Antiinflammatory and Antiarteriosclerotic Effects of Pioglitazone

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Abstract—Peroxisome proliferator-activated receptor-γ (PPARγ) ligands are widely used in patients with insulin resistance and diabetes. Because coronary artery disease is a major complication for such patients, it is important to determine the effects of PPARγ activation on arteriosclerosis. Long-term inhibition of endothelial NO synthesis by administration of N^ω-nitro-L-arginine methyl ester (L-NAME) to rats induces coronary vascular inflammation (monocyte infiltration, monocyte chemoattractant protein-1 [MCP-1] expression) and subsequent arteriosclerosis. We examined the effects of pioglitazone (a PPARγ ligand) in this rat model to determine whether PPARγ activation with pioglitazone inhibits arteriosclerosis by its indirect effects on metabolic conditions or by direct effects on the cells participating to the pathogenesis of arteriosclerosis. We found that pioglitazone did not affect metabolic states, systolic blood pressure, or serum NO levels, but did prevent the L-NAME–induced coronary inflammation and arteriosclerosis. Pioglitazone did not reduce local expression of MCP-1 but markedly attenuated increased expression of the MCP-1 receptor C-C chemokine receptor 2 (CCR2) in lesional and circulating monocytes. PPARγ activation with pioglitazone prevented coronary arteriosclerosis, possibly by its antiinflammatory effects (downregulation of CCR2 in circulating monocytes). Inhibition of the CCR2-mediated inflammation may represent novel antiinflammatory actions of pioglitazone beyond improvement of metabolic state. (*Hypertension*. 2002;40:687-693.)

Key Words: arteriosclerosis ■ leukocytes ■ nitric oxide ■ remodeling

Coronary arteriosclerosis and/or atherosclerosis is increasingly recognized to be a major complication of diabetes. It is the number 1 cause of death in subjects with diabetes.[1,2] Furthermore, prediabetic patients with insulin resistance have high risks for coronary atherosclerosis.[3] Recently, peroxisome proliferator-activated receptor-γ (PPARγ) agonists, oral insulin sensitizers, are used extensively in the treatment of insulin resistance and type 2 diabetes mellitus; therefore, the impact of PPARγ agonists on atherosclerosis is important. PPARγ, a nuclear receptor, is highly expressed in all major cell types participating in vascular injury and atherosclerotic plaque: endothelial cells, macrophages, and vascular smooth muscle cells.[4–6] PPARγ ligands have been shown to inhibit inflammatory and proliferative process. PPARγ ligands inhibited endothelial expression of adhesion molecules or chemokines, which induces monocyte infiltration into the vessel wall.[4,7–9]

Treatment with PPARγ ligand has been shown to reduce atherosclerosis in hypercholesterolemic mice[10–12] and neointimal hyperplasia after balloon injury.[13,14] PPARγ ligands also inhibited neointimal formation of coronary arteries after stent placement in patients with diabetes.[15] Because neointimal formation after vascular injury is usually caused by increased migration and proliferation of vascular smooth

muscle cells, it is suggested that PPARγ ligands might inhibit inflammatory and proliferative process that occurs after vascular injury. In addition, PPARγ ligands improve endothelial dysfunction and activation.[16] In contrast, PPARγ activation might promote vascular disease because it activates the CD36 scavenger receptor on macrophages.[17,18] Furthermore, it remains unclear whether such beneficial vascular effects of PPARγ ligands result from improvement of metabolic disorder or are attributable to direct vasculoprotective actions. Because arteriosclerotic and/or atherosclerotic coronary artery disease is the major cause of death in patients with type 2 diabetes, it is important to determine the effects of the PPARγ agonist on the development of such vascular disease in vivo.

