Original Article

Effects of Pioglitazone on Nitric Oxide Bioavailability Measured Using a Catheter-Type Nitric Oxide Sensor in Angiotensin II–Infusion Rabbit

Toshio IMANISHI[1)], Akio KUROI[1)], Hideyuki IKEJIMA[1)], Katsunobu KOBAYASHI[1)], Seiichi MOCHIZUKI[2)], Masami GOTO[2)], Kiyoshi YOSHIDA[3)], and Takashi AKASAKA[1)]

Recently, peroxisome proliferator–activated receptor γ (PPARγ) ligands have been reported to increase nitric oxide (NO) bioavailability *in vitro* but not *in vivo* because of the difficulty of measuring plasma NO. Here, we investigated the effects of PPARγ on plasma NO concentrations using the newly developed NO sensor in angiotensin II (Ang II)–infused rabbits. Male New Zealand rabbits were randomized for infusion with Ang II, either alone or in combination with pioglitazone (a PPARγ agonist). Plasma NO concentration was measured using the catheter-type NO sensor placed in the aorta. We then infused N^G-methyl-L-arginine (L-NMMA) and acetylcholine (ACh) into the aortic arch to measure the basal and ACh-induced plasma NO concentration. Vascular nitrotyrosine levels were examined by enzyme-linked immunoassay (ELISA). Both an immunohistochemical study and Western blotting were performed to examine the PPARγ and gp91phox expression. The cotreatment with pioglitazone significantly suppressed the negative effects of Ang II, that is, the decreases in basal and ACh-induced NO production and the increase in vascular nitrotyrosine levels. Both the immunohistochemical study and Western blotting demonstrated that pioglitazone treatment enhaced PPARγ expression and greatly inhibited Ang II–induced up-regulation of gp91phox. In conclusion, the PPARγ agonist pioglitazone significantly improved NO bioavailability in Ang II–infused rabbits, most likely by attenuating nitrosative stresses. (*Hypertens Res* 2008; 31: 117–125)

***Key Words*: nitric oxide (NO), peroxisome proliferator–activated receptor agonist, angiotensin II, oxidative stress**

Introduction

Endothelial dysfunction, characterized by impaired endothelial nitric oxide (NO) production is involved in the pathogenesis of atherosclerotic disease and is associated with risk factors for vascular disease, including hypercholesterolemia, hypertension, and diabetes mellitus. Endothelial dysfunction in response to long-term angiotensin II (Ang II) treatment has been shown to be secondary to increased superoxide production within the endothelium, the media, and/or the adventitial layer (*1*, *2*). However, a limitation of these studies is that the release of NO from endothelium could only be inferred from comparisons of vessel relaxation. Therefore, direct *in vivo* measurements of intra-arterial NO concentration in blood would contribute to the detailed evaluation of endothelial function.

It was previously thought that NO, once released from vas-

From the [1)]Department of Cardiovascular Medicine, Wakayama Medical University, Wakayama, Japan; and [2)]Department of Medical Engineering and [3)]Division of Cardiology, Kawasaki Medical School, Kurashiki, Japan.

Address for Reprints: Toshio Imanishi, M.D., Ph.D., Department of Cardiovascular Medicine, Wakayama Medical University, 811–1, Kimiidera, Wakayama 641–8510, Japan. E-mail: t-imani@wakayama-med.ac.jp

Received May 23, 2007; Accepted in revised form July 25, 2007.

cular endothelial cells into the bloodstream, is immediately oxidized or inactivated by dissolved oxygen, oxyhemoglobin and/or oxygen radical species (*3*, *4*). However, growing experimental and clinical evidence suggests that NO remains active in the bloodstream, causing remote vasodilatory responses (*5*, *6*). Several groups have developed high-temporal–resolution methods that use NO sensors for electrochemical measurement (*7*, *8*). These sensors enable us to evaluate dynamic changes in NO concentration in solutions and tissues in response to agonists, NO-generating reagents and physical stimuli (*9*, *10*). However, electrical interference vibration, poor durability of the sensor-tip coatings and other factors have made *in vivo* NO measurement very difficult. To overcome these drawbacks, a new NO sensor, which encloses both the working and reference electrodes within a highly gas-permeable and robust enclosure, has been developed (*11–13*). In addition, we have developed a catheter-type NO sensor (*12*, *13*). Using this sensor, we recently demonstrated that long-term Ang II treatment reduces plasma NO level in a concentration-dependent manner because of the increase in nitrosative stress (*14*).

Peroxisome proliferator–activated receptors (PPARs) are transcription factors belonging to the nuclear superfamily. Emerging evidence indicates that the PPAR signaling pathway plays critical roles in the regulation of a variety of biological processes within the cardiovascular system (*15*). Treatment with PPARγ agonist improves endothelial function in patients with type 2 diabetes (*16*). However, it is not known whether this endothelial protective effect is secondary to improved glucose metabolism by the drug or whether PPARγ agonists exert direct endothelial protection.

Until now, no *in vivo* data have been available on pioglitazone's effect on plasma NO concentration in Ang II–infused rabbits. We used the catheter-type NO sensor to try to elucidate this effect. The present study demonstrates that cotreatment with pioglitazone reversed the Ang II–induced decrease in plasma NO concentration, accompanied by decreased nitrosative stress.

