADPH oxidase and ROS were inhibited by the combination of pioglitazone with candesartan to a greater extent than either monotherapy. On the other hand, cardiovascular SOD was not affected by the combination of pioglitazone and candesartan. Therefore, the benefits of their combination in treatment of hypertensive cardiovascular diseases seem to be at least partially attributed to more inhibition of cardiovascular NADPH oxidase-mediated ROS production. These observations provide the evidence that the combination of pioglitazone with candesartan exerted more beneficial effects on hypertensive cardiovascular injury than either monotherapy.

To further elucidate the underlying molecular mechanism of NADPH oxidase inhibition by pioglitazone, candesartan, and their combination, we determined their effects on NADPH oxidase subunits, Nox1, p22phox, and Rac1 in cardiovascular tissues of SHRSP, because these subunits are involved in cardiac hypertrophy and remodeling, and vascular endothelial dysfunction.17,20–22 In this work, we found that pioglitazone significantly attenuated p22phox (the membrane-bound subunit) and Rac1 (the cytosol regulatory subunit) but did not affect Nox1 (the membrane-bound subunit), thereby showing that the suppression of cardiac and vascular NADPH oxidase activity by pioglitazone was mediated by the inhibition of p22phox expression and Rac1 translocation. On the other hand, candesartan treatment attenuated not only p22phox and Rac1 but also Nox1, indicating differential regulation of NADPH oxidase subunits between pioglitazone and candesartan. Interestingly, the addition of candesartan to pioglitazone led to more reduction of p22phox protein levels in both cardiac and vascular tissues of SHRSP to a greater extent than either monotherapy, supporting the notion that intracellular PPARγ activation coupled with suppression of AT1 receptor is useful for the inhibition of p22phox. Taken together with the fact that p22phox is an essential component of NADPH oxidase activity,23,24 these findings support the notion that more inhibition of cardiovascular NADPH oxidase by the combination of pioglitazone and candesartan than either monotherapy might be attributed to more inhibition of p22phox upregulation in SHRSP.

Study Limitation

In this work, we measured blood pressure using the tail cuff method, rather than by 24-hour monitoring by telemetry or by direct intraarterial recordings.25 Therefore, it cannot be completely excluded that the attenuation of cardiovascular injury by pioglitazone, candesartan, and their combination might be partially dependent of reduction of blood pressure. Furthermore, our present work did not allow us to elucidate the precise localization and role of each NADPH oxidase subunit. Further study is needed to elucidate these points.

In conclusion, we obtained the evidence that pioglitazone at the clinically relevant dose exerts protective effects against hypertensive cardiovascular injury, by reducing NADPH oxidase-mediated ROS. Furthermore, the combination of pioglitazone with candesartan provided more beneficial effects on hypertensive cardiovascular injury than either monotherapy, by reducing NADPH oxidase-mediated ROS more.

Perspectives

The clinical findings on PROspective pioglitAzone Clinical Trial In macroVascular Events (PROactive) Study indicate that pioglitazone reduces cardiovascular events in patients with type 2 diabetes who have a high risk of macrovascular events. Taken together with accumulating experimental findings, PROactive study confirms that pioglitazone has multiple pleiotropic effects on cardiovascular diseases. Our present experimental work provided the evidence for the protective effects of pioglitazone against cardiovascular diseases in hypertension. Furthermore, our work also provided the evidence for the benefit of combination therapy of pioglitazone with candesartan, an antihypertensive drug with multiple pleiotropic effects. Hypertension is often accompanied by insulin resistance or type 2 diabetes in the same patients. Furthermore, importantly, the complication of hypertension and type 2 diabetes synergistically increases the risk of cardiovascular diseases. Therefore, intensive therapy by the combination of antihypertensive drug and antidiabetic drug is very important to prevent cardiovascular diseases. Therefore, our present experimental work is of great clinical relevance and paves the way for the treatment of cardiovascular diseases in hypertension with type 2 diabetes.
Sources of Funding
This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology.

Disclosures
None.