To determine whether PPARγ activation exhibits pro- or antiarteriosclerotic actions in vivo, we examined the effect of the PPARγ activation with pioglitazone in the rat model of long-term inhibition of NO synthesis. In this model, long-term administration of N^ω-nitro-L-arginine methyl ester (L-NAME) induces early inflammation (monocyte infiltration into the coronary vessels) associated with induction of monocyte chemoattractant protein-1 (MCP-1) and causes subsequent arteriosclerosis.[19,20] We have previously demonstrated that (1) increased activity of angiotensin II (increased activity

of ACE and angiotensin II type 1 [AT$_1$] receptor) is critical in the development of such inflammation and arteriosclerosis, and (2) such inflammation is mediated by increased activity of MCP-1 through nuclear factor-κB.[21–24] Thus, some of these pathologic process are similar in human arteriosclerosis. We considered that this rat model is useful to investigate antiarteriosclerotic effects of PPARγ activation, because administration of pioglitazone dose not change serum glucose or insulin levels.

Materials and Methods

Experimental Animals

The study protocol was reviewed and approved by the Committee on the Ethics of Animal Experiments, Kyushu University Graduate School of Medical Sciences. A part of this study was performed at the Kyushu University Station for Collaborative Research and the Morphology Core, Kyushu University School of Medical Sciences.

Twenty-week-old male Wistar-Kyoto rats were obtained from an established colony at the Animal Research Institution of Kyushu University, Faculty of Medicine (Fukuoka, Japan). Several groups of 20-week-old male Wistar-Kyoto rats were studied. The control group received untreated chow and drinking water. The L-NAME group received L-NAME in drinking water (1.0 mg/mL). The L+Pio group received L-NAME in drinking water (1.0 mg/mL) and pioglitazone (3 or 20 mg/kg per day; Takeda Chemical Industries Ltd) in chow. The L+CS866 group received L-NAME in drinking water and CS866 (75 μg/g; Sankyo Pharmaceitocal Co),[25] an AT$_1$ receptor blocker, in chow. Treatment with pioglitazone or CS866 was started 4 days before L-NAME administration was begun.

Serum pioglitazone concentration was measured with the use of high-performance liquid chromatography/mass spectrometry.[26] Serum pioglitazone concentrations in the L+Pio3 and L+Pio20 groups were 0.46±0.13 and 2.16±0.31 μg/mL, respectively. The concentrations of pioglitazone achieved by 3 and 20 mg doses in rats were equivalent to the concentration reported in humans who were administered 15 and 30 mg doses of pioglitazone, respectively. Effectiveness of pioglitazone as a PPARγ ligand was determined by assessing gene expression of lipoprotein lipase (LPL) in adipose tissues by Northern blot analysis; LPL mRNA levels (ratio of LPL to GAPDH relative to control group, which was assigned to be 1.0) in the L-NAME, L+Pio3, and L+Pio20 groups were 1.0±0.1, 2.8±0.2 ($P<0.01$), and 3.8±0.3 ($P<0.01$ versus control and L-NAME group), respectively.

Systolic blood pressure (by the tail-cuff method) was measured on days 3, 7, 14, and 28 of L-NAME treatment. On days 3 or 28, arterial blood was collected after anesthesia, and the rats were killed for morphometric, immunohistochemical, or biochemical analysis.

Biochemical Analysis

Rats were fasted overnight before the collection of the blood samples. Plasma glucose levels were immediately measured on Glutest Pro (Sanwa Kagaku Co). Serum total cholesterol, triglycerides, HDL cholesterol, and insulin levels were detected with commercially available kits (Wako). Serum NO$_x$ (NO$_2$ plus NO$_3$) concentration was measured with use of fluorometric assay kit (Wako).[27] The ACE activity was measured in cardiac tissue by fluorometric assay as described.[19]

Histopathology and Immunohistochemistry

Histopathology and immunohistochemistry were performed as described.[19] Some sections were subjected to immunostaining using antibodies against rat macrophage/monocytes (ED-1, Serotec Inc), proliferating cell nuclear antigen (PCNA, DAKO), MCP-1, transforming growth factor-β_1 (TGF-β1;Biosource International), and C-C chemokine receptor 2 (CCR2) (Santa Cruz Biotechnology Inc). Cell enumeration was performed as described.[19] Evaluation of the medial thickness and perivascular fibrosis of coronary arteries on day 28 was performed as previously described.[19]

Northern Blot Analysis

Northern blot hybridization was performed as described.[20] A rat MCP-1 cDNA probe, rat TGF-β1 probe, and a mouse GAPDH cDNA probe were used.

