Original Contribution

Mechanisms underlying the chronic pioglitazone treatment-induced improvement in the impaired endothelium-dependent relaxation seen in aortas from diabetic rats

Takayuki Matsumoto, Eri Noguchi, Tsuneo Kobayashi, Katsuo Kamata*

Department of Physiology and Morphology, Institute of Medicinal Chemistry, Hoshi University, Shinagawa-ku, Tokyo 142-8501, Japan

Received 5 July 2006; revised 20 November 2006; accepted 28 December 2006

Available online 8 January 2007

Abstract

The objectives of this study were to determine the effects of chronic treatment with pioglitazone, a peroxisome proliferator-activated receptor γ agonist, on the impaired endothelium-dependent relaxation seen in aortas from established streptozotocin (STZ)-induced diabetic rats, and to identify some of the molecular mechanisms involved. Starting at 8 weeks of diabetes, pioglitazone (10 mg/kg) was administered to STZ-induced diabetic rats for 4 weeks. In untreated STZ rats (vs age-matched control rats): (1) ACh-induced relaxation, cGMP accumulation, phosphorylation of the cGMP-dependent protein kinase substrate vasodilator-stimulated phosphoprotein at Ser-239 [an established biochemical end-point of nitric oxide (NO)/cGMP signaling], and Cu/Zn-superoxide dismutase (SOD) expression and SOD activity were all reduced; (2) aortic superoxide generation, nitrotyrosine expression, and NAD(P)H oxidase activity were increased; (3) plasma endothelin-1 (ET-1) and aortic c-Jun (AP-1 component) protein expressions were increased. Pioglitazone treatment markedly corrected the above abnormalities. Collectively, these results suggest that pioglitazone treatment improves endothelium-dependent relaxation by reducing oxidative stress via increased SOD activity, decreased NAD(P)H oxidase activity, and a decreased ET-1 level, and that this decreased ET-1 level may be attributable to an inhibition of the AP-1 signaling pathway.

© 2007 Elsevier Inc. All rights reserved.

Keywords: Aorta; Diabetes; Endothelial dysfunction; Endothelin; Nitric oxide; Oxidative stress; Pioglitazone

Introduction

Endothelial dysfunction plays a key role in the pathogenesis of diabetic vascular disease [1–11]. It has been suggested that the excessive elevations in plasma glucose, low-density lipoprotein (LDL) cholesterol, and superoxide anion that occur in diabetes are causally involved in the development of this dysfunction [12–15].

To date, the mechanisms responsible for mediating endothelial dysfunction in diabetes have not been completely defined, although a considerable body of evidence implicates oxidative stress as an important pathogenic element in its development [2,5,6,10]. Oxidative stress, defined as an increase in the steady-state levels of reactive oxygen species, may occur as a result of increased free-radical generation—an event attributable to a large extent to NAD(P)H oxidase activation within the vascular system [10,16–19]—and/or to a decrease in antioxidant defense mechanisms, such as superoxide dismutase (SOD) [20–22]. In diabetic rats, endothelium-dependent relaxation may be impaired by an excess generation of superoxide, which destroys nitric oxide (NO) [5,6]. Endothelium-derived NO stimulates the production of the

Abbreviations: ACh, acetylcholine; AP-1, activator protein-1; DHE, dihydroethidium; eNOS, endothelial nitric oxide synthase; ET-1, endothelin-1; HDL, high-density lipoprotein; HRP, horseradish peroxidase; KHS, Krebs-Henseleit solution; LDL, low-density lipoprotein; L-NNa, Nε-nitro-L-arginine; NA, noradrenaline; NADH, nicotinamide adenine dinucleotide; NBT, nitroblue tetrazolium; NO, nitric oxide; NOS, nitric oxide synthase; PM, phenylmethylsulfonyl fluoride; PPAR, peroxisome proliferator-activated receptor; SNP, sodium nitroprusside; SOD, superoxide dismutase; SOV, sodium orthovanadate; STZ, streptozotocin; TFA, trifluoroacetic acid; VASP, vasodilator-stimulated phosphoprotein.

* Corresponding author. Fax: +81 3 5498 5856.
E-mail address: kamata@hoshi.ac.jp (K. Kamata)

0891-5849/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
second messenger cGMP by activation of soluble guanylyl cyclase (sGC) in vascular smooth muscle cells, and cGMP alters the properties of several target proteins. For example, it activates cGMP-dependent protein kinase I (cGK), which is essential both for vascular smooth muscle relaxation and for the antiproliferative effects of NO [23]. One of the best-characterized cGK substrates is vasodilator-stimulated phosphoprotein (VASP) [24], originally described in platelets. Within the vascular wall, phosphorylation of VASP at Ser-239 is regulated by NO, and it is a sensitive monitor of defective vascular NO/cGMP signaling, an event associated with endothelial dysfunction [25–28].

Endothelin-1 (ET-1), a potent endothelial-derived vasoconstrictor peptide [29,30], is widely known to stimulate ET\(\alpha\) receptors in vascular smooth muscle cells (to produce vasoconstriction) and ET\(\beta\) receptors on endothelial cells (to produce vasodilation). A perturbation of the balance between ET\(\alpha\)- and ET\(\beta\)-receptor activity may contribute to the pathogenesis of vascular disease [30]. Some, but not all, experimental models of hypertension, atherosclerosis, and diabetes display high levels of circulating ET-1, and exhibit endothelial dysfunction [31–33]. Although ET-1 has been linked to the production of superoxide within the vasculature [34–38], little is known about the precise relationship between these important modulators of vascular function in the diabetic state.

Peroxisome proliferator-activated receptors (PPARs) are a family of ligand-activated transcription factors recently found to be involved in vascular biological homeostasis [39,40]. Indeed, the PPAR-\(\alpha\) and -\(\gamma\) isoforms have been characterized in multiple vascular cell types in rats and humans [40–42]. Moreover, PPAR activators prevent in vitro vascular smooth muscle cell growth and inflammatory responses, and also induce apoptosis, suggesting a potential role in vascular remodeling [40]. Interestingly, PPAR-\(\gamma\) activators are able to suppress ET-1 secretion from both endothelial and vascular smooth muscle cells [43,44]. In addition, ET-1 production by endothelial cells can be activated by many factors, such as insulin or thrombin, through c-jun fixation on the activator protein-1 (AP-1) site of the prepro-ET-1 promoter [45]. Furthermore, Delerive et al. [41] demonstrated in vitro that PPAR-\(\alpha\) and -\(\gamma\) bind c-jun, resulting in an inhibition of ET-1 production.

