Original Article

Pioglitazone Lowers Systemic Asymmetric Dimethylarginine by Inducing Dimethylarginine Dimethylaminohydrolase in Rats

Shu WAKINO, Koichi HAYASHI, Satoru TATEMATSU, Kazuhiro HASEGAWA, Ichiro TAKAMATSU, Takeshi KANDA, Koichiro HOMMA, Kyoko YOSHIOKA, Naoki SUGANO, and Takao SARUTA

Peroxisome proliferator activated receptor-γ (PPARγ) ligands increase nitric oxide (NO) production and reduce systemic blood pressure. Asymmetric dimethylarginine (ADMA) is an endogenous nitric oxide synthase (NOS) inhibitor degraded by the enzyme dimethylarginine dimethylaminohydrolase (DDAH), which has two isoforms, DDAH-I and -II. In order to elucidate the mechanism whereby PPARγ ligands affect NO metabolism, their effects on the DDAH-ADMA pathway were investigated. Six-week-old male Wister-Kyoto rats (WKY) and spontaneously hypertensive rats (SHR) were maintained with or without pioglitazone (PIO), a PPARγ ligand. After 4 weeks, serum ADMA levels and urinary daily NO excretion were analyzed. Tissue DDAH expression was examined by real-time polymerase chain reaction (PCR), immunoblotting, and immunohistochemistry. The results showed that PIO decreased serum ADMA and increased urinary NO excretion in both WKY and SHR. Also in both strains, the expression level of DDAH-II in the kidney was increased at transcriptional levels, although the DDAH-I level was unaffected. PIO lowered blood pressure in SHR, but not in WKY. We also demonstrated that PIO induced DDAH-II protein expression in Marbin-Dubin Canine Kidney (MDCK) cells, a renal tubular cell line. In conclusion, a PPARγ ligand was here found to increase NO production partly by upregulating tissue DDAH-II expression and decreasing systemic ADMA levels. This mechanism constitutes a direct action on renal tubular cells, but is less likely to be responsible for the blood pressure-lowering effects of PPARγ ligands. Since ADMA is one of the risk factors for cardiovascular events, this study provides compelling evidence that PPARγ ligands have the potential for reducing cardiovascular risks. (*Hypertens Res* 2005; 28: 255–262)

***Key Words*: peroxisome proliferator activated receptor-γ ligands, asymmetric dimethylarginine (ADMA), dimethylarginine dimethylaminohydrolase, nitric oxide synthase, Marbin-Dubin Canine Kidney cells**

Introduction

Nitric oxide (NO) is a potent endogenous vasodilator, and is generated by NO synthase (NOS). Derangement of NO production not only impairs endothelium-dependent vasodilation (*1*), but also accelerates the development of vascular lesions (*2*). Indeed, recent studies have indicated that defective endothelial function is a predictive marker for cardiovascular events (*3*). One of the mechanisms that leads to endothelial dysfunction is the accumulation of an endogenous inhibitor of NOS, asymmetrical dimethylarginine (ADMA). It has been demonstrated that the plasma ADMA level is inversely related with NO synthesis in various conditions (*4–6*), and is

From the Department of Internal Medicine, School of Medicine, Keio University, Tokyo, Japan.

Address for Reprints: Shu Wakino, M.D., Department of Internal Medicine, School of Medicine, Keio University, 35 Shinanomachi, Shinjuku-ku, Tokyo 160–8582, Japan. E-mail: swakino@sc.itc.keio.ac.jp

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Table 1. Characteristics of WKY and SHR with or without Pioglitazone

	WKY	WKY+PIO	SHR	SHR+PIO
Body weight (g)	263±8	266±6	254±7	259±10
Glucose (mg/dl)	124±23	112±11	114±23	118±26
Insulin (ng/ml)	1.18±0.40	1.20±0.40	2.85±0.95*	1.58±0.33†
Triglyceride (mg/dl)	29±4	27±4	44±8	21±2†
Free fatty acid (mEq/l)	421±62	248±85	478±63	168±77†
Total cholesterol (mg/dl)	68±3	63±2	45±2**	43±2
Creatinine (mg/dl)	0.24±0.02	0.20±0.01	0.21±0.02	0.22±0.01
Urine protein/creatinine	6.26±1.57	5.76±1.32	8.80±1.47	8.79±3.99