TaqMan Real-Time Reverse Transcription–Polymerase Chain Reaction Analysis

Total RNA was extracted from unfixed hearts or 10 to 20 million peripheral blood mononuclear cells (PBMCs) by guanidinium thiocyanate-phenol-chloroform extraction (Isogen, Nippon Gene Co). Transcripts from 1 μg of total RNA were reverse-transcribed by ReverTra Ace using oligo(dT)10 primer, and the resultant cDNA was amplified by TaqMan real-time reverse transcription–polymerase chain reaction (RT-PCR). The PCR reaction is directly monitored by the ABI Prism 7000 sequence detection system.[28] The PCR primers and TaqMan probes were designed with a software program from Primer Express, version 2.0. Real-time RT-PCR for CCR2 was amplified in the same way with the following primers: sense primer, 5'-ACCTGTTCAACCTGGCCATCT-3'; antisense primer, 5'-AGACCCACTCATTTGCAGCAT-3'; and probe oligonucleotides, 5'-ACCTGCTCTTCCTGCTCACACTCCCA-3'. The GAPDH probe was obtained from Applied Biosystems.

Flow Cytometry Analysis

Rat PBMCs were purified by centrifugation on Lympholyte-M and were washed with ice-cold PBS supplemented with 1% bovine serum albumin and 0.1% sodium azide. The isolated PBMCs (1×10^6) were preincubated with 4 μg of goat IgG for 15 minutes at room temperature and then were incubated with 4 μg of biotin anti-rat Mononuclear Phagocyte (Becton Dickinson Biosciences) and 4 μg of goat anti-rat CCR2 antibody (Santa Cruz Biotechnology Inc) for 30 minutes at 4°C. After washing, cells were stained with 4 μg of phycoerythrin-conjugated streptavidin (Becton Dickinson Biosciences) and 4 μg of fluorescein-labeled mouse anti-goat IgG (Santa Cruz Biotechnology Inc) for 30 minutes at 4°C. Stained cells were analyzed by FACSCalibur instrument using CELL QUEST software (Becton Dickinson Biosciences). In control experiments, fluorescein-conjugated nonspecific goat IgG was used to measure nonspecific binding.

Statistical Analysis

Data are expressed as mean±SE. Statistical analysis of differences was compared by ANOVA and Bonferroni's multiple comparison test. A level of $P<0.05$ was considered statistically significant.

Results

Systolic Arterial Pressure and Serum Metabolic State

The L-NAME group showed a progressive rise in systolic arterial pressure throughout the study (Table 1). Treatment with pioglitazone did not affect the L-NAME–induced increase in systolic blood pressure. Pioglitazone also did not affect blood glucose and serum concentrations of insulin, total cholesterol (TC), triglycerides (TG), and HDL cholesterol (HDL-C) (Table 2).

Effects of Pioglitazone on Local ACE Activity and Serum NO$_x$ Concentration

Cardiac ACE activity was increased in the L-NAME group on day 3 (Table 1). This increased ACE activity was decreased by treatment with pioglitazone. Serum NO$_x$ concentration was significantly decreased in the L-NAME group

TABLE 1. Systolic Blood Pressure and Tissue ACE Activitiy

Group	Systolic Blood Pressure (mm Hg)					LV Tissue ACE Activity (nmol/mg per hr)
	Day 0	Day 3	Day 7	Day 14	Day 28	Day 3
Control	130±7	126±6	128±6	130±7	132±13	0.70±0.02
L-NAME	132±11	173±15**	176±13**	178±12**	197±11**	1.38±0.11**
L+Pio3	135±6	170±9**	176±7**	175±6**	177±10**	0.63±0.05‡
L+Pio20	133±10	170±15**	174±10**	172±17**	174±11**	0.49±0.05‡

Values are mean±SE.

**$P<0.01$ vs control; ‡$P<0.01$ vs L-NAME group.

(Table 2). This decreased serum NO_x was not influenced by treatment of pioglitazone.