The aims of our study were to assess the effects of chronic treatment with pioglitazone, a PPAR-\(\gamma\) agonist, on the impaired endothelium-dependent relaxation seen in aortas isolated from established streptozotocin (STZ)-induced diabetic rats, and to identify some of the molecular mechanisms involved.

Materials and methods

General

The experimental design was approved by the Hoshi University Animal Care and Use Committee, and all studies were conducted in accordance with Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health, and Guide for the Care and Use of Laboratory Animals adopted by the Committee on the Care and Use of Laboratory Animals of Hoshi University (which is accredited by the Ministry of Education, Culture, Sports, Science, and Technology, Japan).

Reagents

Sodium nitroprusside (SNP), STZ, phenylmethylsulfonyl fluoride (PMSF), nitroblue tetrazolium (NBT), (–)-noradrenaline hydrochloride (NA), \(N^\prime\)-nitro-L-arginine (L-NNA), \(\beta\)-nicotinamide adenine dinucleotide (NADH), allopurinol, superoxide dismutase, cytochrome c, dihydroethidium (DHE), and monoclonal \(\beta\)-actin antibody were all purchased from Sigma Chemical Co. (St. Louis, MO), while acetylcholine chloride (ACh) was from Daiichi Pharmaceuticals (Tokyo, Japan). Endothelin-1 was from Peptide Institute, Inc. (Osaka, Japan). Apocynin was from Calbiochem-Novabiochem Corporation (La Jolla, CA). All drugs were dissolved in saline, except where otherwise noted. All concentrations are expressed as the final molar concentration of the base in the organ bath. Horseradish peroxidase (HRP)-linked secondary anti-mouse and anti-rabbit antibodies were purchased from Promega (Madison, WI), while antibodies against endothelial NOS (eNOS), c-Jun, and Mn-SOD were obtained from BD Biosciences (San Jose, CA). Cu/Zn-SOD antibody was from Stressgen (Victoria, BC, Canada), while phospho-VASP (Ser 239) antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). Nitrotyrosine antibody was from Chemicon (Temecula, CA).

Animals and experimental design

Male Wistar rats (8 weeks old and 180–230 g body weight) received a single injection via the tail vein of STZ 65 mg/kg dissolved in a citrate buffer. Age-matched control rats were injected with the buffer alone. Food and water were given ad libitum. To examine the therapeutic effect, not preventive effect, of pioglitazone, some STZ-induced diabetic rats were given pioglitazone (10 mg/kg, p.o., daily) for 4 weeks starting 8 weeks after the STZ injection (i.e., in our established diabetic model). Twelve weeks after the STZ injection, the rats were killed by decapitation under ether anesthesia. Control rats were killed in the same way 12 weeks after receiving their buffer injection. Thus, we studied three groups: controls and pioglitazone-un-treated or -treated diabetic groups.

Measurement of plasma glucose, cholesterol, triglyceride, insulin, and blood pressure

Plasma parameters and blood pressure were measured as described previously [31,46–49].

Measurement of isometric force

Rats were anesthetized with diethyl ether and euthanized by decapitation at 12 weeks after STZ treatment. A section of the aorta from between the aortic arch and the diaphragm was
removed and placed in ice-cold, oxygenated, modified Krebs-Henseleit solution (KHS). This solution consisted of (in mM) 118.0 NaCl, 4.7 KCl, 25.0 NaHCO₃, 1.8 CaCl₂, 1.2 Na₂HPO₄, 1.2 MgSO₄, and 11.0 dextrose. The aorta was prepared by the method described previously by us [15,31,50]. Briefly, it was cleaned of loosely adhering fat and connective tissue and cut into helical strips 2 mm in width and 20 mm in length. The tissue was then placed in a well-oxygenated (95% O₂–5% CO₂ bath of 10 ml KHS at 37 °C. With one end connected to a tissue holder and the other to a force-displacement transducer (Model TB-611T; Nikon Kohden, Tokyo, Japan), the tissue was allowed to equilibrate for 60 min under a resting tension of 1.0 g (determined to be optimal in preliminary experiments). During this period, the KHS in the bath was replaced every 20 min. After equilibration, each aortic strip was contracted with 10⁻⁶ M noradrenaline (NA), and the presence of functional endothelial cells was confirmed by demonstrating relaxation in response to 10⁻⁵ M acetylcholine. For the relaxation studies, the aortic strips were precontracted with an equieffective concentration of NA (5×10⁻⁸–3×10⁻⁷ M). When the NA-induced contraction had reached a plateau level, ACh (10⁻⁹–10⁻⁵ M) or sodium nitroprusside (10⁻¹⁰–10⁻⁵ M) was added in a cumulative manner. After the addition of sufficient aliquots of the agonist to produce the chosen concentration, a plateau response was allowed to develop before the addition of the next dose of the same agonist.

In separate experiments, we investigated the effects of various drugs [NOS inhibitor (L-NNA; 10⁻⁴ M), xanthine oxidase inhibitor (allopurinol; 10⁻³ M), NAD(P)H oxidase inhibitor (apocynin; 10⁻⁴ M), SOD (180 U/ml), or ET-1 (3×10⁻¹⁰–10⁻⁵ M)] on the responses to a relaxant agent. When the NA-induced contraction had reached a plateau level, the relaxant response to ACh (10⁻⁷ M) was examined in a single concentration–effect manner. Each aortic strip was exposed to only one concentration of a given drug. When the effects of the above drugs were examined in aortic strips, each drug was added to the bath 30 min before the administration of NA.