Five rats per group were analyzed for each parameter. Blood was collected at the end of the study. Urine was collected over 24 h. Results were mean±SEM. *$p<0.05$ and **$p<0.01$ *vs.* WKY. †$p<0.05$ *vs.* SHR. WKY, Wister-Kyoto rats; SHR, spontaneously hypertensive rats; PIO, pioglitazone.

elevated in patients with cardiovascular disease, as well as in numerous pathological conditions that are associated with risk factors for cardiovascular disease (*7*, *8*). ADMA is derived from the catabolism of proteins containing methylated arginine residues (*7*, *8*), and is metabolized by an enzyme, dimethylarginine dimethylaminohydrolase (DDAH). Two isoforms of DDAH have been identified: DDAH-I is typically found in tissues expressing NOS I, whereas DDAH-II predominates in tissues containing NOS III. Although DDAH plays an important role in the regulation of systemic ADMA levels (*9*), the regulatory mechanisms for the DDAH expression have not been fully evaluated.

Substantial evidence has been accumulated that both NO and ADMA affect insulin sensitivity (*7*, *10*), and a defect in their regulation has been reported to increase the incidence of cardiovascular events (*8*, *11*). Teleologically, correction of insulin resistance constitutes an important therapeutic target, and recent pharmacological strategies have led to the development of insulin-sensitizing thiazolidinediones, a new class of anti-diabetic agents that possess ligand activity for nuclear hormone receptor peroxisome proliferator activated receptor-γ (PPARγ). Several lines of evidence have demonstrated that PPARγ ligands not only improve insulin resistance, but also exert multiple actions, including antihypertensive actions and anti-inflammatory actions (*12*). PPARγ ligands have also been reported to increase NO production in various tissues by multiple mechanisms (*13*, *14*). However, the effects of PPARγ ligands on ADMA production and the resultant NO production have not been elucidated. Recently, pharmacological intervention with rosiglitazone, a PPARγ ligand, was shown to enhance insulin sensitivity and reduce ADMA levels in normal healthy volunteers (*15*). In addition, analysis of the DDAH-II promoter gene revealed the presence of a PPAR-binding site at the −927 position, implying that PPARγ ligands directly regulate DDAH-II expression at both the transcriptional levels and serum and tissue ADMA levels (*16*).

In the present study, we examined the effects of pioglitazone (PIO), a PPARγ ligand, on circulatory ADMA levels and renal DDAH-I/II expression in normotensive Wistar-Kyoto rats (WKY). In addition, we examined whether the roles of ADMA and DDAH in hypertension were altered in spontaneously hypertensive rats (SHR). We demonstrated that the treatment with PIO increased renal NO production partly by upregulating tissue DDAH-II expression and reducing systemic ADMA levels in both rat strains. We also found that PIO directly upregulated DDAH-II in Marbin-Dubin Canine Kidney (MDCK) cells, a renal tubular cell line, which was considered to be the mechanism for the increase in renal NO production by this agent.

Methods

Animals

Six-week-old male WKY and SHR weighing 130–140 g were used. They were fed a standard rat chow (15 g/day, 0.38% sodium, 0.97% potassium, and 25.1% protein; Nippon Clea, Tokyo, Japan) with or without PIO (160 mg/kg/day) for 4 weeks, and were allowed free access to tap water throughout the experimental protocols. Rats were assigned to four groups: group 1, WKY ($n=5$); group 2, WKY given PIO ($n=5$); group 3, SHR ($n=5$); and group 4, SHR given PIO ($n=5$). PIO was administered by adding the drug to a chow. Systolic blood pressure (SBP) was measured by the tail-cuff method every week. At week 5, the rats in each group were decapitated and the kidneys were harvested. All experiments were performed in accordance with the animal experimentation guidelines of Keio University School of Medicine.