Effects of Pioglitazone on Inflammatory and Proliferative Changes

As we have reported,[22–24] on day 3, attachment of mononuclear leukocytes to endothelium of coronary vessels and their infiltration into the vessel wall were noted in the L-NAME group. The majority of these cells were found to be ED-1–positive monocytes. Nuclear staining with PCNA antibody was observed in some endothelial cells, vascular smooth muscle cells in the media, monocytes, and myofibroblast-like cells. We found no evidence of such inflammation in the control rats.

The number of immunopositive cells per section was significantly greater in L-NAME group than in the control group (Figure 1A). The increases in ED-1–positive cells and PCNA-positive cells were significantly reduced by treatment with low and high doses of pioglitazone (Figure 1A).

Effects of Pioglitazone on Coronary Arteriosclerosis

On day 28, the increases in medial thickening (wall-to-lumen ratio) and perivascular fibrosis of coronary arteries were seen in the L-NAME group. These changes were inhibited by treatment with low and high doses of pioglitazone (Figure 1B and 1C).

TABLE 2. Blood Glucose Levels and Serum Insulin, Lipid, and NOx Concentrations

Group	Glucose (mg/dL)	Insulin (mU/L)	TC (mg/dL)	TG (mg/dL)	HDL-C (mg/dL)	NOx (mmol/L)
Control						
Day 3	103±26	16±6	95±15	25±6	45±9	4.7±0.8
Day 28	103±21	15±7	95±10	25±6	45±8	
L-NAME						
Day 3	93±23	19±6	107±21	31±6	42±8	1.8±0.1**
Day 28	102±8	13±6	100±8	29±5	41±4	
L+Pio3						
Day 3	106±20	19±6	101±13	28±7	44±6	1.5±0.1**
Day 28	90±8	18±3	98±12	25±6	41±5	
L+Pio20						
Day 3	109±19	16±4	103±18	22±6	47±7	1.5±0.1**
Day 28	90±11	13±3	95±6	27±7	40±2	

Values are mean±SE.

**$P<0.01$ vs control.

Effects of Pioglitazone on MCP-1 and TGF-β1 Gene Expression

On day 3, both MCP-1 and TGF-β1 mRNA levels were significantly increased in L-NAME group (Figure 2A and 2B). The increased expression of MCP-1 and TGF-β1 gene was not reduced by treatment with low and high doses of pioglitazone. As we previously reported,[22–24] immunohistochemical study showed the L-NAME–induced increases of MCP-1 and TGF-β1 immunoreactivity in coronary arteries, which were not reduced in rats from the L+Pio group (Figure 2C).

Effects of Pioglitazone on the MCP-1 Receptor Expression in Lesional and Circulating Monocytes

On day 3, CCR2 mRNA levels in PBMC and left ventricle were significantly higher in the L-NAME group (Figure 3A). This upregulation of CCR2 was prevented by treatment with the low and high doses of pioglitazone. We determined the cell type expressing CCR2 in hearts by fluorescence immunohistochemistry (Figure 3B) and found that immunoreactive CCR2 was present exclusively in the monocytes/macrophages that had infiltrated into the intima and adventitia of coronary arteries in the L-NAME group. No such CCR2-positive cells were noted in the control group. The appearance of CCR2-positive cells was significantly reduced by treatment with low and high doses of pioglitazone. The CCR2 mRNA levels in PBMCs and left ventricle, and the number of CCR2-positive cells, were significantly reduced by treatment with CS-866, an AT_1 receptor blocker. The CCR2 immunoreactivity was also observed in endothelial cell layer of coronary arteries, the degrees of which did not differ among the 4 groups.