**Enzyme immunoassay for cGMP**

The cGMP level in the ACh-stimulated aorta was quantified as in our previous paper [46,51]. Briefly, aortic rings from controls and pioglitazone-untreated or -treated diabetic rats were incubated for 30 min at 37 °C in well-oxygenated KHS. The rings were frozen in liquid N₂ after a 5-min addition of ACh (10 μM) or vehicle and then stored at -80 °C. cGMP was subsequently extracted in 6% trichloroacetic acid, followed by neutralization with water-saturated diethyl ether, and enzyme immunoassay (Amersham Biosciences, UK). In this determination of arterial cGMP, we did not employ a phosphodiesterase inhibitor.

**Quantification of superoxide anion by measurement of the amount of NBT reduced and by dihydroethidium staining**

Aortic rings were incubated with NBT to allow the superoxide generated by the tissue to reduce the NBT to blue formazan. The details of the assay have been published previously [50,52,53]. Briefly, aortas from controls and pioglitazone-untreated or -treated diabetic groups were cut into transverse rings 5 mm in length. These were placed for 90 min in 1 ml KHS at 37 °C containing NBT (10⁻⁴ M) in the presence of ACh (10⁻⁷ M). In some diabetic aortic rings, SOD (180 U/ml) or vehicle was pretreated before NBT treatment. The NBT reduction was stopped by the addition of 0.5 N HCl (1 ml). After this incubation, the rings were minced and homogenized in a mixture of 0.1 N NaOH and 0.1% SDS in water containing 40 mg L⁻¹ diethylenetriaminepentaacetic acid. The mixture was centrifuged at 16,000 g for 30 min, and the resultant pellet resuspended in 250 μl of pyridine at 80 °C for 60 min to extract formazan. The mixture was then subjected to a second centrifugation at 10,000 g for 10 min. The absorbance of the formazan was determined spectrophotometrically at 540 nm. The amount of NBT reduced (=quantity of formazan) was calculated as follows: amount of NBT reduced = A × V / (T × Wt × ε × l), where A is the absorbance, V is the volume of pyridine, T is the time for which the rings were incubated with NBT, Wt is the blotted wet weight of the aortic rings, ε is the extinction coefficient (0.71 mmol⁻¹/mm), and l is the length of the light path. The results are reported in picomoles per minute per milligram Wt.

DHE staining was used to evaluate the in situ levels of superoxide in the aorta [25,34,37,54]. Cells are freely permeable to DHE, and in the presence of superoxide it is oxidized to fluorescent ethidium bromide, which is trapped intracellularly by intercalation into the DNA. Frozen, enzymatically intact, 7-μm-thick sections of cross sections of the aorta were incubated with DHE (10 μM) in PBS for 30 min at 37 °C. In some preparations, SOD (180 U/ml) was incubated for 30 min before DHE treatment and was present thereafter. Fluorescent images of ethidium bromide were obtained using an inverted microscope (Nikon ECLIPSE TS100-F; Nikon Co, Tokyo, Japan), and fluorescence was detected using a 590-nm long-pass filter.

**Measurement of nitrite and nitrate**

Concentrations of nitrite (NO₂⁻) and nitrate (NO₃⁻) (NOx) in the plasma were assayed by the method described by us previously [55]. For the determination of plasma NOx, to 0.3 ml of each plasma sample, 0.3 ml of 100% methanol was added, and the sample was then centrifuged at 5000 g for 10 min at 4 °C. Briefly, the NOx in the plasma was separated by means of a reverse-phase separation column packed with polystyrene polymer (NO-PAK, 4.6×50 mm; Eicom, Kyoto, Japan), after which NO₃⁻ was reduced to NO₂⁻ in a reduction column packed with copper-plated cadmium filings (NO-RED; Eicom, Kyoto, Japan). The NO₂⁻ was mixed with a Griess reagent to form a purple azo dye in a reaction coil. The separation and reduction columns and the reaction coil were placed in a column oven set at 35 °C. The absorbance of the product dye at 540 nm was measured using a flowthrough spectrophotometer (NOD-10; Eicom). The mobile phase, which was delivered by a pump at a rate of 0.33 ml/min, was 10% methanol containing 0.15 M
NaCl/NH₄Cl and 0.5 g/L 4 Na-EDTA. The Griess reagent, which was 1.25% HCl containing 5 g/L sulfanilamide with 0.25 g/L N-naphthylethlenediamine, was delivered at a rate of 0.1 ml/min. The concentrations of NO₂⁻ and NO₃⁻ and the reliability of the reduction column were examined in each experiment.

**Measurement of SOD activity in aortic tissue**

An aortic sample was rapidly removed with the endothelium intact, homogenized in 200 μl ice-cold Tris-sucrose buffer containing 10 mM Tris-HCl (pH 7.2), 340 mM sucrose, 1 mM EDTA, 1 mM PMSF, and 10 μg/ml aprotinin, and then incubated for 30 min. Samples were centrifuged (16,000g, 10 min, 4 °C), and the supernatant was used for the measurement of SOD activity. SOD activity was assayed by means of a SOD Assay Kit-WST (Dojindo Lab., Kumamoto, Japan), in which xanthine and xanthine oxidase serve as a superoxide indicator, and a highly water-soluble tetrazolium salt WST-1 (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt) is used as a superoxide indicator. SOD activity was calculated using the standard curve, according to the manufacturer’s instructions.

**Measurement of NAD(P)H oxidase activity in aortic tissue**

An aortic sample was rapidly removed with the endothelium intact, homogenized in 200 μl ice-cold Tris-sucrose buffer containing 10 mM Tris-HCl (pH 7.2), 340 mM sucrose, 1 mM EDTA, 1 mM PMSF, and 10 μg/ml aprotinin, and then incubated for 30 min. Samples were centrifuged (16,000g, 10 min, 4 °C), and the supernatant (5 μg protein) was added to reaction buffer [78 μM cytochrome c, 100 μM NADH (a NAD(P)H oxidase substrate), with or without 250 U/ml SOD], and then incubated at 37 °C for 60 min. NAD(P)H oxidase activity was quantified from the values obtained for absorbance with or without SOD, as previously described [54,56].

