Characterization of an Inhibitory Effect of Pioglitazone on Balloon-Injured Vascular Smooth Muscle Cell Growth

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This study investigates whether pioglitazone could suppress an atherogenic process such as balloon-injured carotid intimal thickening and the proliferation of vascular smooth muscle cells (VSMC). We first examined the effect of pioglitazone to determine whether it could suppress intimal thickening induced by balloon catheterization in Sprague-Dawley rats. After 14 days postcatheterization in the left common carotid artery, the neointimal layers were completely occupied by proliferated VSMC, and the area ratio of neointima to media treated with 10 mg/kg/d of pioglitazone was significantly decreased to 57%. Next, we evaluated the effect of pioglitazone on the proliferation of rat cultured VSMC. Piogliotazone dose-dependently decreased the values of DNA synthesis, total cellular protein content, phosphorylations of extracellular signal-regulated protein kinase 1/2 and mitogen-activated protein kinase kinase 1/2, and proliferative cell nuclear antigen in VSMC. Pioglitazone also inhibited the phosphorylation of Pyk2. We conclude that pioglitazone itself could be effective for suppressing the growth of VSMC and consequent carotid intimal thickening.

PROLIFERATION AND MIGRATION of vascular smooth muscle cells (VSMC) in the neointima is a major event in the formation of atherosclerosis, including the process of restenosis after successful angioplasty.[1-3] Although the mechanism leading to intimal thickening is complicated and remains unclear, it is well accepted that by some action(s), VSMC in the medial layer might be stimulated and transformed from the contractile type to the synthetic type, and then they might even migrate to and proliferate in the intima layer.[2,4,5] The results of these studies have led many investigators to attempt to reduce the incidence of restenosis by pharmacologic interventions or modified procedures, including angiotensin-converting enzyme inhibitors,[6,7] calcium antagonists,[8,9] 3-hydroxy-3-methylglutaryl-coenzyme A reductase inhibitors,[10,11] and heparin.[12] Such trials have met with variable success.

Thiazolidinedione derivatives are novel insulin-sensitizing agents that have been developed for the treatment of insulin resistance, one of the most common abnormalities in non–insulin-dependent diabetes mellitus (NIDDM).[13] These agents can improve glucose metabolism by increasing insulin sensitivity in insulin-sensitive tissues both in patients with NIDDM and in several diabetic animal models.[14-17] Lehman et al[18] have also shown that peroxisome proliferator-activated receptor (PPAR) γ is a molecular target for the adipogenic effects of thiazolidinediones.[18] In addition, we and others have recently shown that some thiazolidinediones may have additional clinical benefits in cardiovascular tissues such as VSMC,[19-21] endothelial cells,[22] and ventricular myocytes.[23] In particular, extensive studies have clarified that thiazolidinediones have an inhibitory effect on calcium channel functions in VSMC by using the patch-clamp method, resulting in improved insulin sensitivity and decreased vascular contractility.[24,25] However, it is not been fully understood whether the inhibition of VSMC growth is a common property of thiazolidinediones both in vivo and in vitro or whether the agents can affect some signal transduction systems at the cytosolic and nuclear levels of VSMC.

To explore these questions, we examined the effects of pioglitazone, a member of the thiazolidinediones, on the carotid intimal thickening in vivo and the mechanism of VSMC growth in vitro in rats.

