Pioglitazone Enhances Cytokine-Induced Apoptosis in Vascular Smooth Muscle Cells and Reduces Intimal Hyperplasia

Yoshiaki Aizawa, MD; Jun-ichi Kawabe, MD, PhD; Naoyuki Hasebe, MD, PhD; Naohumi Takehara, MD, PhD; Kenjiro Kikuchi, MD, PhD

Background—Cytokines induce apoptosis in vascular disease lesions through enhancement of inducible nitric oxide (NO) synthase (iNOS) activation. The thiazolidinediones, novel insulin-sensitizing agents, have been demonstrated to modulate cytokine-induced NO production. We have investigated the role of pioglitazone in the apoptosis of vascular smooth muscle cells (VSMCs) in vitro and developed intimal hyperplasia in vivo.

Methods and Results—Pioglitazone (0.1 to 10 μmol/L) significantly enhanced cytokine-induced expression of iNOS and NO production in a dose-dependent manner in rat VSMCs, but 15-deoxy-D12,14-prostaglandin J2 (up to 10 μmol/L), a native peroxisome proliferator–activated receptor-γ ligand, showed no effect. Pioglitazone also significantly enhanced reduction of cell viability, as evidenced by the increase in the number of TUNEL-positive cells. All of these effects of pioglitazone were blocked by treatment with N-monomethyl-L-arginine, an NO synthesis inhibitor. In an in vivo study with a balloon-injured rat carotid artery, neointimal thickness had reached maximum levels at 2 weeks after injury. Then, rats were fed with or without pioglitazone (3 mg·kg⁻¹·d⁻¹) for an additional week. The ratio of intima to media area of carotid artery was significantly decreased by 30%, and the ratio of apoptotic cells in neointima was significantly increased in pioglitazone-treated rats compared with vehicle-treated control rats.

Conclusions—Pioglitazone enhanced apoptosis in an NO-dependent manner in cytokine-activated VSMCs and induced significant regression of intimal hyperplasia in balloon-injured rat carotid artery. It appears that pioglitazone is a potent apoptosis inducer in vascular lesions, providing a novel pharmacological strategy to prevent restenosis after vascular intervention. (Circulation. 2001;104:455-460.)

Key Words: apoptosis ■ muscle, smooth ■ nitric oxide ■ vessels ■ restenosis

Apoptosis is an important mechanism in the formation of vascular lesions.¹ Increased proliferation and migration of inflammatory cells and vascular smooth muscle cells (VSMCs) is accompanied by heightened apoptosis, which suggests a compensatory balance between cell replication and deletion in maintaining tissue homeostasis.² Previous observations from animal studies suggest that cell proliferation and apoptosis were manipulated independently.¹ In this regard, therapy aimed at cell death is an attractive approach for antirestenotic treatment of vascular intervention.

Nitric oxide (NO) plays various important roles in the biological function of vascular cells. Inducible NO synthase (iNOS), distributed in a variety of cell types including VSMCs, can produce high output of NO on induction with proinflammatory cytokines.³⁻⁵ Excess production of NO in response to cytokines can mediate apoptosis, or programmed cell death.⁶⁻⁸ The thiazolidinediones (which bind to the nuclear receptor, the peroxisome proliferator–activated receptor-γ [PPARγ]) have been viewed primarily as insulin-sensitizing compounds. It is now recognized that thiazolidinediones potentially have beneficial effects on cardiovascular function.⁹ These drugs inhibit growth factor–induced proliferation of VSMCs,¹⁰,¹¹ inhibit smooth muscle cell migration,¹² and attenuate the development of intimal hyperplasia in animal models of balloon-catheter vascular injury.¹¹,¹³,¹⁴ However, it is not known whether thiazolidinediones can induce regression of developed intimal hyperplasia after vascular injury. Recently, it was reported that troglitazone upregulates cytokine-induced iNOS expression in VSMCs.¹⁵ Therefore, we postulated that the thiazolidinediones act on cytokine-activated VSMCs to induce apoptosis, leading to the regression of intimal hyperplasia of vascular lesions. In the present study, we investigated the role of thiazolidinediones on the
apoptosis of cytokine-activated VSMCs in vitro and developed intimal hyperplasia of balloon-injured carotid artery in vivo.

Methods
The animals used in this study were treated in accordance with the guidelines of the Ethics Committee on Laboratory Animals of Asahikawa Medical College.