References
Beneficial Effects of Pioglitazone on Hypertensive Cardiovascular Injury Are Enhanced by Combination With Candesartan
Taishi Nakamura, Eiichiro Yamamoto, Keiichiro Kataoka, Takuro Yamashita, Yoshiko Tokutomi, Yi-Fei Dong, Shinji Matsuba, Hisao Ogawa and Shokei Kim-Mitsuyama

Hypertension. 2008;51:296-301; originally published online December 24, 2007; doi: 10.1161/HYPERTENSIONAHA.107.099044

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2007 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/51/2/296

Data Supplement (unedited) at:
http://hyper.ahajournals.org/content/suppl/2007/12/17/HYPERTENSIONAHA.107.099044.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Hypertension can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Hypertension is online at:
http://hyper.ahajournals.org//subscriptions/
Beneficial effects of pioglitazone on hypertensive cardiovascular injury are enhanced by combination with candesartan

Taishi Nakamura¹, Eiichiro Yamamoto¹, Keiichiro Kataoka¹, Takuro Yamashita¹, Yoshiko Tokutomi¹, Yi-Fei Dong¹, Shinji Matsuba¹, Hisao Ogawa², Shokei Kim-Mitsuyama¹

¹Department of Pharmacology and Molecular Therapeutics, Kumamoto University Graduate School of Medical Sciences, Kumamoto, Japan
²Department of Cardiovascular Medicine, Kumamoto University Graduate School of Medical Sciences, Kumamoto, Japan

Short title: Additive effects of combined pioglitazone and ARB

Address correspondence to: Shokei Kim-Mitsuyama, MD., PhD.
Department of Pharmacology and Molecular Therapeutics,
Kumamoto University Graduate School of Medical Sciences,
1-1-1 Honjyo, Kumamoto 860-8556, Japan
Tel. 81-96-373-5082
Fax. 81-96-373-5082
E mail. kimmitsu@gpo.kumamoto-u.ac.jp
Materials and Methods

Measurement of blood pressure

Blood pressure of conscious rats was measured by the tail cuff method (BP-98A; Softron Co, Tokyo, Japan).

Vessel ring preparation and organ chamber experiments

Isometric tension studies were performed, as previously described by us. In brief, carotid arteries from SHRSP and WKY rats were cut into 5 mm rings with special care to preserve the endothelium, and mounted in organ baths, filled with modified Tyrode buffer aerated with 95 % O\textsubscript{2} and 5 % CO\textsubscript{2} at 37 °C. The preparations were attached to a force transducer, and isometric tension was recorded on a polygraph. Vessel rings were precontracted with L-phenylephrine (10\textsuperscript{-7} mol/L). After the plateau was attained, the rings were exposed to increasing concentrations of acetylcholine (Ach) (10\textsuperscript{-9} mol/L to 10\textsuperscript{-4} mol/L) or sodium nitroprusside (10\textsuperscript{-9} mol/L to 10\textsuperscript{-4} mol/L) to obtain cumulative concentration-response curves.

Cardiac and vascular NADPH oxidase activity

Cardiac and aortic tissues were homogenized with an Ultraturrax T8, centrifuged, and NADPH oxidase activity of the resulting supernatant was measured by lucigenin chemiluminescence in the presence of 10 µM NADPH and 10 µM lucigenin as electron acceptor, as described in detail by us. Protein concentrations were measured by the method of Bradford.

Measurement of cardiac and vascular superoxide

Cardiac tissue and carotid artery, removed from SHRSP or WKY, were immediately frozen in Tissue-Tek O.C.T. embedding medium (Sakura Finetek) and cryostat sectioned (10 µm) directly onto chilled microscope slides. Dihydroethidium (DHE) was used to evaluate superoxide levels of cardiac tissue and carotid artery in situ, as described in detail by us.
DHE fluorescence of arterial and cardiac sections was quantified using Lumina Vision version 2.2, analysis software.