We further analyzed the appearance of CCR2 on circulating monocytes by flow cytometry (Figure 4). The CCR2 expression in monocytes was significantly higher in L-NAME group than in the control group. Treatment with pioglitazone or CS-866 prevented the L-NAME–induced increase in CCR2 expression (Figure 4).
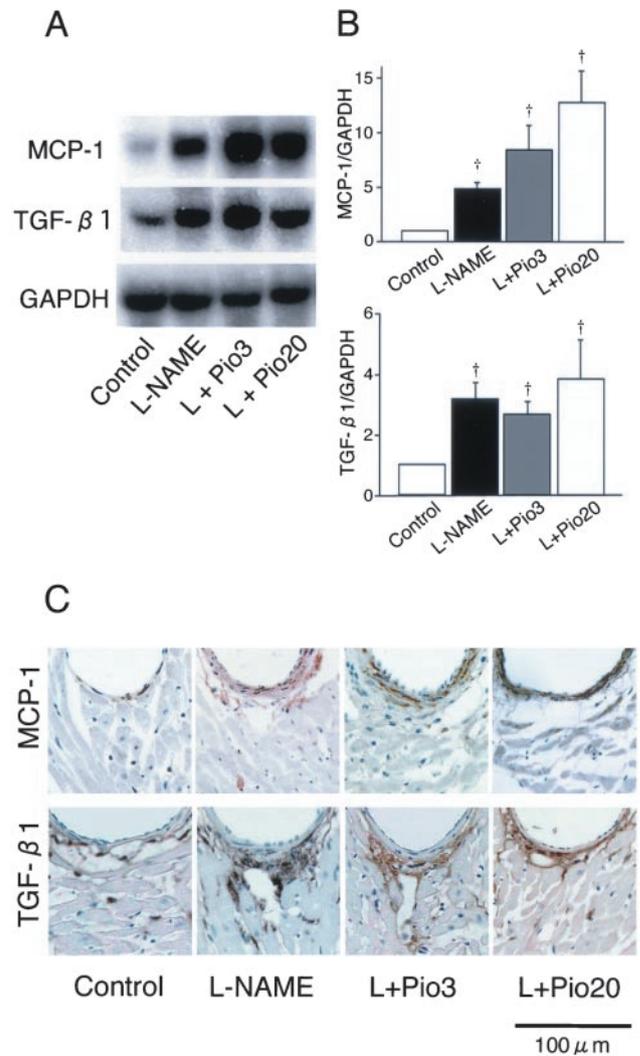
Discussion

We have demonstrated herein that PPARγ activation with pioglitazone prevented coronary arteriosclerosis, possibly by its antiinflammatory action on the MCP-1 receptor (CCR2) in this rat model. Our present data suggest that the antiinflammatory effects of pioglitazone may not be explained by its
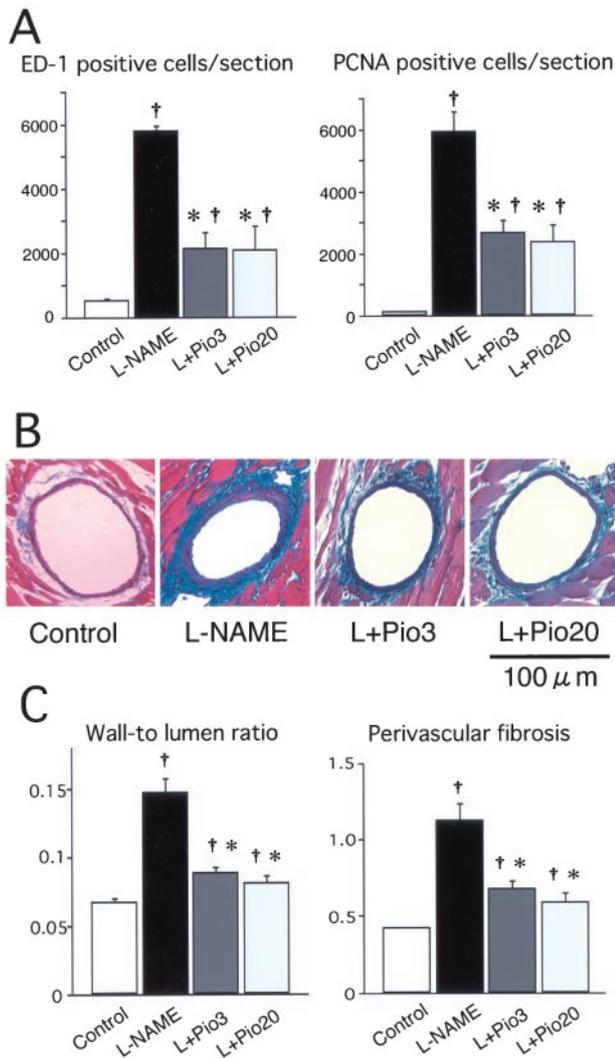
Figure 1. Effect of pioglitazone on early inflammatory and proliferative changes and late arteriosclerosis. A, Coronary artery sections were stained immunohistochemically for ED-1 and PCNA on day 3 of L-NAME treatment. Number of ED-1–positive monocytes infiltrated into the coronary vessels and PCNA-positive cells in the control, L-NAME, L+Pio3, L+Pio20 groups are shown. *$P<0.05$ vs L-NAME group; †$P<0.05$ vs control group; n=7. B, Coronary artery sections stained with Masson's trichrome on day 28. C, Wall-to-lumen ratio and perivascular fibrosis of coronary arteries. *$P<0.05$ vs L-NAME group; †$P<0.05$ vs control group; n=6.