Methods

A Catheter-Type NO Sensor

The integrated architecture and the performance of the catheter-type NO sensor have been described previously (*12*, *13*). In brief, an Amino-700XL NO sensor (Innovative Instruments, Tempa, USA), 700 μm in diameter at the detection tip, was mounted in a 4-Fr catheter (1,200 mm long; Hirakawa Hewtech, Tokyo, Japan) and fixed with silicon adhesive. The tip was coated by soft polyurethane to prevent damage to the vessel wall, and two metal wires were also attached along the detection tip to provide the electrodes with mechanical support. The NO oxidative current was monitored using an NO monitor (model inNO-T, Innovative Instruments). Each sensor was calibrated using NO-saturated pure water as previously described (*11–13*). Briefly, NO-saturated pure water was prepared by bubbling pure NO gas in oxygen-free pure water. Using a gas-tight syringe, 5 μL was injected into a well-stirred saline solution (50 mL) in which the NO sensor was immersed (final NO concentration: 190 nmol/L) as previously described (*11–13*).

Table 1. Final Measures by Groups

Group	MAP, mmHg	HR, bpm	Body weight, kg
Vehicle	70.6±1.3	170±2	2.41±0.05
Pioglitazone	70.3±1.2	166±3	2.45±0.06
Ang II	84.5±2.3*	184±2*	2.39±0.03*
Ang II+pioglitazone	73.1±1.7$^{\#}$	172±2$^{\#}$	2.42±0.04$^{\#}$

Data are the mean±SEM. *$p<0.05$ *vs*. vehicle (control). $^{\#}p<0.05$ *vs*. Ang II alone. MAP, mean arterial pressure; HR, heart rate.

Animal Preparation

The animals were treated in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health. In addition, the study protocol was approved by the Institutional Animal Care and Use Committee of Wakayama Medical University. Male New Zealand rabbits (2.0 to 2.5 kg) were maintained on tap water and a standard diet. The animals were randomized into four groups. Group 1 ($n=6$) received vehicle (0.154 mol/L of NaCl). Groups 2 and 4 ($n=6$ for each) received pioglitazone (oral gavage, 5 mg/kg/day for 14 days). Groups 3 and 4 ($n=6$ for each) were prepared by the infusion of human Ang II (Peninsula Laboratories, San Carlos, USA) at 200 ng/kg per minute *via* an Alzet osmotic minipump (Durect, Cupertino, USA) implanted for 14 days subcutaneously. The rabbits were fed 150 g/day of standard chow (LRC-4; Oriental Yeast, Tokyo, Japan) with water ad libitum. They were anesthetized with xylazine (10 mg/kg intramuscularly), ketamine (50 mg/kg intramuscularly), and pentobarbital sodium (10 mg/kg i.v.), followed by heparin (1,000 units i.v.) for anticoagulation. A catheter for acetylcholine (ACh) infusion was placed in the aortic arch from the external carotid artery and the NO sensor was inserted through the left femoral artery until it reached the abdominal aorta. Aortic blood pressure was simultaneously monitored through a stiff cannula with a strain gauge pressure transducer (Nihon Kohden, Tokyo, Japan). On the day of the experiment, mean arterial pressure (MAP) was measured by a pressure transducer and recorder (model DPM-1B; BioTek Instruments, Winooski, USA) in conscious rabbits *via* an ear artery cannulated under local anesthesia with EMLA cream (2.5% lidocaine and 2.5% prilocaine).

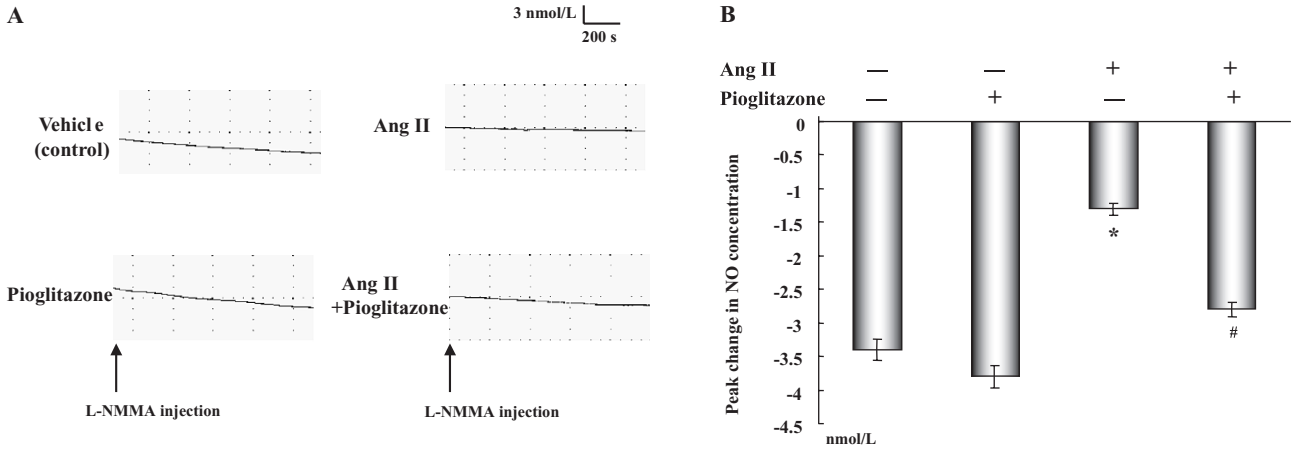

Fig. 1. *Pioglitazone's effects on L-NMMA–induced change in NO concentration and NOS inhibition's effect in the Ang II–infusion model. A: Typical tracings of the basal plasma NO concentration by L-NMMA in the aorta treated with vehicle (control), pioglitazone, Ang II and Ang II+pioglitazone. B: Basal NO concentration measured by peak response in the aorta treated with vehicle (control), pioglitazone, Ang II and Ang II+pioglitazone. Data are the mean±SEM (n=6). *p<0.01 vs. vehicle (control). #p<0.01 vs. Ang II alone.*