MATERIALS AND METHODS

Materials

Pioglitazone was supplied by Takeda Chemical Industries, Ltd (Osaka, Japan). A 2F Forgaty balloon catheter was purchased from Baxter Healthcare (Santa Ana, CA); anti-mouse α–smooth muscle actin (SMA) antibody and trypsin were obtained from Sigma Chemical Co (St Louis, MO), the avidin-biotin-peroxidase complex (ABC) kit from Vectastains (Vector Laboratories, Los Angeles, CA), Dulbecco modified Eagle medium (DMEM) and phosphate buffer saline (PBS) from Nissui Pharmaceutical Co, Ltd (Tokyo, Japan), and fetal bovine serum (FBS) from Gibco BRL (Grand Island, NY). Antibodies specific for extracellular-regulated protein kinase (ERK) 1/2 (p44/p42), as well as antibodies phosphospecific for ERK 1/2 and mitogen-activated protein (MAP) kinase kinase 1/2 (MEK 1/2), were purchased from New England Biolab (Beverly, MA), and antibodies specific for proliferative cell nuclear antigen (PCNA), Pyk2, PPAR-α, PPAR-γ, CD36, and phosphotyrosine (PY99) were obtained from Santa Cruz Biotech (Santa Cruz, CA). [^{3}H]Thymidine, polyvinylfluoride (PVDF) membrane, peroxidase-conjugated immunoglobulins, and an ECL kit were purchased from Amersham (Arlington Heights, IL). Protein A-Sepharose 6MB was purchased from Pharmacia Biotech AB (Uppsala, Sweden), a cytotoxicity detection kit (lactate dehydrogenase [LDH]) from Roche (Mannheim, Germany), and other chemicals from Wako Pure Chemical Industries, Ltd (Osaka, Japan), Nacalai Tesque (Kyoto, Japan), Bio-Rad Laboratories (Richmond, CA), and Sigma.

Protocol of Balloon Catheterization and Histologic Analysis

Male Sprague-Dawley (SD) rats weighing approximately 400 g were divided into control and pioglitazone-treated groups. In the pioglitazone-treated group, pioglitazone suspended in 5% gum arabic solution

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was administered at 10 mg/kg/d via gastric tube to rats (n = 6) once a day for 7 days. Age-matched rats were prepared as the control group (n = 6) and also given 5% gum arabic solution without pioglitazone once a day for 7 days. Under anesthesia with sodium pentobarbital (30 mg/kg intraperitoneally [IP]), balloon catheterization in the left common artery was performed on day 8 as described previously.[20] Briefly, after a minor incision in the left femoral artery, a 2F Fogarty balloon catheter was inserted and manipulated up to the left common carotid artery. The inside space of the balloon was filled with water, and intraluminal passages with 0.02 mL of water were carried out 3 times to denude the endothelium in the left common carotid artery. After catheterization, a 5% gum arabic solution with or without pioglitazone was administered for 14 days.

Two weeks after balloon catheterization, the left common carotid arteries were rapidly removed and divided into 4 cross-sectional pieces at 3-mm intervals for histologic analysis. The right common carotid arteries in all rats were also removed for the measurement of intact medial areas. The samples were fixed with 4% paraformaldehyde for 6 hours and subsequently processed for paraffin embedding. The fixed tissue samples were stained with Elastica-Masson, and the areas of the intima, measured from the internal elastic lamina to the luminal surface, and the medial layer in each section of the left common carotid artery were calculated using a microscopic image analyzing system, XL-10 (Olympus, Tokyo, Japan). The ratio of the intimal area to the medial area was provided as the area ratio of intima/media layer (I/M ratio), and the mean value of each preparation was calculated as 1 value. In addition, immunohistochemical staining was performed with anti-SMA antibody to detect the presence of VSMC-specific structural protein, and bound antibody was visualized by the ABC method and 3,3′-diaminobenzidine tetrahydrochloride (DAB) as chromogen as described previously.[26]

VSMC Culture

Rat VSMC were harvested from the aortas of male SD rats (150 to 200 g) by the medial explant technique and cultured in DMEM containing 10% FBS as described previously.[20] Cells within 10 passages were used for the following studies.