Figure 2. Effect of pioglitazone on cardiac MCP-1 and TGF-β1 gene expression. A, Representative autogram of Northern blot analysis of MCP-1, TGF-β1, and GAPDH mRNA. B, Summary of densitometric analysis of data. Data are expressed as ratio of MCP-1 and TGF-β1 mRNA to GAPDH mRNA relative to control, which was assigned an arbitrary value of 1. C, Coronary artery sections were stained with MCP-1 and TGF-β1 on day 3 of L-NAME treatment. *$P<0.05$ vs L-NAME group; †$P<0.05$ vs control group; n=8.

indirect effects on metabolic state, by antihypertensive effect, or by restoration of NO production.

An important feature that emerged in the present study is that pioglitazone attenuated inflammatory (monocyte infiltration) and proliferative (appearance of PCNA-positive cells) disorders, suggesting that pioglitazone prevented coronary arteriosclerosis by its direct antiinflammatory actions in this model. Because we have previously demonstrated that (1) MCP-1 mediates inflammation and arteriosclerosis, and (2) TGF-β1 mediates fibrosis in this rat model,[22–24] we expected that pioglitazone might suppress increased expression of MCP-1 and TGF-β1. In contrast, pioglitazone did not affect expression of such local factors, indicating that other factors must be involved in antiinflammatory and antiarteriosclerotic actions of pioglitazone. Most previous stud-