Experimental Protocol for Plasma NO Measurement

To measure the endothelium-dependent NO production, 20 μg/kg of ACh was administered at 1 mL/min for 5 min. To inhibit NO synthesis in the endothelium, 5 mg/kg of N^G-methyl-L-arginine (L-NMMA, NO synthase inhibitor) was infused at 1 mL/min for 10 min. The plasma NO concentration in the abdominal aorta was monitored over the entire time course. We measured both ACh-induced and L-NMMA–induced plasma NO production as the peak response in the current from the baseline through the entire period, which is expressed as the "change in NO concentration (nmol/L)," but not the absolute value in total plasma NO concentration (*12–14*). In the present study, we evaluated plasma NO consisting of NO generated from NO synthase (NOS)–dependent pathways and, not NOS-independent pathways.

Tissue Preparation

After treatment, rabbits were anesthetized with pentobarbital (30 mg/kg, i.v.) and perfusion-fixed with 4 % paraformaldehyde in PBS, pH 7.4 (perfusion pressure 100 mmHg). Subsequently, the thoracic aorta was immersion-fixed overnight in 4% paraformaldehyde.

Immunohistochemistry

Thoracic arteries were removed and stored in 10% formalin for less than 48 h. Fixed artery segments were dehydrated and embedded in paraffin, and sections were cut at 5 μm and mounted onto glass slides. PPARγ, gp91phox, and nitrotyrosine were detected immunohistochemically as previously described (*17*). Breifly, the indirect avidin biotin-horseradish-peroxidase visualization method was used (ABC Standard and Elite, Vector Red, Vector Laboratories, Burlingame, USA). Primary antibodies utilized included PPARγ monoclonal antibody (dilution 1:400; Santa Cruz Biotechnology, Santa Cruz, USA), gp91phox polyclonal antibody (1:400; Santa Cruz Biotechnology), and nitrotyrosine monoclonal antibody (1:400; Zymed Laboratories, San Francisco, USA).

Western Blot Analysis

Thoracic aorta was homogenized in a solution containing 62.5 mmol/L Tris-HCl (pH 6.8), 10% glycerol and 2% SDS. After centrifugation, the proteins in the supernatant were quantified. Western blotting was performed under a previously described protocol (*17*). Protein samples (40 μg each) were heated for 5 min at 100°C in sample buffer (62.5 mmol/L Tris-HCl, 10% glycerol, 2% SDS, 5% 2-mercaptoethanol and 0.0025% bromophenol blue), electrophoresed on 12.5% SDS-polyacrylamide gel, then transferred to a PVDF membrane (Millipore, Bedford, USA). The membranes were rinsed with phosphate-buffered saline (PBS) solution, then incubated with primary antibody overnight at 4°C. Following a washout with PBS solution, the membranes were incubated for 1 h at room temperature with secondary antibody plus 1% BSA. A monoclonal antibody against PPARγ (1:100 dilution; Santa Cruz Biotechnology) served as the primary antibody, with peroxidase-conjugated IgG as the secondary antibody (dilution 1:2000). Goat polyclonal antibody against gp91phox (Santa Cruz Biotechnology) was also used at a dilation of 1:100. Immunoreactivity was detected by

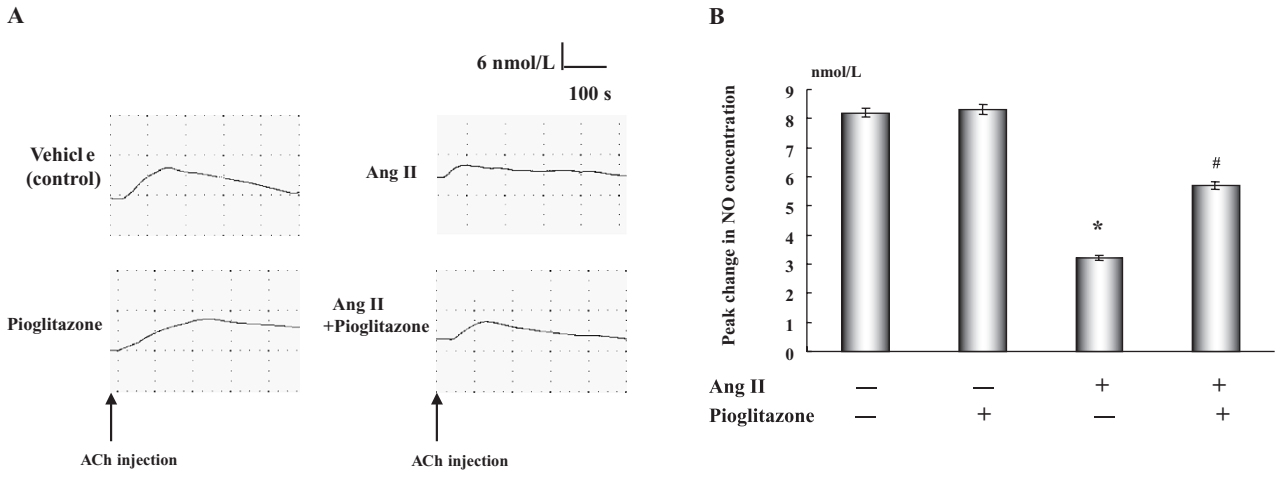

Fig. 2. *Pioglitazone's effects on ACh-induced change in NO concentration and NOS inhibition's effect on the Ang II–infusion model. A: Typical tracings of the plasma NO concentration induced by ACh in the aorta treated with vehicle (control), pioglitazone, Ang II and Ang II+pioglitazone. B: ACh-induced increase in the plasma NO concentration measured by peak response in the aorta treated with vehicle, pioglitazone, Ang II, and Ang II+pioglitazone. Data are expressed as absolute values. Bars represent mean±SEM. *$p<0.01$ vs. control. $^{\#}p<0.01$ vs. Ang II alone.*

enhanced chemiluminescence (ECL; Amersham, Sunnyvale, USA) using Hyperfilm (Amersham Pharmacia Biotech, Buckinghamshire, UK). The profile of each band was plotted using NIH Image software (National Institutes of Health, Bethesda, USA), and the densitometric intensity corresponding to each band was measured as an intensity value. The protein intensities of both PPARγ and gp91phox were normalized using α-tubulin expression.