**Preparation of cardiac and vascular protein extracts and Western blot analysis**

Our detailed method of Western blot analysis has been described previously. We used a subcellular proteome extraction kit (S-PEK) to extract cytosol and membrane/organelle fractions from cardiac and vascular fragmented frozen tissue, according to the manufacturer’s instructions. After membrane extracts of cardiac and vascular tissues and molecular weight protein standards (Precision Plus Protein Standards, Bio-Rad Laboratories, Inc.) were subjected to sodium-dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and electric transfer to polyvinylidene difluoride membrane, the membranes were probed with specific antibodies. The following primary antibodies were used: rabbit anti-p22 phox (x 2,000, Santa Cruz B); rabbit anti-Mox1 (x 1,000, Santa Cruz B); mouse anti-Rac1 (x 5,000, upstate); rabbit anti-Cu/Zn SOD antibody (x 5,000, Stressgen Biotechnologies); mouse anti-GAPDH antibody (x 10,000, Santa Cruz Biotechnologies); anti-Na+/K+ATPase antibody (x 2,000, Upstate). After the blots were incubated with secondary antibody, the antibody was visualized using an enhanced chemiluminescence method ECL western blotting detection system (ECL Plus; Amersham Biosciences). The intensity of the bands was quantified using NIH Image analysis software v1.61. In Western blot analysis of membrane protein extracts, Na+/K+ATPase was used as the internal control.

**NOS activity (arginine-to-citrulline conversion)**

The activity of Ca2+-dependent nitric oxide synthase (NOS) (eNOS) of aortic tissue was determined by measuring the conversion of [3H]-arginine to [3H]-citrulline, using a NOS assay kit (Cayman CHEMICAL).

**Histological examination and immunohistochemistry**

The hearts were fixed with 4% formalin overnight, embedded in paraffin, cut into 4 µm
Nakamura et al.  *Additive effects of combined pioglitazone and ARB*

thick coronal sections, and stained with Sirius Red F3BA (0.5% in saturated aqueous picric acid, Aldrich Chemical Company) for assessment of cardiac interstitial fibrosis. The area of fibrosis was assessed by using Lumina Vision version 2.2 analysis software. For assessment of cardiac macrophage infiltration, the heart sections were immunostained with anti-ED-1 antibody (BMA Biomedicals AG) (working dilution 1:500) for identification of monocytes/macrophages, as described by us. The number of ED-1-positive cells was counted in 10 sections in individual rats; and the average of ED-1 positive cell number was obtained in individual rats.

**RNA extraction and complimentary DNA synthesis**

Total RNA was extracted from frozen left ventricular tissue, according to the manufacturer’s suggested protocol. Frozen left ventricular tissue samples were homogenized using ISOGEN regent (NIPPON GENE, Tokyo, Japan), followed by phenol-guanidinium thiocyanate-chloroform extraction and ethanol precipitation. Each sample was treated with RNase-free DNase. Total RNA integrity and concentration were determined from spectrophotometric optical density measurement (260 and 280 nm). The purified RNA was stored at -80°C until use. One microgram of total RNA was reverse transcribed to first-strand cDNA and contaminated genomic DNA was removed, using QuantiTect® Reverse Transcription Kit (QIAGEN Inc., Hilden, Germany) according to the manufacturer’s recommended protocol.

**Quantitative real time RT-PCR**

Two step Real time PCR was performed using Thermal Cycler Dice® Real Time System (TaKaRa Bio Inc., Shiga, Japan) with SYBR Green I detection and melting temperature analysis. cDNA was amplified using SYBR® Premix Ex Taq™ (Perfect Real Time) PCR kit (TaKaRa Bio Inc.) with specific primers for target sequences (described in online Table S1) or glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Amplification
conditions included 10 seconds at 95°C, and run for 40 cycles at 95°C for 5 seconds and 60°C for 30 seconds, and then dissociation 15 seconds at 95°C and 30 seconds at 60°C on the Thermal Cycler Dice® Real Time System. To confirm amplification specificity the PCR products from each primer pair were subjected to a melting curve analysis. After PCR cycling, samples were heated to 95°C for 15 seconds and 65°C for 30 seconds and then heated to 95°C at a linear transition rate of 0.1 °C/s. Fluorescence of the samples was monitored continuously while the temperature was increasing. SYBR Green I is released upon denaturation, which results in a decreasing fluorescence of the signal. The threshold cycle (Ct) value, which was determined using second derivative maximum method, was normalized to the respective housekeeping GAPDH (Applied Biosystems, California, U.S.A) Ct value and relatively calculated by setting a calibrator sample in each run using standard curve method.

**Measurement of plasma insulin**

Plasma insulin levels were quantified by using the commercial ELISA kits from MORINAGA.