**Enzyme immunoassay for ET-1**

Plasma samples taken 12 weeks after injection of STZ or buffer were extracted using Amprep C2 columns (Amersham International plc., Buckinghamshire, UK). The columns were equilibrated by washing with 2 ml methanol followed by 2 ml water. Each plasma sample (1 ml) was acidified with 0.25 ml 2 M HCl, centrifuged at 10,000g for 5 min at room temperature, and then loaded onto the column. The column was washed with 5 ml of 0.1% trifluoroacetic acid (TFA), and immunoreactive ET-1 was eluted with 2 ml of 80% methanol containing 0.1% TFA. Then, the eluent was dried down in a centrifugal evaporator, care being taken not to overdry the pellet. Measurement of the plasma ET-1 concentration was carried out using a commercially available ET-1 ELISA system (Amersham Biosciences UK Ltd, Buckinghamshire, England).

**Western blotting**

The level of several proteins was quantified using immunoblotting procedures, essentially as described before [48]. For the detection of P-VASP, the aorta was cleaned of loosely adhering fat and connective tissue, and cut into rings (5 mm long) under ice-cold KHS. Rings were incubated for 30 min at 37 °C in well-oxygenated KHS, frozen in liquid N₂ after a 5-min addition of Ach (10 μM), and then stored at −80 °C. Aortic tissues were homogenized in ice-cold lysis buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.5 mM EDTA, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM DTT, 100 μg/ml PMSF, 1 μg/ml aprozin, 2 μg/ml leupeptin, and 100 μM SOV. The lysate was cleared by centrifugation at 16,000g for 10 min at 4 °C. The supernatant was collected, and the proteins were solubilized in Laemmli’s buffer containing mercaptoethanol. The protein concentration was determined by means of a bicinchoninic acid (BCA) protein assay reagent kit (Pierce, Rockford, IL). Samples (20 or 40 μg/lane) were resolved by electrophoresis on 7.5, 12, or 15% SDS-PAGE gels and then transferred onto polyvinylidene difluoride (PVDF) membranes. Briefly, after blocking the residual protein sites on the membrane with Immuno-Block (Dainippon-Pharm., Osaka, Japan), the membrane was incubated with anti-eNOS (140 kDa; 1:1000), anti-c-Jun (39 kDa; 1:1000), anti-Mn-SOD (25 kDa; 1:1000), anti-Cu/Zn-SOD (20 kDa; 1:1000), anti-phospho VASP (53 kDa; 1:200), anti-nitrotyrosine (30 kDa; 1:1000), or anti-β-actin (42 kDa; 1:5000) in blocking solution. HRP-conjugated, anti-mouse or anti-rabbit antibody was used at a 1:10,000 dilution in Tween PBS, followed by detection using SuperSignal (Pierce). To normalize the data, we used β-actin as a housekeeping protein. The optical densities of the bands on the film were quantified using densitometry, with correction for the optical density of the corresponding β-actin band.

**Immunohistochemistry**

The level of nitrotyrosine protein was assayed using immunohistochemical procedures, essentially as described before [57]. Some strips from aortas were embedded in O. C. T. Compound (Sakura, Torrance, CA). After a wash with this compound, slides were treated with 10 mmol/L citric acid and then microwave-heated (for 1 min) to recover antigenicity. Nonspecific binding was blocked by treatment with a drop of normal horse serum in Block ace overnight at 4 °C. For the detection of P-VASP, the aorta was cleaned of loosely adhering fat and connective tissue, and cut into rings (5 mm long) under ice-cold KHS. Rings were incubated for 30 min at 37 °C in well-oxygenated KHS, frozen in liquid N₂ after a 5-min addition of Ach (10 μM), and then stored at −80 °C. Aortic tissues were homogenized in ice-cold lysis buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.5 mM EDTA, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM DTT, 100 μg/ml PMSF, 1 μg/ml aprozin, 2 μg/ml leupeptin, and 100 μM SOV. The lysate was cleared by centrifugation at 16,000g for 10 min at 4 °C. The supernatant was collected, and the proteins were solubilized in Laemmli’s buffer containing mercaptoethanol. The protein concentration was determined by means of a bicinchoninic acid (BCA) protein assay reagent kit (Pierce, Rockford, IL). Samples (20 or 40 μg/lane) were resolved by electrophoresis on 7.5, 12, or 15% SDS-PAGE gels and then transferred onto polyvinylidene difluoride (PVDF) membranes. Briefly, after blocking the residual protein sites on the membrane with Immuno-Block (Dainippon-Pharm., Osaka, Japan), the membrane was incubated with anti-eNOS (140 kDa; 1:1000), anti-c-Jun (39 kDa; 1:1000), anti-Mn-SOD (25 kDa; 1:1000), anti-Cu/Zn-SOD (20 kDa; 1:1000), anti-phospho VASP (53 kDa; 1:200), anti-nitrotyrosine (30 kDa; 1:1000), or anti-β-actin (42 kDa; 1:5000) in blocking solution. HRP-conjugated, anti-mouse or anti-rabbit antibody was used at a 1:10,000 dilution in Tween PBS, followed by detection using SuperSignal (Pierce). To normalize the data, we used β-actin as a housekeeping protein. The optical densities of the bands on the film were quantified using densitometry, with correction for the optical density of the corresponding β-actin band.
Statistical analysis

Data are expressed as the means±SE. When appropriate, statistical differences were assessed by Dunnett’s test for multiple comparisons after a one-way analysis of variance (ANOVA), a probability level of \( P<0.05 \) being regarded as significant. Statistical comparisons between concentration–response curves were made using a two-way ANOVA, with Bonferroni’s correction for multiple comparisons being performed post hoc (\( P<0.05 \) again being considered significant).

Results

General parameters

As indicated in Table 1, the plasma glucose, cholesterol, and triglyceride levels were all significantly higher in STZ rats than in age-matched control rats. Body weight, systolic blood pressure, and heart rate were significantly lower in STZ rats than in control rats. Plasma HDL cholesterol levels were similar between STZ rats and control rats. Treatment with pioglitazone
did not alter plasma glucose, HDL cholesterol, body weight, or blood pressure in our established diabetic rats, but it significantly lowered plasma triglyceride and elevated heart rate (compared with untreated STZ rats).