Cell Culture and Treatments
VSMCs were prepared from thoracic aorta of 6-week-old Sprague-Dawley rats by an explant technique. The cells were cultured in DMEM containing 10% fetal bovine serum (FBS). Cells were grown to subconfluence and made quiescent by incubation in medium containing 0.5% FBS and 0.05% bovine serum albumin and indicated concentrations of pioglitazone (Takeda Chemical Industries Ltd, Tokyo, Japan), troglitazone (Sankyo Co, Tokyo, Japan), 15-deoxy-Δ12,14-prostaglandin J2 (15d-PGJ2; Calbiochem-Novabiochem Co), or vehicle (DMSO, <0.01% in final solution) for 16 hours. Cells were then stimulated with rat interleukin (IL)-1β (Genzyme Corp) and/or interferon (IFN)-γ (PeproTech Ltd) for 48 hours. For all experiments, passages 4 to 8 of subcultured cells were used.

Nitrite and Nitrate Assay
Synthesis of the stable NO metabolites nitrite and nitrate was determined in culture supernatants. Nitrate was reduced to nitrite by nitrate reductase (0.4 U/mL) in the presence of 10 mmol/L β-NADPH. Then, total nitrite accumulation was measured with a Griess reaction kit (Dojindo Laboratory).

Western Blot Analysis
Cells were homogenized in lysis buffer (20 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L phenylmethylsulfonyl fluoride, 5 U/mL egg white trypsin inhibitor, and 10 μg/mL leupeptin) and centrifuged at 12 000 g for 20 minutes at 4°C. Equal amounts of protein (5 mg) from the supernatants were separated on 7.5, 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L phenylmethylsulfonyl fluoride, 5 U/mL egg white trypsin inhibitor, and 10 μg/mL leupeptin) and centrifuged at 12 000 g for 20 minutes at 4°C. Equal amounts of protein (5 mg) from the supernatants were separated on SDS 6% polyacrylamide gels and blotted onto nitrocellulose membrane. Western blot analysis was performed with a polyclonal anti-iNOS antibody (Santa Cruz Biotechnology Inc) as described previously. Relative intensity of iNOS expression to the iNOS expression induced by IL-1β and IFN-γ was quantified by densitometry.

MTT Assay
A modified assay kit (Chemicon International Inc) based on the mitochondria-dependent reduction of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) to formazan was used to quantify cell viability. Values of MTT assay in nontreated control cells were set at 100%.

Measurements of Apoptosis
Apoptotic cells in both culture cells and arterial tissue were detected in situ by the terminal transferase-mediated fluorescein-conjugated dUTP nick end labeling (TUNEL) method (Boehringer Mannheim) and immunohistochemistry with anti–single-stranded DNA (ssDNA). TUNEL-positive cells were detected with diaminobenzidine according to the manufacturer’s instructions. Cells were also visualized with immunohistochemistry by staining of smooth muscle–specific α-actin. To quantify apoptosis, the percentage of apoptotic cells in the total cell population was calculated by counting all cells from 5 random microscopic fields at a magnification of ×200.

Rat Balloon-Injury Model
Male Sprague-Dawley rats (10 weeks old) were used in the present study. All surgical procedures were performed under general anesthesia by an injection of sodium pentobarbital (40 mg/kg IP). Balloon-catheter injury was accomplished with a Fogarty 2F balloon catheter as described previously. At 2 weeks after balloon injury, the rats were randomly divided into 3 groups: nontreated control group, pioglitazone-treated group, and vehicle-treated group. For nontreated control rats, the right and left common carotid arteries were excised and then snap-frozen in liquid nitrogen. Serial cryostat sections (5 μm) were fixed for 10 minutes in cold acetone at −20°C. For the other groups, pioglitazone suspended in 0.5% methylcellulose solution or the methylcellulose solution alone was administered orally (3 mg·kg⁻¹·d⁻¹) by stomach tube for 1 more week. Then, the carotid arteries of the 2 groups were extracted as described above. These sections were stained with hematoxylin and eosin or analyzed by immunohistochemistry.

Immunohistochemistry
Arterial sections were incubated with rabbit anti-ssDNA (Dako Japan Co Ltd) and mouse anti–proliferating cell nuclear antigen (PCNA; Dako Co) overnight at 4°C. Subsequently, sections were incubated with peroxidase conjugated secondary antibodies for 60 minutes at room temperature and the chromogenic substrates, diamobenzidine for ssDNA or PCNA and 4-Cl-1-naphthol for α-actin. Nuclei were counterstained in 0.5% methyl green.

Morphometry
Arterial morphometry was performed by the method described by Paigen et al with minor modification. To measure intimal and medial areas of injured carotid arteries, 4 cross sections of each artery spaced at 0.5-mm intervals were stained with hematoxylin and eosin. The cross-sectional intimal and medial areas of a lesion in a given photomicrograph were determined with image-analysis software (NIH IMAGE, NIH Research Service Branch). Then, the ratio of average intimal area to medial area was calculated for each artery.