ies that investigated inflammatory aspects of vascular disease focused exclusively on lipid- or stress-induced changes in inflammation-driving factors, such as MCP-1 in cells of the arterial wall,[29,30] whereas pathobiological changes in CCR2 have not been attracted attention. The monocyte/macrophage infiltration and atherosclerotic lesion formation were less in CCR2$^{-/-}$ and apoE$^{-/-}$ mice than in apoE$^{-/-}$ mice.[31] We found here that pioglitazone prevented the L-NAME–induced increase in CCR2 expression in lesional and circulating monocytes.
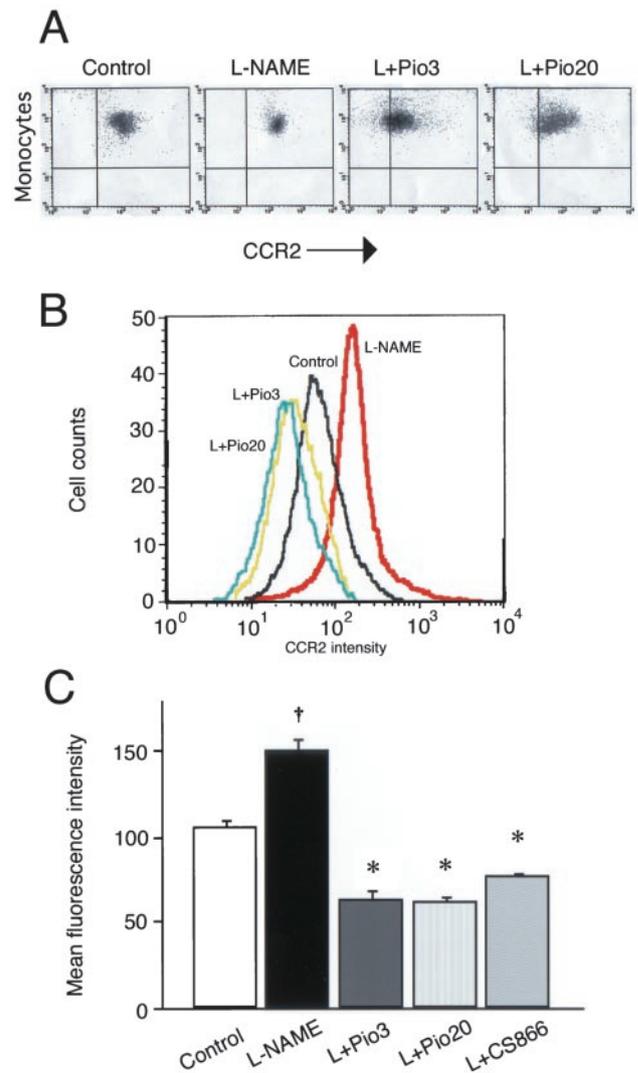
Our present data are in accordance with recent data by Han et al,[10] who demonstrated inhibition of monocyte CCR2 expression by rosiglitazone in LDL receptor mice. The increase in CCR2 intensity by $\approx$40% in L-NAME group and its prevention by pioglitazone are implicated to be pathophysiologically significant findings, because prior reports have shown that small changes (ie, 20%) in CCR2 expression in monocytes result in
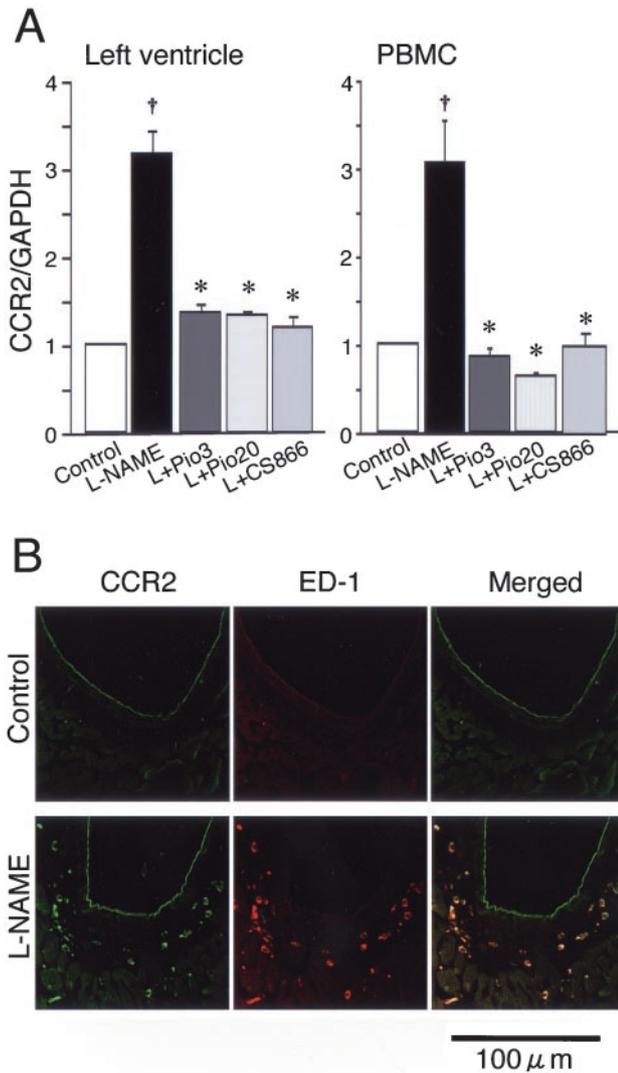
Figure 3. Effect of pioglitazone on CCR2 gene expression. A, CCR2 gene expression by real-time RT-PCR in left ventricular tissues and PBMCs. Data are expressed as ratio of CCR2 mRNA to GAPDH mRNA relative to control, which was assigned an arbitrary value of 1. *$P<0.05$ vs L-NAME group; †$P<0.05$ vs control group; n=7 to 8. B, Immunofluorescence images of coronary arteries. Indirect immunofluorescence was performed on paraffin-fixed tissue section, using anti-rat CCR2 antibody or anti-rat ED-1. The bound antibody is visualized with a fluorescein-conjugated secondary antibody (green fluorescence) or rhodamine-conjugated secondary antibody (red fluorescence): CCR2 immunofluorescence (left; labeled green with fluorescein), ED-1 immunofluorescence (middle; red with rhodamine), and their overlap (right; yellow). CCR2 located exclusively at infiltrated monocyte/macrophages, resulting in overlap with ED-1 (yellow).