Relaxation responses to ACh and SNP

In order to demonstrate endothelial dysfunction in diabetic animals and its modulation by chronic pioglitazone treatment, we assessed the vasodilator response to the endothelium-dependent activator ACh. When the NA (5 × 10⁻⁸ – 3 × 10⁻⁷ M)-induced contraction had reached a plateau, ACh (1 × 10⁻⁸ – 1 × 10⁻⁵ M) or SNP (1 × 10⁻¹⁰ – 1 × 10⁻⁵ M) was added cumulatively (Fig. 1). In aortic strips from age-matched control rats, ACh caused a concentration-dependent relaxation, with the maximum response at 10⁻⁵ M. This relaxation was significantly weaker (Fig. 1A), and the sensitivity to ACh significantly lower (Fig. 1B), in strips from STZ-induced diabetic rats. Aortic strips from STZ-induced diabetic rats chronically treated with pioglitazone (10 mg/kg, p.o., daily for 4 weeks) showed improved ACh-induced relaxation (Figs. 1A and 1B). The endothelium-independent relaxation responses induced by SNP did not differ significantly among the three groups (Figs. 1C and 1D). These results indicate a dysfunction of the endothelium in diabetic animals and an improvement of this dysfunction by chronic pioglitazone treatment.

Biochemical analysis of NO/cGMP signaling pathway

In view of the published evidence that the NO/cGMP signaling pathway plays a major role in endothelium-dependent relaxation in the aorta [5,6], we performed a biochemical analysis of this pathway in aortas from our three groups of rats.

The basal (i.e., vehicle-treated) concentration of cGMP was lower in rings from diabetic rats than in those from age-matched control rats (Fig. 2A). In both age-matched control and diabetic rats, ACh increased the cGMP level in aortic rings, although the increase was greater in the former than in the latter group (Fig. 2A). The increment in the ACh-stimulated cGMP level was significantly greater in aortic rings from chronically pioglitazone-treated diabetic rats than in those from untreated diabetic rats (Fig. 2A).

Phosphorylation of VASP at Ser-239 (P-VASP) is a reliable monitor of NO/cGMP signaling [25–28]. Therefore, to look for a correlation between endothelial dysfunction in the diabetic aorta and the integrated signal of the NO/cGMP pathway, we examined the levels of phosphorylated VASP in aortic rings after pretreatment with ACh (Fig. 2B). The amount of P-VASP was markedly reduced in such aortas from diabetic rats (vs their controls). The amount of P-VASP was significantly greater in rings from pioglitazone-treated diabetic rats than in those from diabetic rats not treated with pioglitazone.

Determination of NO synthesis as plasma nitrite and expression of aortic eNOS protein

Since the plasma level of nitrite (an oxidation product of NO) correlates with the level of NO biosynthesis [58,59] and since the nitrate/nitrite ratio is often used as an indirect marker of oxidative stress [60], we measured the levels of nitrite and nitrate in rat plasma as a measure of NO synthesis. Moreover, we used Western blot analysis to examine whether the aortic expression of eNOS protein is changed by chronic pioglitazone treatment (Fig. 3). The plasma nitrite level was lower in untreated and chronically pioglitazone-treated STZ-diabetic rats than in age-matched control rats. The nitrate/nitrite ratio was significantly greater in untreated STZ rats than in the controls. Chronic administration of pioglitazone to diabetic rats significantly reduced this increased nitrate/nitrite ratio (Fig. 3A). The aortic expression of eNOS protein did not differ among the three groups (Fig. 3B).

Expression of nitrotyrosine protein

The reaction between NO and superoxide results in the formation of peroxynitrite, which then nitrates tyrosyl residues...
in proteins to yield 3-nitrotyrosine [9,61]. Since nitrotyrosine accumulation is a widely accepted biochemical marker of increased generation of reactive oxygen/nitrogen species [9,22,61], we performed immunohistochemistry and Western blotting for nitrotyrosine in aortas from our three groups of rats. By comparison with their controls, aortas from STZ-induced diabetic rats showed increased positive immunostaining for nitrotyrosine (Fig. 4A). In the pioglitazone-treated diabetic rats, staining for nitrotyrosine was greatly decreased (vs untreated diabetes) (Fig. 4A). Next, we examined the expression of nitrotyrosine protein by Western blot (Fig. 4B). Use of anti-nitrotyrosine antibody allowed detection of immunoreactive proteins with a molecular weight of 30 kDa, as previously reported [57,61]. The expression of such nitrotyrosine protein was significantly increased in aortas from STZ-induced diabetic rats (vs the controls), and this increase was completely normalized by chronic pioglitazone treatment (Fig. 4B).

Effects of various oxidative stress-related agents on the time course of ACh-induced relaxation

To investigate the possible mechanisms underlying the endothelial dysfunction seen in diabetic rats and its normalization in chronically pioglitazone-treated individuals, we examined the effects of various oxidative stress-related agents on the time course of the relaxation induced by ACh (10⁻⁷ M), a method reported previously [20]. ACh (10⁻⁷ M) caused a rapid, long-lasting relaxation of aortic strips from the age-matched controls (Figs. 5A and 5B). The relaxation response to ACh (10⁻⁷ M) was more transient in diabetic rats and the maximal relaxation was decreased (Figs. 5A and 5B). The ACh (10⁻⁷ M)-induced relaxation was significantly greater in aortic rings from chronically pioglitazone-treated diabetic rats than in those from untreated diabetic rats (Figs. 5A and 5B). This ACh-induced relaxation was completely blocked by L-NNA (10⁻⁴ M) treatment in all three groups (Fig. 5C). Although the ACh-induced relaxation was significantly rescued by SOD (180 U/ml) treatment in STZ-diabetic strips, SOD (180 U/ml) had no effect on the ACh-induced relaxation in aortic strips from either the age-matched controls or the pioglitazone-treated diabetic rats (Fig. 5C). Similarly, apocynin (10⁻⁴ M) improved the ACh-induced relaxation in STZ-diabetic aortas, but had no effect on control strips (Fig. 5C). On the other hand, preincubation with allopurinol (10⁻³ M) had no effect on ACh-induced relaxation in aortic strips from either age-matched control rats or STZ-induced diabetic rats (Fig. 5C).