Statistical Analysis
Results are reported as mean±SEM. Statistical significance was determined by 1-way ANOVA followed by protected least significant difference Fisher’s test, and values of P<0.05 were considered statistically significant.

Results
Pioglitazone Enhances Cytokine-Induced NO Production and iNOS Expression in VSMCs
IL-1β dose dependently (1 to 20 ng/mL) increased NO production, reaching a maximum effect at 10 ng/mL IL-1β (data not shown). With a combination of IL-1β (10 ng/mL) and IFN-γ (10 ng/mL), NO production was synergistically enhanced. As shown in Figure 1, the NO production induced by IL-1β and IFN-γ was enhanced by pioglitazone or troglitazone in a dose-dependent manner. The half-maximal concentrations of these thiazolidinediones were ~1 μmol/L. 15d-PGJ2, the natural ligand for PPARγ, did not have any effect on NO production induced by a combination of IL-1β and IFN-γ. Thiazolidinediones or 15d-PGJ2 (up to 10 μmol/L) alone did not result in any change in production of NO (data not shown).

As shown in Figure 2, iNOS expression was detected in IL-1β–treated but not nontreated cells. Treatment with a combination of IL-1β and IFN-γ caused a synergetic increase in the level of iNOS protein. Pioglitazone significantly enhanced cytokine-induced iNOS protein expression, whereas pioglitazone alone did not have any effect on iNOS expression.
Pioglitazone Stimulates Cytokine-Induced Apoptosis of VSMCs
As shown in Figure 3, treatment with IL-1β (10 ng/mL) or IFN-γ (10 ng/mL) alone slightly but significantly decreased cell viability in the mitochondrial respiration assay (MTT reduction assay).20 These effects were not blocked by 1 mmol/L N-nitro-L-arginine (L-NMMA). A combination of these cytokines synergistically reduced cell viability. Pioglitazone (10 μmol/L) enhanced the effects of cytokine-induced cell damage in a dose-dependent manner (Figure 3). Cotreatment of L-NMMA with cytokines partially blocked cytokine-induced cell damage and totally canceled the effects of pioglitazone (Figure 3).

Cytokine-treated cells with or without 1 mmol/L L-NMMA were stained in situ by the TUNEL method (Figure 4). Very few cells in the control group were TUNEL positive, whereas many cells treated with IL-1β and IFN-γ showed positive TUNEL staining. Pretreatment with pioglitazone (10 μmol/L) increased the number of TUNEL-positive cells (from 5.2±1.0% to 13.6±2.9%; n=6; P<0.05). In the presence of L-NMMA, TUNEL-positive cells were significantly decreased in VSMCs treated with cytokines (0.9±0.2%; n=6; P<0.01) or cytokines plus pioglitazone (1.9±0.9%; n=6; P<0.05). Importantly, cytotoxic or apoptotic effects were not detected in VSMCs treated with pioglitazone alone up to 10 μmol/L for 72 hours (Figures 3 and 4).

Pioglitazone Induces Apoptosis and Regression of Intimal Hyperplasia Induced by Balloon Injury in Rat Carotid Artery
We next examined whether pioglitazone induced apoptosis and reduced the mass of hyperplastic intima in vivo. Representative hematoxylin-and-eosin–stained cross sections from injured carotid arteries are shown in Figure 5. Neointimal thickness had almost reached its maximum after 2 weeks of balloon injury. Quantitative analysis of injured artery segments showed that pioglitazone-fed rats had ~30% less neointimal area to medial area ratio than rats in the vehicle-treated group (Figure 5D). The target specificity of pioglitazone was further evidenced by the lack of change in medial layer dimensions (data not shown).

As shown in Figure 6, apoptotic cells in hyperplastic intima were ~6% of the whole intimal cells at 2 weeks after balloon injury. After an additional week, the ratio of apoptotic cells remained at a low level. Pioglitazone significantly increased apoptotic cells in the neointima compared with the vehicle-treated group, but apoptotic signal was not detected either in the normal intimal and medial areas or even the adventitial area of arterial rings regardless of treatment with pioglitazone. We also examined the degree of proliferation in neointima as assayed by immunostaining for PCNA.21 Only 15% of PCNA-positive cells were detected at 2 weeks after balloon injury, and the number of proliferative cells was slightly decreased after an additional week.
was no significant difference among pioglitazone- and vehicle-treated groups (data not shown).