Measurement of DNA Synthesis in VSMC

The assay was performed as described previously,[27] with minor modifications. VSMC were plated at a density of 5×10^4 cells per well on a 12-well culture plate and grown in DMEM containing 10% FBS for 48 hours. The medium was then changed to DMEM containing 0.2% bovine serum albumin (BSA) for starvation. After 24 hours of incubation, the media were aspirated, and the cells were cultured in DMEM containing 3% FBS with various concentrations (final concentrations 10, 20, 40, 70, and 100 μmol/L) of pioglitazone solubilized in dimethylsulfoxide (DMSO) for 16 hours. Then, 1 μCi of [^{3}H]thymidine was added to each well and incubated for another 4 hours. At the end of the incubation, the cells were washed, and a high molecular mass of [^{3}H]thymidine was precipitated by 5% trichloroacetic acid at 4°C for 20 minutes. The precipitate was washed and solubilized in 500 μL of a mixture of 0.5N NaOH and 0.1% sodium dodecyl sulfate (SDS). After the solution had been neutralized with 250 μL of 1N HCl, radioactivity was measured in a liquid scintillation counter. Protein concentrations were measured by the method of Lowry et al.[28]

Growth Assay in VSMC

The cells were prepared as mentioned above and starved with DMEM containing 0.2% BSA for 24 hours. The media were then aspirated and cultured in DMEM containing 5% FBS with various concentrations (final concentrations 10, 40, and 100 μmol/L) of pioglitazone solubilized in DMSO. After incubation for the indicated number of days (0, 1, 3, and 5 days), the media were aspirated, and the cells were washed twice with ice-cold PBS. The cells were then solubilized in 1 mL of a mixture of 0.5% SDS and 0.5N NaOH. After the solution had been neutralized with 500 μL of 1N HCl, total cellular protein content was measured by the method of Lowry et al.[28] Those media were replaced every 24 hours during this experiment.

Immunoblot Analysis of ERK 1/2, MEK 1/2, and Pyk2 in VSMC

After being starved with DMEM containing 0.2% BSA for 24 hours at 37°C, the cells were cultured in DMEM containing 3% FBS with various concentrations (final concentrations 10, 20, 40, 70, and 100 μmol/L) of pioglitazone solubilized in DMSO for another 24 hours at 37°C. The following experimental procedure was performed as described previously.[29] The cells were washed 3 times with ice-cold PBS containing 1 μmol/L sodium orthovanadate and solubilized with 0.5 mL of a lysis buffer consisting of 20 mmol/L HEPES, 5 mmol/L $MgCl_2$, 25 mmol/L KCl, 1 mmol/L Na_3SO_4, 1 mmol/L sodium molybdate, 10 mmol/L β-glycerophosphate, 5 mmol/L tetrasodium pyrophosphate, 250 mmol/L sucrose, 1 mmol/L adenosine 5′-triphosphate (ATP), 2 mmol/L phenylmethylsulfonyl fluoride (PMSF), 0.1 mg/mL aprotinin, and 1% Triton-X. The total cellular lysates were centrifuged at 10,000*g* to remove insoluble materials, and then protein concentrations were determined by the method of Bradford.[30] The lysates were solubilized in a Laemmli buffer, and the samples were separated by 10% SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to PVDF membranes. Alternatively, the above lysates were incubated with 10 μL of anti-Pyk2 antibody overnight at 4°C. Then, protein A-Sepharose 6MB was used to precipitate the immune complexes and was incubated for another 2 hours at 4°C. The pellets were washed twice with ice-cold PBS, the bound proteins were then solubilized in a Laemmli buffer, and the complexes were separated by 7.5% SDS-PAGE and transferred to PVDF membranes.

After blocking overnight, the membranes were incubated with the primary antiphosphospecific antibody for ERK 1/2, MEK 1/2, or antiphosphotyrosine antibody overnight at 4°C and/or with the antibody for ERK 1/2 or Pyk2 for 1 hour at room temperature. The blots were washed and then incubated with an anti-rabbit or anti-mouse peroxidase-conjugated immunoglobulin for 1 hour at room temperature. After this washing, sites of antibody binding were visualized using the ECL Western blotting detection system and quantified with a densitometer.