**Statistics**

Results were expressed as mean ± SEM. Statistical significance was determined by one way ANOVA, followed by Fisher’s PLSD test, using StatView for Windows (SAS Institute, Inc. Cary, U.S.A.). In all tests, differences were considered statistically significant at the value of P less than 0.05.
References


Nakamura et al.  *Additive effects of combined pioglitazone and ARB*

**Figure Legends**

**Figure S1.** Effects of pioglitazone, candesartan, and their combination on blood pressure of SHRSP, during 4 weeks of the treatment

Abbreviations used: V, SHRSP treated with vehicle; P(1), SHRSP treated with pioglitazone at the dose of 1 mg/kg/day; P(2), SHRSP treated with pioglitazone at the dose of 2 mg/kg/day; C, SHRSP treated with candesartan at the dose of 0.3 mg/kg/day; P(1)+C, SHRSP subjected to the combination therapy of pioglitazone at the dose of 1 mg/kg/day and candesartan at the dose of 0.3 mg/kg/day; W, Wistar-Kyoto rats. Values are means ± SEM (n=5 in W, n=8 in V, n=6 in P(1), n=4 in P(2), n=6 in C, n=6 in P(1)+C).

**Figure S2** Effects of pioglitazone, candesartan, and their combination on LV interstitial fibrosis of SHRSP

Abbreviations used were the same as in Figure S1. These panels show representative microphotograph stained with Sirius Red F3BA of each group. Magnification x 200. Bar = 100 µm.

**Figure S3.** Effects of pioglitazone, candesartan, and their combination on LV TGFβ1 mRNA expression of SHRSP

Abbreviations used were the same as in Figure S1. LV TGFβ1 mRNA levels in individual samples were corrected for GAPDH mRNA levels. Values are means ± SEM (n=6 in W, n=8 in V, n=5 in P(1), n=3 in P(2), n=6 in C, and n=6 in P(1)+C).

**Figure S4.** Effects of pioglitazone, candesartan, and their combination on LV superoxide of SHRSP

Abbreviations used were the same as in Figure S1. The panels indicate representative confocal images stained with dihydroethidium fluorescence in LV section from each group. Magnification x 200. Bar = 100 µm.

**Figure S5.** Effects of pioglitazone, candesartan, and their combination on arterial
endothelium-independent relaxation of SHRSP

Abbreviations used were the same as in Figure S1. Carotid arterial relaxation was observed with the addition of various concentrations of S-nitroso-N-acetylpenicillamine (SNAP) to obtain the dose-response curve of vascular relaxation. Values are means ± SEM (n=3-5).

**Figure S6.** Effects of pioglitazone, candesartan, and their combination on vascular superoxide of SHRSP

Abbreviations used were the same as in Figure S1. The panels indicate representative confocal images stained with dihydroethidium fluorescence in carotid artery from each group. The increase in DHE fluorescence of SHRSP was observed in both endothelium and vascular smooth muscle cells and all the interventions homogeneously reduced DHE fluorescence in vascular tissues of SHRSP. Magnification x 200. Bar = 100 µm.

**Figure S7.** Effects of pioglitazone, candesartan, and their combination on vascular eNOS activity of SHRSP

Abbreviations used were the same as in Figure S1. Values are means ± SEM (n=5 in W, n=8 in V, n=6 in P(1), n=4 in P(2), n=6 in C, n=6 in P(1)+C).

**Figure S8.** Effects of pioglitazone, candesartan, and their combination on NADPH oxidase subunits, Nos1 and Rac1, in the heart (A) and the aorta (B) of SHRSP

Abbreviations used were the same as in Figure S1. Upper panels in each figure indicate representative Western blot. Nox1 and Rac1 protein levels in individual samples were normalized to Na⁺/K⁺ATPase protein levels. Values are means ± SEM (n=4).