Measurement of Vascular Nitrotyrosine

Peroxynitrite is a strong oxidant formed in the reaction between NO and superoxide under atherosclerotic stimuli. The peroxynitrite subsequently reacts with proteins resulting in nitrotyrosine. As a stable end product of peroxynitrite-mediated oxidation/nitration, nitrotyrosine can be used as a surrogate index of *in vivo* uncoupled NOS-dependent damage. Nitrotyrosine count, a footprint of *in vivo* $ONOO^-$ formation, was measured using the NWLSS™ nitrotyrosine enzyme-linked immunoassay (ELISA) kit (Northwest Life Science Specialties, Vancouver, USA) according to the manufacturer's protocol (*18*).

Measurement of Tetrahydrobiopterin in Aortic Segments

Measurement of tetrahydrobiopterin (BH_4) by high-performance liquid chromatography with fluorescence detection is beased on the quantification of biopterin, a highly fluorescent BH_4 analogue (*19*). The oxidation of BH_4 to biopterin under acidic conditions is quantitative. Under basic conditions, however, BH_4 is further oxidized to nonfluorescent compounds. Thus, BH_4 concentrations are calculated from the difference in biopterin levels measured under these conditions. Frozen aortic segments from vehicle (control-), pioglitazone-, Ang II–, and Ang II+pioglitazone–treated rabbits were cryopulverized and divided into two fractions of known weight. One fraction was suspended in HCl (0.25 mL, 0.1 Eq/L), and the other was suspended in NaOH (0.3 mL, 0.1 Eq/L). Each fraction was sonicated twice on a water/ice bath for 1 min by use of 25% sonicator full-power potency to break open the cells. After a 90-min incubation at room temperature, 50 μL of a 50% ascorbate solution was added to remove excess iodine solution and then centrifuged at 14,000 rpm for 10 min to remove tissue debris. After adjustment of pH to 4.0 with HCl, supernatants were injected onto a Kromasil C-18 coluum equilibrated with phosphate buffer (0.15 mmol/L, pH 6.4), and the biopterin level was analyzed by authentic standards.

Statistical Analysis

All data were expressed as mean±SEM based on at least six independent experiments. Statistical analyses for the comparison of ACh-induced changes in plasma NO concentration were conducted by paired *t*-test. Differences in values between groups and treatments were tested using one way ANOVA with repeated measurements. A p value of <0.05 was considered significant.

Results

Calibration of Sensors

The basic performance of the integrated catheter-type NO sensors was previously reported (*12*, *13*). The NO sensors showed no noticeable response to changes in concentrations of oxygen, ACh, L-NMMA, or solution mixing, indicating a high specificity for NO (data not shown). The mean peak response in NO value of the seven sensors used in the present study was 330±11 pA/nmol/L. This value is comparable to the values obtained with the original *in vivo* sensor (*11*).

Effect of Pioglitazone's Effect on Hemodynamic Parameters

Ang II–treated rabbits showed a significant increase in MAP. This effect was significantly reduced by cotreatment with pioglitazone (Table 1). Similarly, Ang II–treated rabbits showed a significant increase in HR, and this effect likewise was significantly abolished by cotreatment with pioglitazone (Table 1).

Pioglitazone's Effect on Basal Plasma NO Concentration

Ang II's effect on basal plasma NO concentration was evaluated using a NO synthesis inhibitor, L-NMMA. Ang II affected basal plasma NO concentration: the decrease in that concentration by L-NMMA infusion was significantly lower in the Ang II group than in the vehicle (control) group, indicating a lower basal NO bioavailability (Fig. 1). In contrast, Ang II's inhibitory effect on basal plasma NO concentration was significantly reversed by cotreatment with pioglitazone (Fig. 1).

Pioglitazone's Effect on the ACh-Induced Increase in Plasma NO Concentration

Endothelial function was monitored with ACh-induced NO synthesis. Intra-aortic infusion of ACh (4 μg/kg/min for 5 min) increased plasma NO concentration: this concentration was significantly lower than that with Ang II treatment compared with control (Fig. 2). In contrast, cotreatment with pioglitazone significantly reversed the Ang II–induced decrease in ACh-induced NO production, although the increase in plasma NO concentration with pioglitazone alone was similar to that in the control (Fig. 2).