Immunoblot Analysis of Nuclear Proteins in VSMC

Extraction of nuclear proteins was performed as described previously.[31] After being starved with DMEM containing 0.2% BSA for 24 hours at 37°C, the cells were cultured in DMEM containing 3% FBS with various concentrations (final concentrations 10, 20, 40, 70, and 100 μmol/L) of pioglitazone solubilized in DMSO for another 36 hours at 37°C. The cells were scraped and transferred to a microfuge tube. After being centrifuged at 10,000*g* for 10 seconds, the cells were suspended in 400 μL of ice-cold buffer A (10 mmol/L HEPES-KOH [pH 7.9, 4°C], 1.5 mmol/L $MgCl_2$, 10 mmol/L KCl, 0.5 mmol/L dithiothreitol [DTT], 0.2 mmol/L PMSF) by flicking the tube. The lysates were put on ice for 10 minutes and then vortexed for 10 seconds and centrifuged at 10,000*g* for 10 seconds. The pellet was resuspended in 50 μL of ice-cold buffer C (20 mmol/L HEPES-KOH [pH 7.9, 4°C], 25% glycerol, 420 mmol/L NaCl, 1.5 mmol/L $MgCl_2$, 0.2 mmol/L ethylenediaminetetraacetic acid [EDTA], 0.5 mmol/L DTT, 0.2 mmol/L PMSF) and incubated on ice for 20 minutes. After being centrifuged at 10,000*g* for 2 minutes to remove insoluble materials, the supernatant fractions were used for the determination of protein concentrations by the method of Bradford.[30] These samples were solubilized in a Laemmli buffer, separated by 12% SDS-PAGE, and trans-

ferred to PVDF membranes. After being blocked overnight, the membranes were incubated with antibody for PCNA, PPAR-α, PPAR-γ, or CD36 at room temperature for 1 hour. The blots were treated to visualize the sites of antibody binding by the same method and quantified as described.

Other Measurements

Blood samples were taken from the inferior vena cava for measurement of paroxysmal plasma glucose (PPG), immunoreactive insulin (IRI), and lipid levels on the day rats were killed. Plasma glucose and IRI levels were examined by a glucoseoxidase method and a polyethylene glycol method, respectively. Total cholesterol and triglyceride were measured by an enzymatic method, and high-density lipoprotein cholesterol (HDL-C) was measured by a precipitation method modified with the phosphotungstate-$MgCl_2$ method.

Statistical Analysis

Results are expressed as means ± SE. Statistical significance was estimated by 1-way analysis of variance (ANOVA) for the comparison of the 2 groups, and the differences were considered significant at $P < .05$.

RESULTS

Body Weight and Levels of Glucose, IRI, and Lipids

No rats died during the experimental run. Treatment with pioglitazone modified neither rat body weight (control group [C], 408.0 ± 11.6 g; pioglitazone-treated group [P], 413.2 ± 25.1 g) nor serum levels of PPG (C, 159.4 ± 29.8 mg/dL; P, 160.7 ± 12.9 mg/dL), IRI (C, 19.8 ± 7.6 μU/mL; P, 15.9 ± 1.7 μU/mL), total cholesterol (C, 48.1 ± 6.5 mg/dL; P, 52.4 ± 5.9 mg/dL), triglyceride (C, 46.8 ± 16.2 mg/dL; P, 43.3 ± 12.9 mg/dL), and HDL-C (C, 38.1 ± 5.2 mg/dL; P, 40.8 ± 5.2 mg/dL).

Effect of Pioglitazone on Histologic findings and Value of I/M Ratio

The intimal thickening layers consisted of abundant infiltrated cells. Immunohistochemical staining with anti-SMA antibody showed that diffusely positive immunoreactivities completely occupied the intimal thickening layers of all preparations (Fig 1). The findings showed that these layers in our experiment were composed of VSMC, and we observed a decrease in the amount of positively stained cells in these layers with the treatment of pioglitazone.

Changes in the I/M ratio are shown in Fig 2. Compared with the I/M ratio in the pioglitazone-treated group, that of rats without pioglitazone in the control group was significantly higher (P, 0.40 ± 0.07, n = 6; C, 0.70±0.07, n = 6; $P < .05$). Therefore, treatment with pioglitazone resulted in a distinct change in the size of intimal thickening. The areas of the medial layer in the catheterized and intact common carotid arteries did not differ significantly between the 2 groups (data not shown).