Biochemical Analyses

Plasma homocysteine was determined by the high performance liquid chromatography (HPLC) method using a modification of the assay described by Vester *et al*. (*17*). Blood samples were collected in EDTA tubes. Plasma was separated at 4°C by centrifugation and frozen at −70°C. Plasma concentrations of ADMA were determined by HPLC using pre-column derivatization with *o*-phthalaldehyde by a modification

of a previously described method (*18*). Urinary nitrites/nitrates (NOx) concentrations were evaluated using the Griess reaction (*19*). Blood samples were obtained after 12-h starvation at the end of the experiments. Plasma glucose was measured by the oxidase method, and plasma insulin by means of an enzyme-linked immunosorbent assay kit. Serum free fatty acid (FFA), cholesterol and triglyceride levels were determined by a nonesterified fatty acid (NEFA) SS test (Eiken, Tokyo, Japan), cholesterol L-type test and triglyceride L-type test (Wako, Osaka, Japan), respectively.

mRNA Isolation, cDNA Synthesis, and Real-Time Polymerase Chain Reaction (PCR)

Total RNA was isolated from the kidneys with Trizol Reagent (Invitrogen, Carlsbad, USA). Total RNA (50 ng) was reverse-transcribed for cDNA synthesis with a SuperScript First-Strand Synthesis System (Invitrogen) for the quantification of mRNA expression of DDAH-I/II and endothelial NOS. Real-time PCR was performed using an ABI PRISM-7700 sequence detector (PE Applied Biosystems, Tokyo, Japan). SYBER Green I Dye (PE Applied Biosystems) was used to detect the PCR reaction. Each set of primers yielded a single amplified PCR product with a sequence identical to one published in GenBank. The sequences for the forward and reverse primers were 5′-catggctgggcctaacctaat-3′ and 5′-tgagtttgtcat agcggtggtc-3′ for DDAH-I, 5′-cagctgctgactgcctctttc-3′ and 5′-aggtaccagggtgacatcagaga-3′ for DDAH-II, and 5′-ggttgat cctgccagtagcatatg-3′ and 5′-ggccgtgcgtacttagacatg-3′ for glyceraldehydes-3-phosphate dehydrogenase (GAPDH), respectively.

Immunoblotting

Excised kidney tissues were snap frozen and stored at −80°C. After tissues were lysed and sonicated in solubilization buffer, immunoblot analysis was performed as previously described (*20*), with some modifications. Blots were incubated with specific antibodies against DDAH-II (Abcam Inc., Cambridge, USA), endothelial NOS (eNOS; Transduction Laboratories, Lexington, USA), neuronal NOS (nNOS; Transduction Laboratories) and inducible NOS (iNOS; Transduction Laboratories) and developed using appropriate secondary antibodies and chemiluminescence (Amersham Biosciences, Buckinghamshire, UK).

Confocal Microscopy

Kidneys were snap frozen in liquid nitrogen and embedded in OCT compound (Sakura Tissue-Tek, McGaw Park, USA), and cryosectioning was performed at a thickness of 5 μm. The slides containing the sectioned tissues were dehydrated in 0.01% sodium bicarbonate at pH 7.4. Tissue was incubated with anti-DDAH-II antibodies at a dilution of 1:200 in 5% nonfat milk in PBS for 2 h at 37°C. The secondary antibody was monoclonal donkey anti-goat IgG conjugated to fluorescein isothiocyanate (Santa Cruz Biotechnology, Santa Cruz, USA) at a 1:50 dilution in PBS containing 0.5% BSA for 1 h. The sections were washed three times with PBS and mounted in Dako fluorescent mounting medium (Dako, Carpinteria, USA). Primary antibody was omitted and run in parallel in negative controls. Immunolabeled sections were examined through the use of confocal laser scanning microscopy (LSM-510; Carl Zeiss, Oberkochen, Germany) at an excitation wavelength of 488 nm.

Cell Culture

In order to examine whether PIO induces the expression of DDAH-II through its direct action on renal tubular cells, we performed *in vitro* experiments using MDCK cells, a renal tubular cell line. MDCK cells were obtained from RIKEN Cell Bank (Ibaraki, Japan) and cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) containing 10% FBS (Irvine Scientific, Santa Anna, USA), 100 units/ml penicillin, 100 mg/ml streptomycin, and 200 mmol/l L-glutamine to reach 70% confluence. After overnight serum starvation, the cells were pretreated with PIO at concentrations of 10 nmol/l, 100 nmol/l and 1 μmol/l. Twenty-four hours after the treatment with PIO, cell lysates were obtained and analyzed by immunoblotting with the antibody against DDAH-II as described above. All treatments were performed in three independent experiments.