Aortic superoxide generation

Since superoxide has been shown to decrease NO bioavailability [2,5,6,10,14,17], we examined aortic superoxide generation. This was done by staining with DHE (superoxide-sensitive dye) [25,34,37,54] and by measuring the amount of NBT reduced [50,52,53]. Increased nuclear staining, indicating enhanced superoxide production, was evident in aortic tissues from STZ-induced diabetic rats compared with those from age-matched control rats (Fig. 6Aa vs Fig. 6Ab). Chronic administration of pioglitazone to diabetic rats greatly reduced this staining (Fig. 6Ab vs Fig. 6Ac). In STZ-diabetic aortas, the antioxidant SOD (180 U/ml) prevented the increase in DHE staining (Fig. 6Ab vs Fig. 6Ad). Moreover, to judge from the amount of NBT reduced (Fig. 6B), the

Fig. 3. Analysis of the plasma level of nitrite and the nitrate/nitrite ratio and of eNOS protein expression in aortas obtained from age-matched controls, untreated diabetic rats, and chronically pioglitazone-treated diabetic rats. Details are given under Materials and methods. (A) Plasma level of nitrite and the nitrate/nitrite ratio in age-matched controls, untreated diabetic rats, and chronically pioglitazone-treated diabetic rats. Each column represents the mean±SE of twelve determinations. *P<0.05, **P<0.01, ***P<0.001 vs. control group, ##P<0.01 vs. untreated diabetic group. (B, upper panel) Representative Western blots for eNOS and β-actin. Proteins (20 μg) were subjected to 7.5% PAGE and then transferred to polyvinylidene difluoride membranes. These were then incubated with a primary antibody specific either for eNOS (140 kDa) or for β-actin (42 kDa), and also with a secondary anti-mouse antibody. (B, lower panel) Bands were quantified as described under Materials and methods. Ratios were calculated for the optical density of eNOS over that of β-actin. Values are each the mean±SE of five determinations.
superoxide level was significantly greater in aortic rings from diabetic rats than in those from the controls. This increased superoxide level in the STZ-induced diabetic aorta was significantly reduced by SOD (180 U/ml) treatment (Fig. 6C). Chronic administration of pioglitazone to diabetic rats normalized the increased superoxide level, the difference from the untreated diabetic group being significant at $P < 0.01$ (Fig. 6B).

**SOD protein expression and SOD activity**

Intracellular levels of superoxide are controlled not only by the rate of superoxide production but also by its rate of degradation [5,6,22]. SOD, which constitutes the major enzymatic mechanism for superoxide degradation, catalyzes the conversion of superoxide to $\text{H}_2\text{O}_2$. Hence, we examined whether the expression of the protein for Mn-SOD or Cu/Zn-SOD, and SOD activity, might be altered by chronic pioglitazone treatment (Fig. 7). Application of Western blot analysis to aortic homogenates from age-matched controls, untreated diabetic, and chronically pioglitazone-treated diabetic rats revealed the following. The expressions of Mn-SOD and Cu/Zn-SOD were lower in diabetic rats than in control rats (Figs. 7A and 7B). After chronic treatment with pioglitazone, the Mn-SOD level tended to be higher than that in untreated diabetic rats (although statistical significance was not reached) while the level of Cu/Zn-SOD was higher than that in untreated diabetic rats [$P < 0.001$; the Cu/Zn-SOD level being then similar to that seen in the controls] (Figs. 7A and 7B). Moreover, the aortic total SOD activity was significantly lower in STZ-diabetic rats than in age-matched control rats, and this decreased SOD activity was significantly improved by chronic pioglitazone treatment (Fig. 7C).

**NAD(P)H oxidase activity**

Since recent biochemical studies have shown that an NAD(P)H oxidase is the major source of superoxide in the vascular wall [16–19,22], we investigated the aortic activity of NAD(P)H oxidase in our three groups of rats (Fig. 8). Aortic NAD(P)H oxidase activity was greater in diabetic rats than in the controls, and it was significantly decreased by the chronic administration of pioglitazone.

**Quantification of plasma ET-1 and its contribution to endothelium-dependent relaxation**

ET-1 is thought to act locally in a paracrine and autocrine fashion to regulate vascular tone and endothelial functions. Moreover, we reported previously that an increased plasma level of ET-1 is associated with the endothelial dysfunction seen in our diabetic model [31,32]. Thus, to investigate the possible mechanisms underlying the improved ACh-induced relaxation observed in chronically pioglitazone-treated diabetic rats, we measured the level of ET-1 in plasma samples taken from all three groups. As shown in Fig. 9A, the plasma ET-1 level was greater in diabetic rats than in the controls, and it was completely normalized by the chronic administration of pioglitazone.

**Expression of c-Jun protein**

The AP-1 signaling pathway plays an important role in ET-1 gene expression [41,45]. To help determine whether there might be a relationship between PPAR-γ and the increase in ET-1 described above, we investigated the expression of c-Jun protein in aortic homogenates from our three groups. As shown in Fig. 10, the expression of this protein was greater in diabetic rats than in the controls, and it was completely normalized by the chronic administration of pioglitazone.
Discussion