These findings indicate that pioglitazone appears to have an inhibitory effect on the intimal thickening induced by balloon catheterization.

Effect of Pioglitazone on Cultured VSMC Growth

To characterize the effect of pioglitazone on rat VSMC growth in vitro, we first examined the mitogenic response by an [^{3}H]thymidine incorporation assay. As shown in Fig 3, the values of [^{3}H]thymidine incorporation were inhibited by the treatment with pioglitazone dose dependently. At more than 40 μmol/L of pioglitazone, the values were significantly decreased compared with those in the control group ($P < .01$). In addition, cell viability was more than 98%, as determined by the exclusion of 0.2% Trypan blue at even 100 μmol/L of pioglitazone.

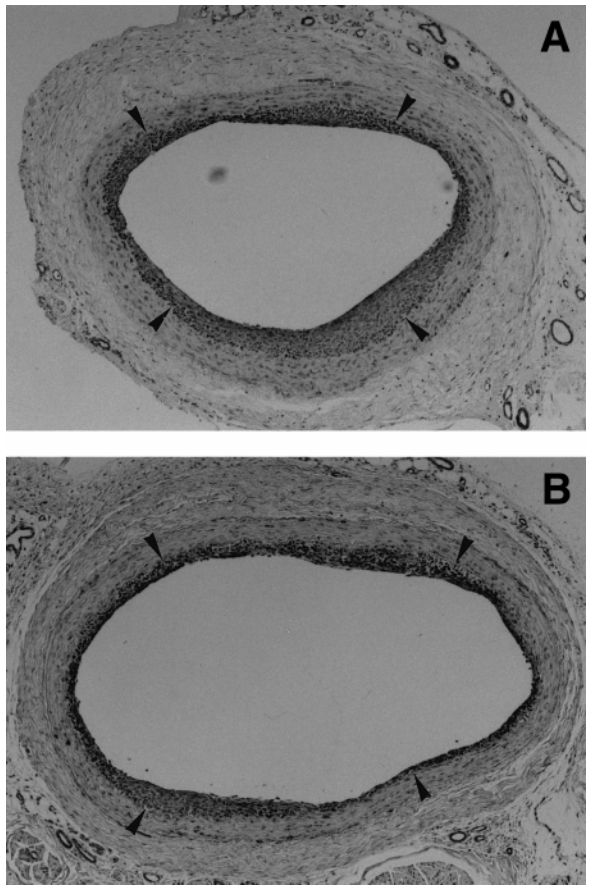

Fig 1. Cross-sections of rat left common carotid artery 14 days after balloon catheterization. Immunohistologic stainings with anti-SMA antibody diluted 1:2,000 with PBS(−) and developed by the ABC method. DAB, 0.4 mg/mL, was used as the counterstain of the ABC method, and the antibody-positive staining is shown as black. (A) Untreated rat. (B) Rat treated with 10 mg/kg/d of pioglitazone. Arrowheads indicate the position of the internal elastic lamina. The preparations were examined under 20× magnification.

Next, we also examined changes in total cellular protein concentration (Fig 4). The values in the control group were gradually increased to 102.3 ± 2.5 μg/mL on day 3 and 199.2 ± 2.8 μg/mL on day 5 by stimulation with 5% FBS. Compared with the control group, values in the pioglitazone-treated groups were decreased dose dependently. The values at 10 μmol/L pioglitazone were significantly decreased even on day 3 ($P < .05$), indicating that pioglitazone itself prevented VSMC growth. We also measured the values of LDH from the above cultured media to estimate the cytotoxicity of pioglita-