Statistical Analysis

Data are expressed as the mean±SEM. Results are analyzed by 2-way ANOVA, followed by Newman-Keuls post hoc test. p values <0.05 were considered statistically significant.

Results

Baseline Characteristics in SHR, WKY and PIO-Treated Rats

Four-week treatment with PIO did not alter body weights in either WKY or SHR (Table 1). Blood pressure was higher in SHR than in age- and sex-matched WKY, and PIO significantly lowered blood pressure in SHR but not in WKY (Fig. 1A). Although fasting glucose levels were not different among the four groups, fasting insulin levels were higher in SHR than in WKY (Table 1), suggesting an insulin-resistant state in SHR (*21*, *22*). PIO restored the fasting insulin level in SHR. Serum levels of triglyceride and FFA were decreased by the treatment with PIO in SHR. Serum total cholesterol levels were lower in SHR than in WKY, a finding consistent with the previous report (*23*). Neither the serum creatinine nor the urinary protein/creatinine level was different between WKY and SHR. Finally, PIO had no effect on these parameters in either strain.

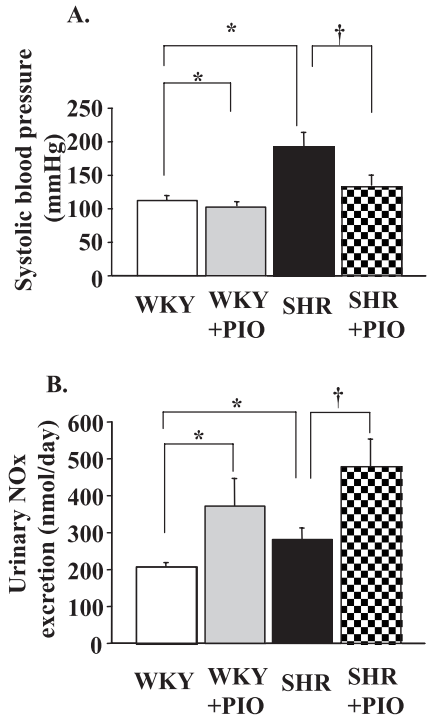

Fig. 1. *Effects of pioglitazone (PIO) on systemic blood pressure and urinary nitric oxide (NOx) excretion in Wister-Kyoto rats (WKY) and spontaneously hypertensive rats (SHR). Four weeks after the treatment with PIO, systolic blood pressure (measured by the tail-cuff method) (A) and daily urinary NOx excretion (B) were measured. Results are presented as the mean±SEM (*n = *5). *p < *0.05* vs. *WKY.* †p < *0.05* vs. *SHR.*

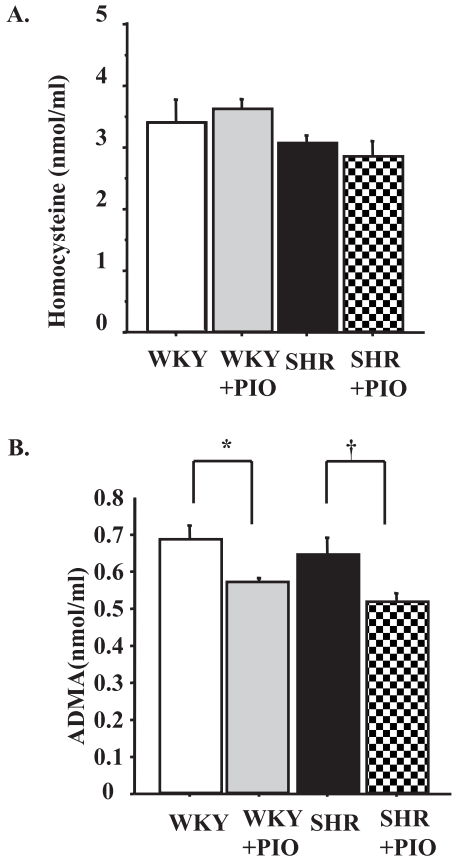

Fig. 2. *Effects of pioglitazone (PIO) on serum levels of homocysteine and asymmetrical dimethylarginine (ADMA) in Wister-Kyoto rats (WKY) and spontaneously hypertensive rats (SHR). Four weeks after the treatment with PIO, serum levels of homocysteine (A) and ADMA (B) were measured. Results are presented as the mean±SEM (*n = *5). *p < *0.05* vs. *WKY.* †p < *0.05* vs. *SHR.*