Pioglitazone's Antinitrative Effect in Ang II–Infused Rabbits

Having demonstrated that pioglitazone preserved plasma NO concentration in Ang II–treated aortas, we investigated its effect on vascular nitrative stress associated with Ang II. To this end, we determined the vascular nitrotyrosine content by ELISA and by immunohistochemical study. As illustrated in Fig. 3A, strong nitrotyrosine staining was detected in endothelial cells from Ang II–infused rabbits, and pioglitazone treatment reduced nitrotyrosine staining. Quntitative ELISA results indicated that vascular nitrotyrosine content in Ang II–

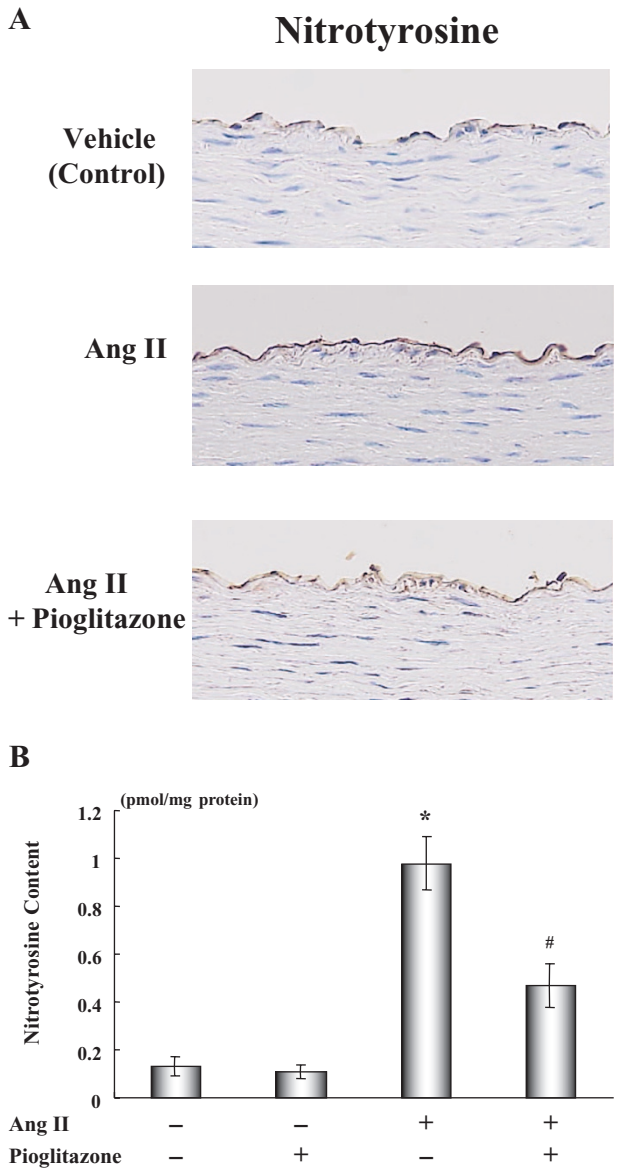

Fig. 3. *A: Representative immunohistochemisty of nitrotyrosine staining (a marker of peroxynitrite) of thoracic aorta from vehicle (control), pioglitazone, Ang II, and Ang II+pioglitazone. B: Vascular nitrotyrosine content measured by ELISA in aorta treated with vehicle, pioglitazone, Ang II, and Ang II+pioglitazone. Data are expressed as the mean±SEM (n=6). *p<0.01 compared with the control group. #p<0.01 compared with the Ang II–treated group.*