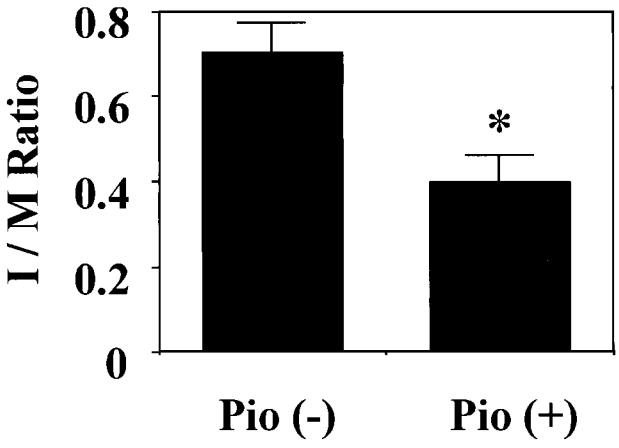

Fig 2. Effect of pioglitazone on the value of the I/M ratio from rat left common carotid artery 14 days after balloon catheterization. Pio (−), untreated-rat group (n = 6); Pio (+), rats treated with 10 mg/kg/d of pioglitazone group (n = 6). Values are means ± SE, and statistical significance was assessed by ANOVA; *$P < .05$.

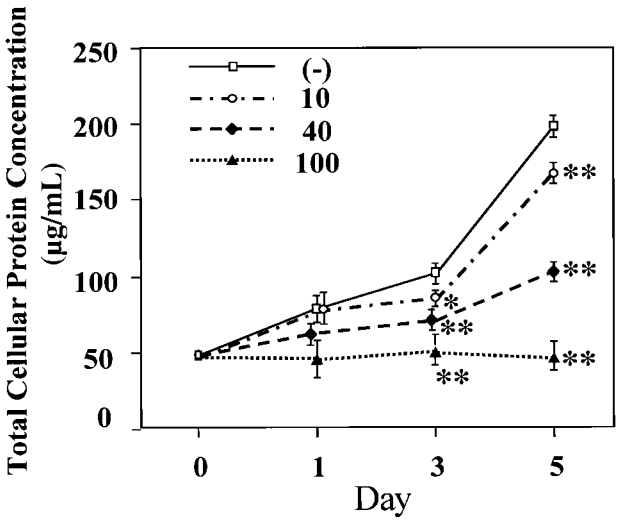

Fig 4. Effect of pioglitazone on changes in total cellular protein concentrations in rat VSMC. The concentrations were determined as described in Materials and Methods. The results were derived from 3 separate experiments, and each experiment was performed in triplicate. Each bar represents mean ± SE; *$P < .05$, **$P < .01$ *v* pioglitazone (−).

zone, but these values were not significantly elevated even in 100 μmol/L pioglitazone on day 5 (data not shown).

Effect of Pioglitazone on Phosphorylation of ERK 1/2 and Its Upstream, MEK 1/2, in VSMC

We examined whether pioglitazone could affect the activation of ERK 1/2 (p44/42) in rat VSMC by immunoblot analysis. The upper panel of Fig 5A shows the effect of pioglitazone on the phosphorylation of ERK 1/2. Compared with the control band, the levels of phosphorylated ERK 1/2 were dose-dependently decreased by treatment with pioglitazone and significantly suppressed to 66.4% ± 5.4% at 40 μmol/L, 54.4% ± 7.0% at 70 μmol/L, and 39.9% ± 4.2% at 100 μmol/L (lower panel of Fig 5A). In contrast, protein levels of ERK 1/2 were not changed by treatment with pioglitazone (data not shown).

To confirm the identity of the effect of pioglitazone on the signal-transduction pathway of ERK, we further examined the phosphorylation of MEK 1/2, an upstream kinase of ERK 1/2,[32] in rat VSMC. As shown in Fig 5B, the levels of phosphorylated MEK 1/2 were also decreased dose dependently by treatment with pioglitazone and significantly suppressed to 77.4% ± 4.8% at 40 μmol/L, 71.0% ± 4.3% at 70 μmol/L, and 66.7% ± 4.5% at 100 μmol/L (lower panel of Fig 5B).

These data clearly demonstrate that pioglitazone can prevent cell growth via a MEK-ERK cascade in rat VSMC.