Effects of PIO on NO Production, Sodium Excretion, and Serum Levels of Homocysteine and ADMA

Urinary NOx excretion was increased in male SHR at 10 weeks of age compared with age- and sex-matched WKY (*24*, *25*) (Fig. 1B). Four-week treatment with PIO enhanced the NOx excretion in both WKY and SHR. Serum homocysteine levels were not different among the four groups (Fig. 2A). Serum levels of ADMA were the same in WKY and SHR. Four-week treatment with PIO significantly decreased the serum ADMA level in both strains (Fig. 2B).

Renal Expression of eNOS, nNOS and iNOS

In order to elucidate the mechanisms for the increased excretion of NOx by the PPARγ ligand, we first examined the expressions of three isoforms of NOS (eNOS, nNOS and iNOS) in the kidney. As previously reported (*25*), the expression levels of eNOS were upregulated in SHR in comparison with those in WKY. PIO had no effects on eNOS expression in either WKY or SHR (Fig. 3A). Similar results were obtained for nNOS and iNOS expression levels (Fig. 3B and C, respectively).

Expression of DDAH-I and DDAH-II in the Kidney

Since ADMA is an endogenous inhibitor for NOS and treatment with PIO decreases the serum ADMA levels, the reduced circulatory ADMA can result in increased NOx production in the kidney. In order to elucidate the mechanism for the decrease in ADMA by PIO, the expression levels of an ADMA-degrading enzyme, DDAH, in the kidney were measured with the use of three different techniques, *i.e.*, real-time PCR, immunoblotting, and immunohistochemistry. Real-time PCR revealed that DDAH-II mRNA levels in the kidney were increased by PIO treatment in both strains (Fig. 4A, b), although the mRNA expression levels of DDAH-I were not changed in PIO-treated rats (Fig. 4A, a). Consistent with these findings, immunoblotting and immunohistochemistry demonstrated that PIO induced protein expression of DDAH-II in

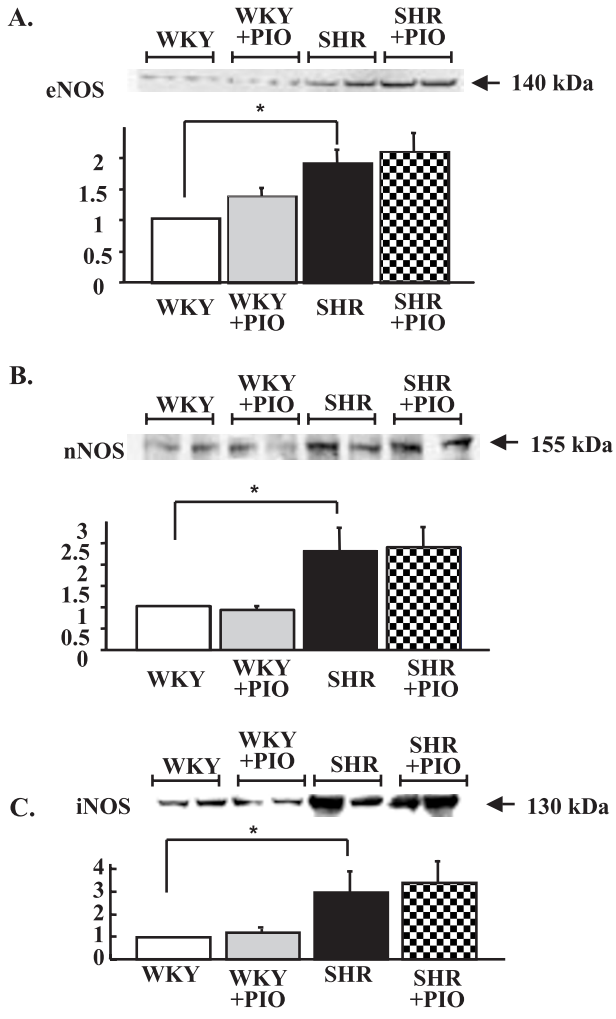

Fig. 3. *Effects of pioglitazone (PIO) on protein expression of endothelial nitric oxide synthase (eNOS), neuronal nitric oxide synthase (nNOS) and inducible nitric oxide synthase (iNOS) in the kidney. Four weeks after the treatment with PIO, tissue homogenates of the kidneys from each rat group were obtained and immunoblotting was performed by using antibodies against eNOS (A), nNOS (B) and iNOS (C). The results are representative of three independent experiments.* *p<*0.05* vs. *WKY. WKY, Wister-Kyoto rats; SHR, spontaneously hypertensive rats.*