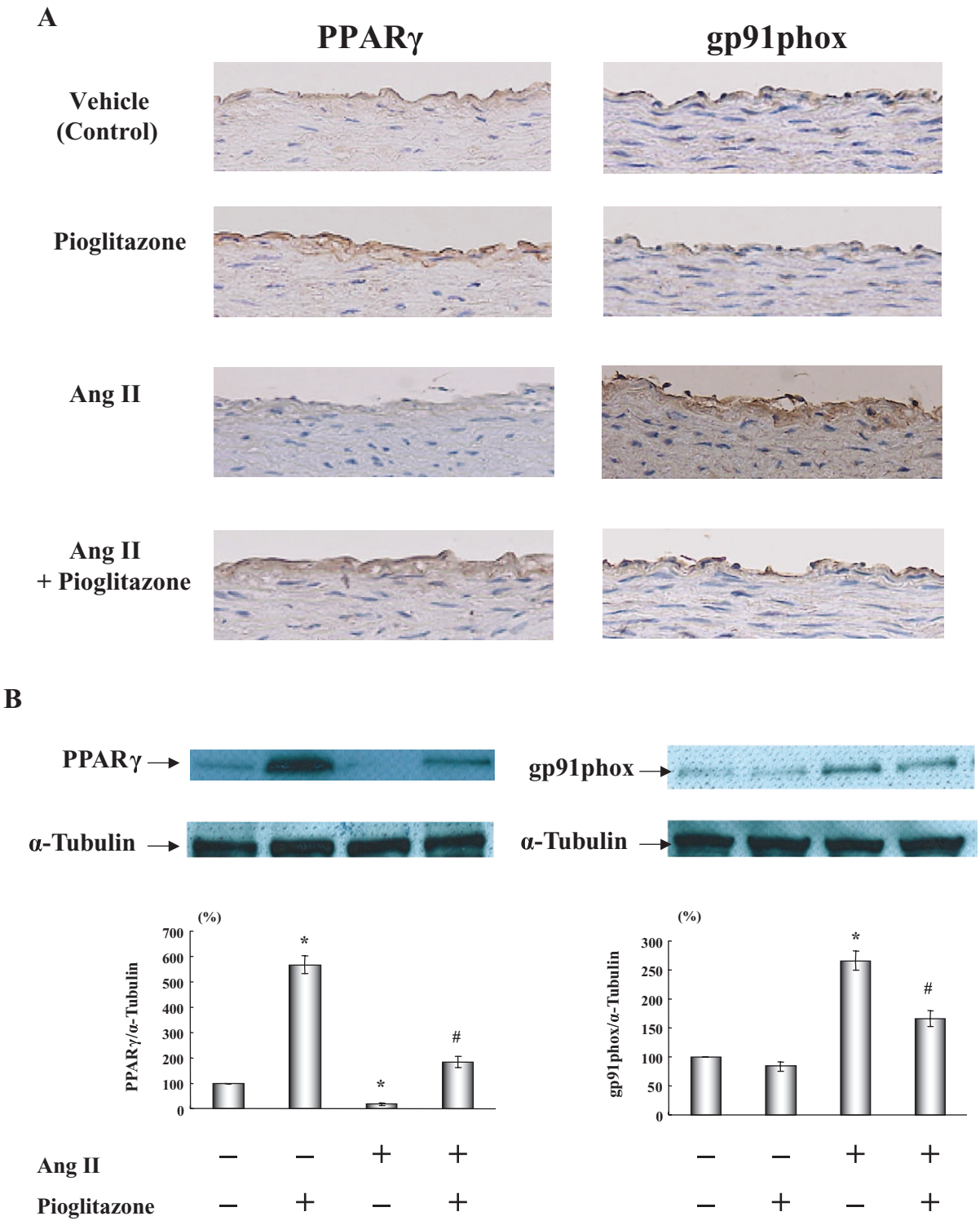

Fig. 4. *A: Representative immunohistochemistry of PPARγ staining (left) or gp91phox staining (right) of thoracic aorta from vehicle (control), pioglitazone, Ang II, and Ang II+pioglitazone. B: Representative immunoblotting analysis (upper) and densitometry analysis (lower) of PPARγ (left) or gp91phox (right) of thoracic aorta from vehicle (control), pioglitazone, Ang II, and Ang II+pioglitazone. Data are the mean±SEM (n=6). *p<0.01 compared with the control group. #p<0.01 vs. Ang II–treated group.*

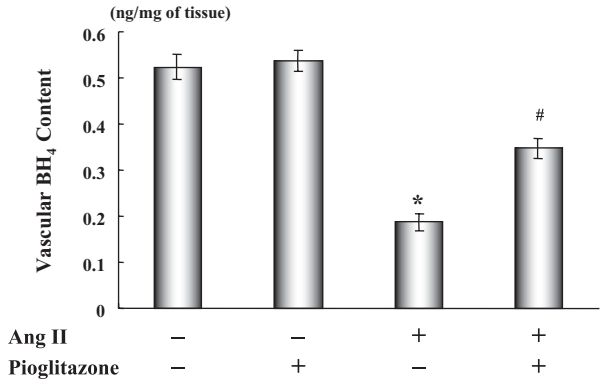

Fig. 5. *BH_4 levels in the aorta treated with vehicle (control), pioglitazone, Ang II, and Ang II+pioglitazone. Data are expressed as the mean±SEM ($n=6$). *$p<0.01$ compared with the control group. #$p<0.01$ compared with the Ang II–treated group.*

infused rabbits was significantly higher than that in control, and pioglitazone administration markedly reduced this increase in vascular nitrotyrosine content (Fig. 3B).

Pioglitazone's Effect on PPARγ and gp91phox Expression

To determine whether or not Ang II may alter PPARγ expression and whether or not *in vivo* pioglitazone treatment may up-regulate PPARγ expression, both immunohistochemical study and Western blotting were performed. As shown in Fig. 4A (left), in thoracic arteries isolated from control rabbits, PPARγ is expressed in endothelial cells. As expected, *in vivo* pioglitazone treatment markedly up-regulated PPARγ expression in endothelial cells. On the other hand, in thoracic arteries isolated from Ang II–infused rabbits, PPARγ expression in endothelial cells was virtually abolished, while cotreatment with pioglitazone improved the Ang II–induced inhibitory effects (Fig. 4A). Similarly, Western blotting analysis has shown that long-term Ang II treatment significantly decreased PPARγ expression compared with vehicle (control) or pioglitazone (Fig. 4B). Cotreatment with pioglitazone markedly increased the Ang II–induced down-regulation of PPARγ expression. In addition, immunohistochemical study (Fig. 4A) as well as Western blotting analysis (Fig. 4B) demonstrated that long-term Ang II treatment dramatically increased gp91phox expression, while cotreatment with pioglitazone markedly decreased the Ang II–induced up-regulation of gp91phox expression.

Pioglitazone's Effect on BH_4 Levels

Because BH_4 is of fundamental importance in normal endothelial NO synthase, we examined pioglitazone's effects of pioglitazone on vascular BH_4 levels. We observed that long-term Ang II treatment significantly decreased vascular BH_4 levels compared with vessels that received either vehicle (control) or pioglitazone alone (Fig. 5). Cotreatment with pioglitazone significantly reversed the Ang II–induced decrease in vascular BH_4 levels (Fig. 5).

Discussion

The present study confirmed our previous reports (*14*) that Ang II induces endothelial dysfunction by increased nitrosative stress. Moreover, using a novel catheter-type NO sensor, we demonstrated for the first time that pioglitazone markedly improved NO bioavailability in the Ang II–infused rabbit model. In addition, we made a novel observation that pioglitazone inhibited gp91phox expression, and attenuated toxic $ONOO^-$ formation in Ang II–infused rabbits. We also provided direct evidence in an *in vivo* animal model that Ang II downregulates and a PPARγ agonist upregulates PPARγ expression in vascular tissue.

In our previous study, we measured direct *in vivo* endothelium-derived NO with a catheter-type NO sensor in a rabbit model (*13*). The sensor was shown to be stable and specific to NO because direct exposure to either ACh or L-NMMA solution did not significantly change the baseline current (data not shown). The NO sensor also showed no noticeable change in the baseline current because of solution mixing, suggesting that fluid (blood) motion did not affect it (data not shown). Thus, this system has high specificity for NO, and the monitored current reflected the change in the plasma concentration of NO released from the endothelium after ACh and L-NMMA infusions.