In the present study, we examined whether the impaired endothelium-dependent relaxation seen in aortas isolated from established diabetic rats might be improved by chronic pioglitazone treatment. Since we wished specifically to address the possible association between the observed changes in endothelium-dependent relaxation and changes in NO/cGMP signaling and oxidative stress in this artery, we chose the established STZ rat as our model. We did this because in this chronic diabetic model we and others have demonstrated: (1) an impairment of ACh-induced endothelium-dependent relaxation [1,14,15,20,31,50], (2) evidence of a reduction in the production of cGMP [1], (3) evidence of an increment in the generation of superoxide [4,13,20,50], and (4) decreased expressions of the mRNAs for PPAR-α and PPAR-γ in the aorta [31]. In this chronic diabetic model, the plasma lipid and glucose concentrations are both increased [14,15,31,47–50]. When pioglitazone was administered for 4 weeks to such established STZ rats, there was no significant effect on blood glucose levels, so its beneficial effects are clearly unrelated to a correction of the hyperglycemia. Many studies from different laboratories have demonstrated that blood pressure is altered in animal models of diabetes. However, the results have not been consistent (increase [62], decrease [63], no change [64]). Moreover, Majithiya et al. noted an increase in systolic blood pressure in STZ (55 mg/kg, iv)-induced diabetic Sprague-Dawley rats, and also noted that pioglitazone administration to such rats lowers blood pressure [65]. In contrast, in the present study systolic blood pressure was lower in STZ-diabetic rats than in the age-matched controls. The reasons for this discrepancy are not clear, but such variability is generally attributed to differences in the diabetogen used, the sex, species, and/or strain of the animals, the animal breeder, the duration of the hyperglycemia, and/or the blood pressure-measuring techniques and the experimental conditions. Pioglitazone treatment did not alter the systolic blood pressure in our diabetic rats (Table 1), an observation supported by previous reports that pioglitazone treatment does not influence systolic blood pressure in several states [66,67].

A novel, intriguing, and potentially important finding made in this study was that the modest increase in endothelium-dependent relaxation seen in aortas isolated from pioglitazone-

Fig. 5. Effects of various agents on acetylcholine (ACh; 10−7 M)-induced relaxation in aortic strips obtained from age-matched controls, untreated diabetic rats, and chronically pioglitazone-treated diabetic rats. (A) Typical records showing the effects of ACh-induced relaxation in NA (5 × 10−8–3 × 10−7 M)-precontracted aortic strips. (B) Time course of changes in relaxant responses to ACh in aortic strips from age-matched controls, untreated diabetic rats, and chronically pioglitazone-treated diabetic rats. Each data point represents the mean±SE from six experiments ***P<0.001 vs. age-matched control group. ###P<0.001 vs. untreated diabetic rats. (C) Effects of various agents [SOD (180 U/ml), apocynin (10−4 M), allopurinol (10−3 M), or L-NNA (10−4 M)] on ACh-induced relaxation in aortic strips obtained from age-matched controls (left panel), untreated diabetic rats (middle panel), and chronically pioglitazone-treated diabetic rats (right panel). Each data point represents the mean±SE from four (L-NNA) or six (SOD, apocynin, allopurinol) experiments. †††P<0.001 vs. corresponding vehicle-treated group.
treated STZ rats was associated with evidence of marked changes in oxidative stress. We therefore attempted to identify some of the molecular mechanisms involved. Our results show that STZ treatment of rats leads to impaired ACh-induced endothelium-dependent vasodilation. This seems likely to be a consequence of a reduced bioavailability of endothelium-derived NO, since (a) the plasma nitrite level (oxidation product of NO and correlated with NO biosynthesis [58,59]) was decreased compared to that in the controls, despite eNOS expression showing no change, and (b) the plasma nitrate/nitrite ratio (often used as an indirect marker of oxidative stress [60]) was increased. The above idea is supported by our previous findings, namely: (a) that in the ACh-stimulated aorta, the nitrite level was lower but the nitrate level was higher in STZ-induced diabetic rats than in the age-matched controls, and (b) that acute incubation of STZ-diabetic aortas in vitro with SOD normalized the production of both nitrite and nitrate (i.e., increased nitrite and decreased nitrate vs the SOD-untreated diabetic group) [55]. In line with this interpretation, we found that both ACh-stimulated cGMP production and cGK activity, as determined by the level of P-V ASP [25,28], were markedly decreased in the STZ-diabetic aorta. In this study on the aorta, we found that in STZ rats chronically treated with pioglitazone (vs untreated STZ rats): (1) ACh-induced endothelium-dependent relaxation was normalized, (2) ACh-stimulated cGMP production was significantly increased, and (3) the ACh-stimulated P-VASP level was significantly increased. These results suggest that pioglitazone improves endothelium-dependent relaxation in the diabetic aorta via an enhancement of the NO/cGMP signaling pathway. Several possible mechanisms might underlie a reduction in NO/cGMP signaling in the diabetic aorta, such as (a) a decrease in NO release (or production) from the endothelium, (b) a decreased reactivity of the vascular smooth muscle to NO, and/or (c) an increase in the oxidative degradation and inactivation of NO. In the present study, the SNP-induced endothelium-independent relaxation was not different among the three groups (controls and pioglitazone-untreated and -treated STZ rats). Interestingly, the plasma nitrite level was not altered, but the nitrate/nitrite ratio was decreased in pioglitazone-treated diabetic rats (versus untreated diabetic rats). Moreover, we found that the aortic nitrotyrosine level (a widely accepted biochemical marker of increased generation of reactive oxygen/nitrogen species [9,22,61]) was greatly increased in untreated diabetic rats and that this increased nitrotyrosine level was reduced by pioglitazone treatment. Thus, we suggest that the functional changes observed in endothelium-dependent relaxation in the diabetic aorta may be due to an alteration in NO bioavailability via increased oxidative stress (see below), rather than to an altered sensitivity of the vascular smooth muscle to NO.