the kidney (Fig. 4B and C, respectively). To the extent that the kidney is one of the major organs that metabolize ADMA (*7*), the decrease in serum ADMA in PIO-treated rats was ascribed to the induction of an ADMA-degrading enzyme, DDAH-II, in the kidney.

PIO Upregulated the Protein Expression of DDAH-II in MDCK Cells

Although *in vivo* data indicate that PIO induces the expression of DDAH-II in the kidney at the transcriptional level but not through secondary effects, it remains unclear whether this effect is mediated by direct actions on renal tubular cells. As shown in Fig. 5, DDAH-II protein expression was induced by the treatment with PIO in a renal tubular cell line, MDCK, in a dose-dependent manner, suggesting that PIO induced DDAH-II in the kidney through its direct effect on renal tubular cells.

Discussion

In the present study, we demonstrated that 4-week treatment with a PPARγ ligand, PIO, increased urinary NOx excretion (Fig. 1B). Furthermore, this agent reduced serum ADMA (Fig. 2B). Since ADMA is an intrinsic inhibitor of NOS (*7*, *8*), the decreased level of ADMA would be responsible in part for the increase in urinary NOx. Of note, PIO induced increases in urinary NOx excretion and decreases in ADMA in both WKY and SHR, whereas blood pressure was reduced only in SHR (Fig. 1A). These findings would militate against the premise that renal NO and NOS expressions constitute a major determinant of the pathogenesis of hypertension in SHR, but rather would suggest that these expressions contribute to the compensatory mechanisms to preserve renal function in SHR. In this regard, we have recently demonstrated that PIO reduces the stimulated Rho-kinase activity in the vascular tissue from SHR, but not WKY (*26*). It appears therefore that multiple actions mediate the beneficial effects of PPARγ ligands in hypertension. Alternatively, the serum ADMA concentration observed in this study may not have reached a level sufficient to affect systemic blood pressure. Achan *et al*. (*27*) indicated that a serum ADMA level of 2 μmol/l (2 nmol/ml) was required to induce substantial cardiovascular effects. Furthermore, Matsuoka *et al*. (*28*) reported that urinary ADMA excretion was lower in SHR than in WKY, suggesting that serum ADMA levels had no influence on systemic blood pressure. Nevertheless, the possibility that ADMA functions as a local modulator cannot be eliminated (*7*, *8*).

Of interest, the present study indicates that the expression of three NOS isoforms is higher in kidneys from SHR than in those from WKY at the age of 11 weeks. This observation is consistent with a previous report by Vaziri *et al*. (*25*) that demonstrated increased NO production and upregulation of iNOS and eNOS protein expression in both prehypertensive (8-week-old) and hypertensive (12-week-old) SHR. On the other hand, at 20–25 weeks of age, the renal expression of nNOS is higher in SHR, but the distribution and the expression of both eNOS and iNOS are similar in SHR and WKY (*29*). With advanced age (63 weeks), untreated SHR show lower urinary NOx excretion and depressed renal NOS protein expression compared to untreated WKY, and these effects may be associated with the development of renal injury (*30*). Taken together, these findings indicate that renal NOS expression levels in SHR vary depending on the age of the rat used.

Several lines of evidence have shown that PPARγ ligands stimulate NO production both *in vitro* (*13*) and *in vivo* (*14*).