The present study showed that pioglitazone improved Ang II–induced events such as decrease in basal and ACh-induced NO production and increased vascular nitrotyrosine levels. There are several potential mechanisms by which pioglitazone may exert its endothelial protection in Ang II–infused rabbits. Increasing evidence indicates that NADPH oxidase is the predominant superoxide source in both endothelial and smooth muscle cells (*20*, *21*). Several studies have demonstrated that Ang II type 1 (AT_1) receptor activation by Ang II stimulates NADPH oxidase, resulting in the generation of superoxide in vascular cells and eventually, NO degradation (*22*, *23*). In the present study, we provided direct evidence that *in vivo* pioglitazone treatment markedly attenuated Ang II–induced upregulation of gp91phox, a membrane-bound subunit of NADPH oxidase, in Ang II–infused rabbits. Therefore, the present experiment demonstrated that pioglitazone at least in part achieves its endothelial protection *via* inhibition of superoxide formation and subsequent NO degradation. However, the mechanisms by which pioglitazone improved Ang II–induced oxidative stress could not be clarified in the present study. Because several studies have demonstrated that the PPARγ ligand inhibited AT_1 receptor expression (*24–26*), one may speculate that pioglitazone's inhibitory effects on Ang II–induced oxidative stress may be secondary to down-

regulation of AT_1 receptors, resulting in less NO degradation. We could not elucidate pioglitazone's effect on AT_1 receptor expression in the present study, because there is no available antibody against AT_1 receptor in rabbits so far. Further studies will be needed on this important subject.

Emerging evidence suggests that in addition to oxidative stress, nitrative stress also plays a role in endothelial dysfunction. In the present study, we demonstrated that pioglitazone treatment improved NO bioavailability in Ang II–infused rabbits, accompanied by diminished vascular production of peroxynitrite, a reactive oxygen species (ROS) generated from a rapid reaction of NO with superoxide. However, one may argue these results. That is, if there is loss of NO due to decreased production from endothelial NOS (eNOS), how is peroxynitrite formed? The discrepancy between NO and peroxynitrite may likely be due to differences in measurement sites. In the present study, the NO sensor was placed in the abdominal aorta. On the other hand, vascular peroxynitrite was measured in the thoracic aorta. Most NO, once released from vascular endothelial cells into the bloodstream, is immediately degraded by superoxide, if it exists. We measured remaining plasma NO, which reflects *in vivo* NO bioavailability, in the abdominal aorta. This might lead to the discrepancy between NO and peroxynitrite. As pointed out by Gori *et al*. (*27*), peroxynitrite is a strong stimulus for the oxidation of the eNOS cofactor BH_4 to dihydrobiopterin (BH_2). The resulting intracellular BH_4 deficiency may convert eNOS from a NO to a superoxide-producing enzyme, which may further increase oxidative stress in vascular tissue in a positive-feedback fashion. In fact, we showed in the present study that Ang II–induced intracellular BH_4 depletion was significantly improved by cotreatment with pioglitazone. This finding is in line with our recent findings that exogenous NO suppresses flow-induced, endothelium-derived NO production by superoxide released from uncoupled NOS because of intracellular BH_4 depletion (*28*). Taken together, the findings indicate that pioglitazone exerted significant endothelial protective effects by reducing both oxidative stress and nitrative stress. However, in the present study, we could not elucidate the extent to which either superoxide-induced NO degradation or eNOS uncoupling plays an important role in pioglitazone's effect.

In the present study, pioglitazone prevented hypertension as well as tachycardia in Ang II–infused rabbits. Because several studies have demonstrated that PPARγ ligands, such as pioglitazone, inhibited AT_1 receptor expression (*24–26*), we can speculate that pioglitazone's effects on Ang II–induced hypertension as well as tachycardia may be secondary to down-regulation of AT_1 receptor. However, in the present study, we could not elucidate pioglitazone's effect on AT_1 receptor expression, because there is no available antibody against AT_1 receptor in the rabbit so far. Further studies will be needed on these clinically important topics.

In conclusion, we elucidated for the first time that PPARγ agonist pioglitazone exerted significant endothelial protective effects by inhibiting Ang II–induced nitrosative stress.

Perspectives

The catheter-type NO sensor has potential use in the study of the pharmacologic modulation of NO, enabling the measurement of basal and ACh-induced NO production in the aorta of experimental animal models. Furthermore, it may also be possible to apply the sensor to the clinical diagnosis of endothelial dysfunction, *i.e.*, reduced endothelium-derived NO availability in the cardiovascular system.