**Figure S9.** Effects of pioglitazone, candesartan, and their combination on Cu/Zn SOD in the heart (A) and the aorta (B) of SHRSP

Abbreviations used were the same as in Figure S1. Upper panels in each figure indicate representative Western blot. Cu/Zn SOD protein levels in individual samples were
normalized to GAPDH protein levels. Values are means ± SEM (n=4).
**Online Table S1.** The sequence of primers used in the real-time PCR assays

<table>
<thead>
<tr>
<th>Target</th>
<th>Primer</th>
<th>Sequence</th>
<th>Nucleotide Position</th>
<th>Tm</th>
<th>Acc No.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>F</td>
<td>5'-GTCTCCAGAACAAATCCACGATG-3'</td>
<td>159-180</td>
<td>61.8</td>
<td>NM031545</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>5'-AAGCGCTGTCTTGAGACCTAA-3'</td>
<td>281-302</td>
<td>62.6</td>
<td></td>
</tr>
<tr>
<td>BNP</td>
<td>F</td>
<td>5'-AAGAAGTCACCCGCGTGCTA-3'</td>
<td>729-748</td>
<td>58.4</td>
<td>NM021578</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>5'-TGTTGATGTCTTTTGTTTGTCA-3'</td>
<td>775-798</td>
<td>55.3</td>
<td></td>
</tr>
</tbody>
</table>

F, forward sequence; R, reverse sequence. Acc No. indicates GenBank accession number.
**Online Table S2.** Effects of pioglitazone, candesartan, and their combination on body weight, blood glucose, and plasma insulin of SHRSP

<table>
<thead>
<tr>
<th>Groups</th>
<th>Body weight (g)</th>
<th>Blood glucose (mg/dl)</th>
<th>Plasma insulin (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>W (n=5)</td>
<td>392±5*</td>
<td>147±17</td>
<td>3.5±0.4</td>
</tr>
<tr>
<td>V (n=8)</td>
<td>314±3</td>
<td>143±15</td>
<td>3.1±0.2</td>
</tr>
<tr>
<td>P(1) (n=6)</td>
<td>313±3</td>
<td>129±16</td>
<td>3.0±0.5</td>
</tr>
<tr>
<td>P(2) (n=4)</td>
<td>306±14</td>
<td>157±32</td>
<td>3.0±0.3</td>
</tr>
<tr>
<td>C (n=6)</td>
<td>324±7</td>
<td>130±14</td>
<td>3.3±0.1</td>
</tr>
<tr>
<td>P(1)+C (n=6)</td>
<td>317±3</td>
<td>119±12</td>
<td>3.4±0.3</td>
</tr>
</tbody>
</table>

Abbreviations used: V, SHRSP treated with vehicle; P(1), SHRSP treated with pioglitazone (1 mg/kg/day); P(2), SHRSP treated with pioglitazone (2 mg/kg/day); C, SHRSP treated with candesartan (0.3 mg/kg/day); P(1)+C, SHRSP subjected to the combination therapy of pioglitazone (1 mg/kg/day) and candesartan (0.3 mg/kg/day); W, Wistar-Kyoto rats. Values are mean±SEM. * P<0.01 vs V.
Figure S1

Blood pressure vs. Duration of treatment (weeks)

- W
- V
- P(1)
- P(2)
- C
- P(1)+C

Blood pressure (mmHg) vs. Duration of treatment (weeks)
Figure S2

W  V  P(1)

P(2)  C  P(1)+C
Figure S3

LV TGFβ1/GAPDH mRNA

* P<0.01 vs V

P<0.01

P<0.01

P<0.05
Figure S4

W  V  P(1)

P(2)  C  P(1)+C
Figure S5

Vascular relaxation

SNAP (−log mol/L)

 (%)
Figure S6

W  V  P(1)

P(2)  C  P(1)+C
Figure S7

![Graph showing NOS activity (14C-citrulline) with statistical significance notes: * P<0.01 vs V, □ P<0.01. The x-axis represents different treatments (W, V, P(1), P(2), C, P(1)+C), and the y-axis represents activity (x10^2 cpm).]
Figure S8

(A) Heart

* P<0.01 vs V □ P<0.01

Nox1
- 68 kDa
Rac1
- 21 kDa
Na+/K+ATPase
- 100 kDa

(B) Aorta

* P<0.01 vs V □ P<0.01

Nox1
Rac1
Na+/K+ATPase

![Bar charts showing protein levels with statistical significance](image)
Figure S9

(A) Cu/Zn SOD
GAPDH

(B) Cu/Zn SOD
GAPDH

Vascular Cu/Zn SOD/GAPDH

LV Cu/Zn SOD/GAPDH

* P<0.01 vs V

Vascular Cu/Zn SOD/GAPDH

* P<0.01 vs V