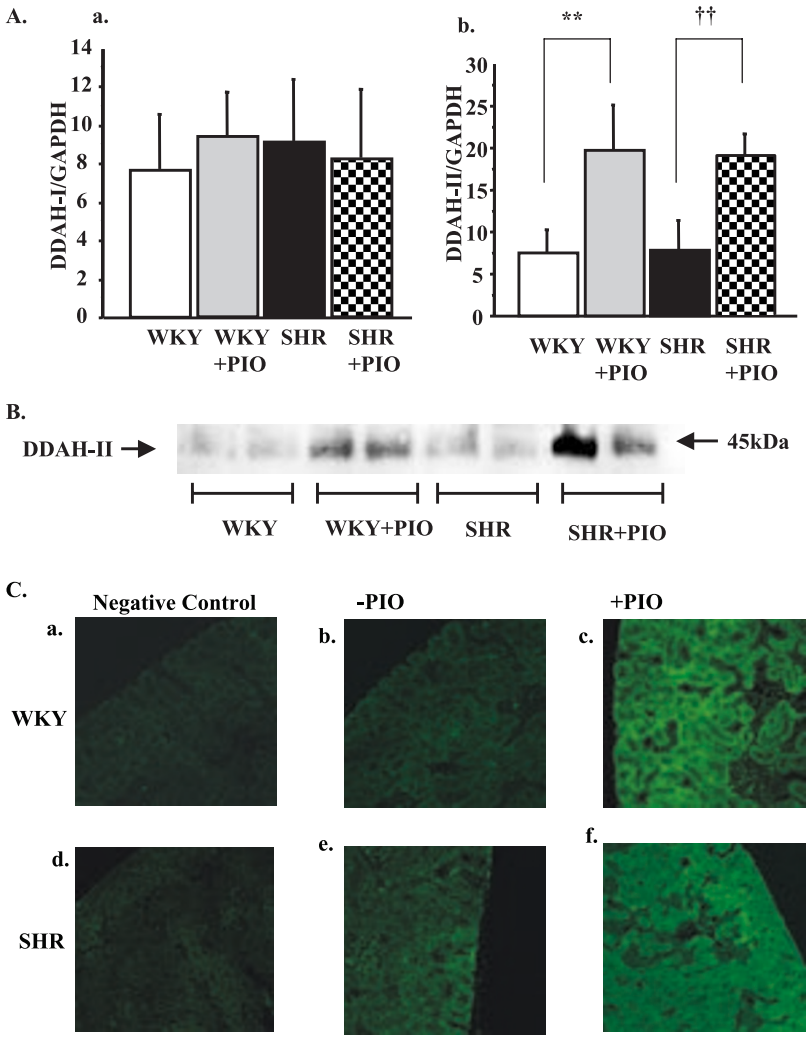

Fig. 4. *Effects of pioglitazone (PIO) on the expression of dimethylarginine dimethylaminohydrolase (DDAH)-I and DDAH-II. A: Four weeks after the treatment with PIO, mRNA expressions of DDAH-I (a) and DDAH-II (b) in the kidneys were measured by real-time PCR. Results are presented as the mean±SEM (*n = *4). **p<*0.01* vs. *WKY.* ††p<*0.01* vs. *SHR. B: Immunoblotting was performed by using the tissue homogenates of the kidneys from each rat group. C: Fluorescent immunohistochemical assays for DDAH-II were performed by using the kidneys from WKY (b), WKY treated with PIO (c), SHR (e), and SHR treated with PIO (f). The results for negative controls for the kidneys from WKY (a) and SHR (d) are also shown. All results are representative of three independent experiments. WKY, Wister-Kyoto rats; SHR, spontaneously hypertensive rats.*

We have also demonstrated that in obese Zucker rats, in which renal NOx production is suppressed compared with that in lean Zucker rats, troglitazone causes partial restoration of urinary NOx excretion (*31*). Since the expression of both eNOS and nNOS in the kidney has been shown to be induced in PIO-treated obese rats (*14*) and PPARγ proteins are widely expressed along the nephron segments (*32*), we have explored the possibility that PPARγ upregulates the renal NOS. Nevertheless, neither eNOS nor nNOS was induced in the kidney by the 4-week treatment with PIO in the present study (Fig. 3A and B, respectively). It is unclear why the expressions of eNOS and nNOS were unaltered in our study. Calnek *et al.* (*13*) have reported that PPARγ ligands stimulate NO release from endothelial cells through a transcriptional mechanism unrelated to eNOS expression. This observation suggests the presence of a mechanism other than NOS expression. Thus, the effect of the PPARγ ligands on NOS expression may vary depending on the experimental settings.