Discussion

This study demonstrates for the first time that pioglitazone enhances apoptosis and induces regression of developed intimal hyperplasia in balloon-injured carotid artery. The proinflammatory cytokine-mediated apoptosis of VSMCs has been identified in atherosclerosis and restenosis after angioplasty. In previous studies, the combination of IL-1β and IFN-γ synergistically induced NO production, resulting in apoptosis in VSMCs. In the present study, we showed that pioglitazone augmented cytokine-induced NO production by 60% and also enhanced apoptosis in VSMCs. The excess amount of NO production in VSMCs was well correlated with the extent of apoptosis, although NO may be either antiapoptotic or proapoptotic depending on the circumstances. In the present study, the NOS inhibitor L-NMMA blocked apoptosis even with pioglitazone treatment. Therefore, we suggest that pioglitazone induces apoptosis in VSMCs by enhancement of cytokine-induced NO production.

Neointimal mass in the injured artery is determined by the balance between apoptosis and cell replication. It is known that thiazolidinediones also inhibit growth factor–induced proliferation of VSMCs. However, in the present study, pioglitazone did not show any inhibitory effects on proliferation in the neointima. According to a previous study with the same animal model, balloon-catheter injury induces maximal proliferation and apoptosis in intimal lesion within the first week, and then both proliferation and apoptosis decline to basal levels. In the present study, pioglitazone was supplied 2 weeks after balloon injury. At that time, intimal hyperplasia had reached maximum levels (Figure 5), and the ratio of proliferative and apoptotic cells was balanced at a low level (Figure 6C). Thus, it is suggested that the antiproliferative effect of pioglitazone is almost negligible and that the regression of intimal hyperplasia might be mediated mainly through apoptosis-inducing effects in this experimental condition. However, both effects (the antiproliferative and apoptosis-inducing effects) of thiazolidinediones on VSMCs might effectively cooperate to inhibit the progression of arterial hyperplasia in vivo.

The thiazolidinediones are high-affinity ligands for PPARγ, a ligand-activated transcription factor of the steroid hormone receptor superfamily. It has been recognized that PPARγ exists in diverse cell types and tissues, including aortic VSMCs. We showed that pioglitazone enhanced cytokine-induced iNOS expression and produced an excess of NO in VSMCs. This effect of pioglitazone seems to be independent of PPARγ, because native PPARγ-binding ligand, 15d-PGJ2, had no effect on cytokine-induced NO production in VSMCs. These data are in good agreement with the recent study reported by Hattori et al.
stratified that troglitazone upregulates NO synthesis in VSMCs by prolonging the half-life of iNOS mRNA rather than activating its transcription, showing no effect on nuclear factor-κB activity, which is critical for the transcription of iNOS. These effects are also independent of PPARγ. Alternative unknown signaling mechanisms, including other intracellular target molecules to the thiazolidinediones, could account for the presented effects of the thiazolidinediones.

Recently, it was reported that the native PPARγ ligand, 15d-PGJ2, and synthetic ligands, including the thiazolidinediones, inhibit the production of NO and cytokines such as IL-1β in activated macrophages, in which PPARγ is upregulated.27,28 The effect of pioglitazone on VSMCs is dependent on the presence of cytokines released mainly from activated macrophages. Thus, it may be argued that the effect of pioglitazone on VSMCs is modified by its action as PPARγ ligand on macrophage in the neointimal lesion. However, as discussed above, the effect of pioglitazone on VSMCs appears to be independent of PPARγ. Half-maximal concentrations (EC50) of troglitazone and 15d-PGJ2 on inhibition of cytokine synthesis in macrophages were ∼10 and 1 μmol/L, respectively.27,28 The difference in these EC50s may be due to the different affinities to PPARγ. By contrast, in the present study, the EC50 of pioglitazone or troglitazone on the enhancement of cytokine-induced NO in VSMC was ∼1 μmol/L, whereas 15d-PGJ2 (up to 10 μmol/L) had no effect. According to previous data derived with the same method of drug supplementation in rats, expected peak concentration (Cmax) of pioglitazone in serum is <10 μmol/L.29 Indeed, expression of IL-1β in neointimal lesions was still detected in the pioglitazone-treated group (data not shown). Therefore, pioglitazone could act on cytokine-activated VSMCs more effectively than on macrophages in the neointimal lesion in this condition. However, these effects of the thiazolidinediones might be beneficial against formation of neointimal lesions. It remains to be clarified how these different effects of the thiazolidinediones actually work and interact with each other on arterial lesions in vivo.

In conclusion, our present observations suggest that pioglitazone enhances apoptosis in cytokine-activated VSMCs in an NO-dependent manner and induces significant regression of hyperplasia in balloon-injured vascular lesions. It appears that pioglitazone is a potent apoptosis inducer in vascular lesions, providing a novel pharmacological strategy to prevent restenosis after vascular intervention.

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