Figure 4. Effect of pioglitazone on CCR2 surface expression of circulating monocytes, which was determined by flow cytometry. A, CCR2 surface expression on monocytes shown by 2-color immunofluorescence using anti-rat mononuclear phagocyte and anti-rat CCR2 antibody. B, Overlays of 4 histograms that illustrate control group (black line), L-NAME group (red line), L+Pio3 group (yellow line), and L+Pio20 group (blue line). C, Summary of mean log fluorescence intensity, which was calculated as an expression index of CCR2 on the surface of the circulating monocytes. *$P<0.05$ vs L-NAME group; †$P<0.05$ vs control group; n=6.

biologically meaningful (2×) enhancement in chemotaxis activity.[10,32] These findings suggest that in addition to MCP-1, the enhanced expression and activity of CCR2 in lesional and circulating monocytes contributed not only to monocyte recruitment into the arterial wall by MCP-1 but also to monocyte activation (production of growth factors and/or cytokines).

We have previously shown the critical role of local activity of angiotensin II (increased activity of tissue ACE and AT$_1$ receptor) in the development of vascular inflammation and arteriosclerosis induced by long-term inhibition of NO syn-

thesis.[19,22] Therefore, our present data on prevention of local ACE activation by pioglitazone may be an interesting observation. Because previous reports by us[22] and others[33,34] showed that ACE and AT$_1$ receptor activities are increased in lesional monocytes in experimental and human arteriosclerotic lesions, pioglitazone might have decreased local ACE activity secondary to its antiinflammatory effects. Further studies are needed to elucidate the molecular mechanism involved in decreased ACE activity by pioglitazone.

Our present observation of inhibition of enhanced expression of CCR2 by AT$_1$ receptor blocker may imply that an AT$_1$ receptor might be involved in the pathogenesis of upregulation of CCR2 in the present study. Compared with the regulation of

MCP-1 expression by angiotensin II, a relationship between angiotensin II and CCR2 expression has not extensively been investigated. A recent in vitro study demonstrated increased CCR2 expression by oxidative stress.[32,35] Because oxidative stress has been shown to mediate various proinflammatory and proarteriosclerotic actions of angiotensin II,[36] and because oxidative stress is increased through increased activity of angiotensin II in this rat model of long-term inhibition of NO synthesis,[37] it is possible that oxidative stress driven by angiotensin II might mediate the upregulation of CCR2 in the present study. Further studies are needed to elucidate the mechanism of downregulation of CCR2 by angiotensin II receptor blockade. A caveat to interpret our present data is that the observed antiinflammatory effect of pioglitazone was dependent or independent of PPARγ activity. We showed an in vivo dose-related response of pioglitazone in increasing LPL mRNA, whereas pioglitazone at the low and high doses exhibited equivalent antiinflammatory effect. This discrepant action of pioglitazone suggests that the observed antiinflammatory actions may occur independent of PPARγ activity as reported by Chawla et al[38] Further studies are needed to determine the molecular mechanisms by which pioglitazone downregulated CCR2 in monocytes in vivo.

Perspectives

Pioglitazone prevented coronary arteriosclerosis, possibly by its antiinflammatory effects. The antiinflammatory and anti-arteriosclerotic effects of pioglitazone may be mediated by downregulation of CCR2 in circulating and lesional monocytes. Inhibition of the CCR2-mediated inflammation may represent novel antiinflammatory actions of pioglitazone beyond metabolic effects.

Acknowledgments

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