The present study further examined the mechanism by which the PPARγ ligand reduced ADMA and increased NOx. Because DDAH metabolizes ADMA, this enzymatic pathway may be responsible for the induction of these changes by the PPARγ ligands. Thus, in the present study, we have demonstrated that 4-week treatment with PIO markedly upregulated DDAH-II, but not DDAH-I, expression in the kidney (Fig. 4). Furthermore, such upregulation was observed in cultured

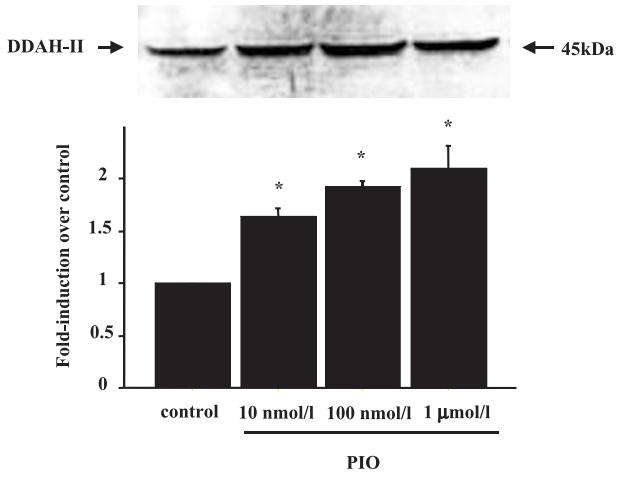

Fig. 5. *Pioglitazone (PIO) induces the expression of dimethylarginine dimethylaminohydrolase (DDAH)-II in Marbin-Dubin Canine Kidney (MDCK) cells. MDCK cells were serum-starved for 24 h and stimulated with PIO at concentrations of 10 nmol/l to 1 μmol/l for 24 h. Total cell lysates were analyzed by immunoblotting using antibody against DDAH-II. The results are presented as the mean±SEM (*n = *4). *p < *0.05* vs. *controls.*

MDCK cells (Fig. 5), indicating a direct effect of the PPARγ ligand on renal tubular cells. These data are consistent with a recent genome analysis demonstrating the presence of PPRE (PPAR responsive element) in the promoter region of DDAH-II (*16*). It has been shown that DDAH-II is regulated by various factors, including low density lipoprotein (LDL)-cholesterol (*33*), interleukin (IL)-1β (*34*) and retinoic acid (*35*). The present study provides strong evidence for a novel mechanism whereby DDAH is upregulated and then the NO synthesis pathway is stimulated. Finally, although we did not evaluate the endothelial function in PIO-treated SHR or WKY, PIO has been reported to improve endothelial function in various pathological settings (*36*, *37*). Since DDAH-II is expressed in endothelial cells (*16*) and modulates eNOS activity, the ADMA-lowering effects of PIO could provide beneficial effects on endothelial function.

Of note, a recent study in healthy human subjects has revealed a close relationship between circulating ADMA concentrations and insulin resistance (*15*). This study also showed that the decrease in ADMA by treatment with a PPARγ ligand, rosiglitazone, was attributed partly to the amelioration in insulin sensitivity. Nevertheless, our data failed to demonstrate a correlation between serum ADMA and fasting insulin levels (Table 1), suggesting a direct action of PIO on the ADMA production rather than an indirect action through the change in insulin sensitivity. Finally, two previous studies have demonstrated that serum ADMA levels are elevated in hypercholesterolemic rabbits (*38*) and humans (*39*). Boger *et al*. (*33*) demonstrated that LDL-cholesterol upregulated the synthesis of ADMA in human endothelial cells through the induction of protein arginine methyl transferase (PRMT), an enzyme associated with ADMA production. Nevertheless, in the present study PIO did not affect either total cholesterol levels (Table 1) or LDL-cholesterol concentrations (data not shown), which would seem to preclude an important role of these lipids in ADMA levels.

Accumulating evidence has demonstrated a close association between elevated ADMA levels and cardiovascular risk factors. For example, a recent study demonstrated that a systemic increase in ADMA produces adverse cardiovascular effects in humans, including an increase in blood pressure, reduced heart rates and reduced cardiac output (*29*). Therefore, the decrease in ADMA levels by treatment with PPARγ ligands may contribute to cardiovascular risk reduction. Although a clinical trial should be awaited, our data suggest that the use of PPARγ ligands may constitute a novel therapeutic strategy in the field of cardiovascular disease.

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