A considerable body of evidence now suggests that the impairment of endothelium-dependent relaxation seen in diabetes involves inactivation of NO by superoxide [5,6,10,22]. Production of superoxide leads to inactivation of NO, and dismutation of free radicals has been found to improve the impaired endothelium-dependent relaxation seen in experimental models of diabetes [13]. Indeed, we previously reported that NO is metabolized by superoxide to NO₃⁻, not just to NO₂⁻.
and that the resulting rapid inactivation of NO may be responsible for the impairment of endothelium-dependent relaxation seen in aortic strips from STZ-induced diabetic rats [55]. In the present study, in the STZ-induced diabetic aorta, the decreased ACh-induced relaxation was significantly rescued by SOD treatment, as reported previously [20]. However, the ACh-induced relaxation did not respond to SOD treatment in the pioglitazone-treated diabetic group or in the controls. Moreover, the aortas of chronically pioglitazone-treated diabetic rats exhibited increases in both Cu/Zn-SOD expression and SOD activity, as well as decreased superoxide generation. These results suggest that the effect of pioglitazone on the endothelial dysfunction seen in the STZ-diabetic aorta may be, at least in part, attributable to a reduction in the superoxide level via increased Cu/Zn-SOD activity. This speculation is supported by the finding that PPAR-γ activators increase Cu/Zn-SOD expression in primary endothelial cells [68], and further supported by the finding of Hwang et al. [42] that PPAR-γ ligands stimulate the activity and protein expression of Cu/Zn-SOD in endothelial cells. Although the molecular basis for the PPAR-γ ligand-induced increases in SOD expression has not been defined, the identification of a functional PPRE in the Cu/Zn-SOD promoter suggests that Cu/Zn-SOD gene expression may be stimulated directly by PPAR-γ activation [69,70].

Recent biochemical studies have shown that NAD(P)H oxidase is the major source of superoxide in the vascular wall [16–19]. Moreover, several reports have demonstrated that the stimulation of superoxide production by ET-1 is mediated through activation of NAD(P)H oxidase [35–39]. In the present study, in the STZ-induced diabetic aorta, the decreased ACh-induced relaxation was significantly increased by apocynin (an inhibitor of NAD(P)H oxidase) but not by allopurinol (an

---

Fig. 7. Analysis of Mn-SOD or Cu/Zn-SOD protein expression and total SOD activity in aortas obtained from age-matched controls, untreated diabetic rats, and chronically pioglitazone-treated diabetic rats. (A) Representative Western blots for Mn-SOD or Cu/Zn-SOD and β-actin. Proteins (20 μg) were subjected to 15% PAGE and then transferred to polyvinylidene difluoride membranes. These were then incubated with a primary antibody specific for either Mn-SOD (25 kDa) or Cu/Zn-SOD (20 kDa), or for β-actin (42 kDa), and also with a secondary anti-mouse or anti-rabbit antibody. (B) Bands were quantified as described under Materials and methods. Ratios were calculated for the optical density of Mn-SOD or Cu/Zn-SOD over that of β-actin. Values are each the mean±SE of four (Mn-SOD) or five (Cu/Zn-SOD) determinations. (C) Total SOD activity was measured as described under Materials and methods. Each column represents the mean±SE of eight experiments. *P<0.05, ***P<0.001 vs. control group. †P<0.05, ###P<0.001 vs. untreated diabetic group. T. Matsumoto et al. / Free Radical Biology & Medicine 42 (2007) 993–1007
inhibitor of xanthine oxidase). Moreover, the NAD(P)H oxidase activity was increased (versus the controls) and this increased NAD(P)H oxidase activity was significantly decreased by pioglitazone treatment. Furthermore, when aortic strips from control rats were preincubated with ET-1 (3 × 10^{-10} M), this did not cause tension development, but instead significantly decreased the ACh-induced relaxation. We have previously reported that: (1) the plasma ET-1 level is increased in STZ-induced diabetic rats, and that this increase may be due to an overexpression of the mRNA for ppET-1 [32,71], (2) the overproduction of ET-1 seen in STZ-induced diabetes results from the hyperglycemia, not from any increase in LDL cholesterol or triglyceride [72], (3) the expression of the mRNA for p22^phox^, a NAD(P)H oxidase subunit, is significantly increased in STZ-induced diabetic rats, an increase that is completely preventable by the chronic administration of J-104132 (a potent orally active mixed antagonist of ETA and ETB receptors) [50], and (4) prolonged treatment with ET-1 impairs endothelial function in the aorta both in nondiabetic rats and in rats with established STZ-induced diabetes, an effect that may be related to superoxide generation and to PI3-kinase activity [36]. Moreover, we recently demonstrated that chronic administration of bezafibrate (a PPAR-α agonist) to our established STZ-induced diabetic rats normalized both the mRNA for ppET-1 and the plasma concentration of ET-1, while improving endothelium-dependent relaxation [31]. In that paper, we also demonstrated that in aortic segments, the expressions of the mRNAs for PPAR-α and PPAR-γ were downregulated in STZ-induced diabetic rats [31]. In the present study, both the plasma ET-1 level and the expression of aortic c-Jun (an AP-1
component) were significantly increased in our established diabetic model, and these abnormalities were more or less completely normalized by chronic treatment with pioglitazone. These results—which seem to be supported by previous findings showing that PPAR-α and PPAR-γ ligands regulate ppET-1 gene expression and transcription through AP-1 and nuclear factor-kappa B (NF-κB) [41,73,74]—suggest that down regulation of PPAR-α and PPAR-γ in the aorta may contribute, through the ET-1 system, to the endothelial dysfunction seen in diabetes mellitus. Thus, our results strongly suggest first that ET-1 plays an important role in the endothelial dysfunction seen in diabetic states, and secondly that pioglitazone normalizes this dysfunction by improving the abnormal ET-1 system.

In conclusion, our study demonstrates that in established STZ-diabetic rats, chronic pioglitazone treatment improves endothelium-dependent aortic relaxation by reducing oxidative stress via an increase in Cu/Zn-SOD, a suppression of NAD(P)H oxidase activity, and a decrease in the ET-1 level, and that this decrease in ET-1 may be attributable to an inhibition of the AP-1 signaling pathway. We believe that our findings should stimulate further interest in pioglitazone as a potential therapeutic drug for use against diabetes-associated vascular disease.

Acknowledgments

We thank N. Yoshizawa, T. Shinohara, and K. Hotta for technical help. This study was supported in part by the Ministry of Education, Culture, Sports, Science and Technology, Japan, by the Promotion and Mutual Aid Cooperation for Private Schools of Japan, and by the Mochida Memorial Foundation for Medical and Pharmaceutical Research (Japan).

References

[27] Schulz, E.; Tsilimisgas, N.; Rinze, R.; Reiter, B.; Wendl, M.; Oelze, M;