References

1. Rajagopalan S, Kurz S, Munzel T, *et al*: Angiotensin II–mediated hypertension in the rat increases vascular superoxide production *via* membrane NADH/NADPH oxidase activation. Contribution to alternations of vasomotor tone. *J Clin Invest* 1996; **97**: 1916–1923.
2. Pagano PJ, Clark JK, Cifuentes-Pagano ME, Clark SM, Callis GM, Quinn MT: Localization of a constitutively active, phagocyte-like NADPH oxidase in rabbit aortic adventitia: enhancement by angiotensin II. *Proc Natl Acad Sci U S A* 1997; **94**: 14483–14488.
3. Dinerman JL, Lowenstein CJ, Snyder SH: Molecular mechanisms of nitric oxide regulation. *Circ Res* 1993; **73**: 217–222.
4. Wennmalm A, Benthin G, Petersson AS: Dependence of the metabolism of nitric oxide (NO) in healthy human whole blood or the oxygenation of its red cell haemoglobin. *Br J Pharmacol* 1992; **106**: 507–508.
5. Schechter AN, Gladwin MT: Hemoglobin and the paracrine and endocrine functions of nitric oxide. *N Engl J Med* 2003; **348**: 1483–1485.
6. Stamler JS, Jia L, Eu JP, *et al*: Blood flow regulation by *S*-nitrosohemoglobin in the physiological oxygen gradient. *Science* 1997; **276**: 2034–2037.
7. Malinski T, Taha Z: Nitric oxide release from a single cell measured *in situ* by a porphyrinic-based microsensor. *Nature* 1992; **358**: 676–678.
8. Shibuki K: An electrochemical microprobe for detecting nitric oxide release in brain tissue. *Neurosci Res* 1990; **9**: 69–76.
9. Pinsky DJ, Patton S, Mesaros S, *et al*: Mechanical transduction of nitric oxide synthesis in the beating heart. *Circ Res* 1997; **81**: 372–379.
10. Vallance P, Patton S, Bhagat K, *et al*: Direct measurement of nitric oxide in human beings. *Lancet* 1995; **345**: 153–154.
11. Mochizuki S, Himi N, Miyasaka T, *et al*: Evaluation of basic performance and applicability of a newly developed *in vivo* nitric oxide sensor. *Physiol Meas* 2002; **23**: 261–268.
12. Mochizuki S, Miyasaka T, Goto M, *et al*: Measurement of acetylcholine-induced endothelium-derived nitric oxide in aorta using a newly developed catheter-type nitric oxide sensor. *Biochem Biophy Res Commun* 2003; **306**: 505–508.
13. Neishi Y, Mochizuki S, Miyasaka T, *et al*: Evaluation of bioavailability of nitric oxide in coronary circulation by direct measurement of plasma nitric oxide concentration. *Proc Natl Acad Sci U S A* 2005; **102**: 11456–11461.
14. Imanishi T, Kobayashi K, Kuroi A, Mochizuki S, Goto M,

Akasaka T: Effects of angiotensin II on nitric oxide bioavailability measured using a catheter-type nitric oxide sensor. *Hypertension* 2006; **48**: 1058–1065.
15. Bishop-Bailey D: Peroxisome proliferator–activated receptors in the cardiovascular system. *Br J Pharmacol* 2000; **129**: 823–834.
16. Caballero AE, Saouaf R, Lim SC, *et al*: The effects of troglitazone, an insulin sensitizing agent, on the endothelial function in early and late type 2 diabetes: a placebo-controlled randomized clinical trial. *Metabolism* 2003; **52**: 173–180.
17. Imanishi T, Jonathan M, Quoc H, Kevin D, Schwartz SM, Han DKM: Expression of cellular FLICE-inhibitory protein in human coronary arteries and in a rat vascular injury model. *Am J Pathol* 2000; **156**: 125–137.
18. Ter Steege JCA, Koster-Kamphuis L, Van Straaten EA, Forget P, Buurman WA: Nitrotyrosine in plasma of celiac disease patients as detected by a new sandwich ELISA. *Free Radic Biol Med* 1998; **25**: 953–963.
19. Fukushima T, Nixon JC: Analysis of reduced forms of biopterin in biological tissues and fluids. *Anal Biochem* 1980; **102**: 176–188.
20. Guzik TJ, Mussa S, Gastaldi D, *et al*: Mechanisms of increased vascular superoxide production in human diabetes mellitus: role of NAD(P)H oxidase and endothelial nitric oxide synthase. *Circulation* 2002; **105**: 1656–1662.
21. Sorescu D, Weiss D, Lassegue B, *et al*: Superoxide production and expression of Nox family proteins in human atherosclerosis. *Circulation* 2002; **105**: 1429–1435.
22. Griendling KK, Minieri CA, Ollerenshaw JD, Alexander RW: Angiotensin II stimulates NADH and NADPH oxidase activity in cultured vascular smooth muscle cells. *Circ Res* 1994; **74**: 1141–1148.
23. Lassegue B, Sorescu D, Szocs K, *et al*: Novel gp91phox homologues in vascular smooth muscle cells: nox1 mediates angiotensin II–induced superoxide formation and redox-sensitive signaling pathways. *Circ Res* 2001; **88**: 888–894.
24. Takeda K, Ichiki T, Tokunou T, *et al*: Peroxisome proliferator-activated receptor gamma activators downregulate angiotensin II type 1 receptor in vascular smooth muscle cells. *Circulation* 2000; **102**: 1834–1839.
25. Sugawara A, Takeuchi K, Uruno A, *et al*: Transcriptional suppression of type 1 angiotensin II receptor gene expression by PPARγ in vascular smooth muscle cells. *Endocrinology* 2001; **142**: 3125–3134.
26. Sugawara A, Takeuchi K, Uruno A, *et al*: Differential effects among thiazolidinediones on the transcription of thromboxane receptor and angiotensin II type 1 receptor gene. *Hypertens Res* 2001; **24**: 229–233.
27. Gori T, Mark SS, Kelly S, Parker JD: Evidence supporting abnormalities in nitric oxide synthase function induced by nitroglycerin in humans. *J Am Coll Cardiol* 2001; **38**: 1096–1101.
28. Mochizuki S, Sipkema P, Goto M, *et al*: Exogenous NO suppresses flow-induced endothelium-derived NO production because of depletion of tetrahydrobiopterin. *Am J Physiol Heart Circ Physiol* 2005; **288**: H553–H558.