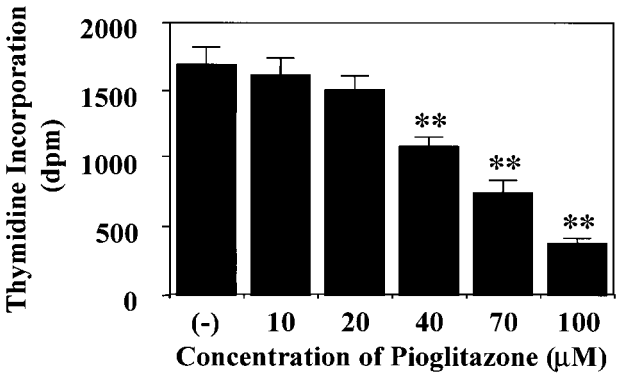

Fig 3. Effect of pioglitazone on DNA synthesis in rat VSMC. The mitogenic response (DNA synthesis) was determined, as described in Materials and Methods. The results were derived from 4 separate experiments, and each experiment was performed in triplicate. Each bar represents mean ± SE; **$P < .01$ *v* pioglitazone (−).

Effect of Pioglitazone on the Phosphorylation of Pyk2 in VSMC

To evaluate the effect of pioglitazone on the upstream level of a MEK-ERK cascade in VSMC, we further characterized the role of Pyk2, which activates the MEK-ERK signal-transduction pathway in response to elevation of intracellular calcium concentrations.[33,34] Compared with the control band, the levels of phosphorylated Pyk2 were also decreased dose dependently by treatment with pioglitazone and significantly suppressed to 75.6% ± 6.0% at 40 μmol/L, 50.4% ± 3.2% at 70 μmol/L, and 41.8% ± 4.7% at 100 μmol/L (Fig 6A). In contrast, the protein levels of Pyk2 were not changed by treatment with pioglitazone (Fig 6B).

Effect of Pioglitazone on the Levels of Nuclear Proteins in VSMC

To estimate the inhibitory effect of pioglitazone on VSMC growth in the nuclear signaling level, we further investigated the levels of nuclear proteins. As shown in Fig 7, the protein level of PCNA was dose-dependently attenuated by treatment with pioglitazone. The levels were also significantly decreased to 75.5% ± 5.5% at 40 μmol/L, 58.7% ± 8.3% at 70 μmol/L,

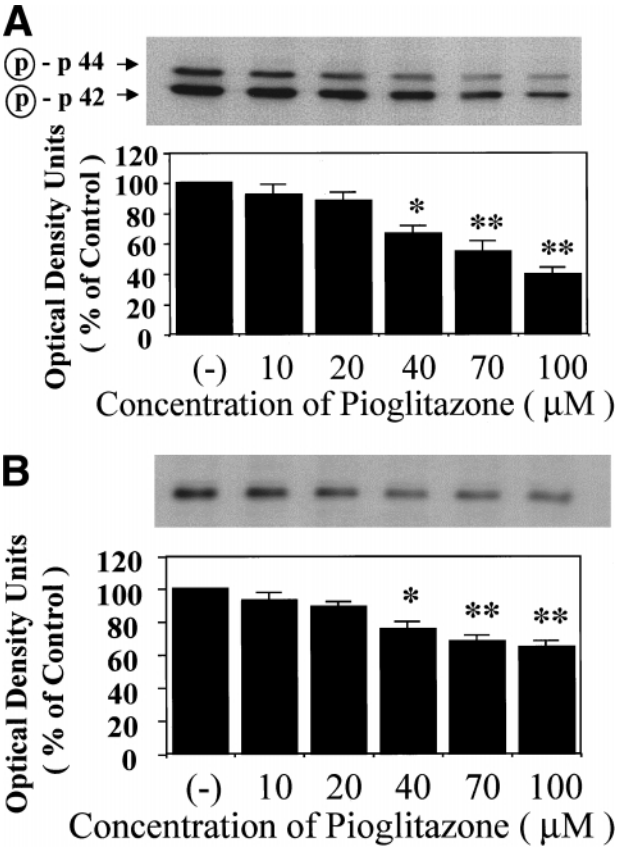

Fig 5. Effect of pioglitazone on the phosphorylations of (A) ERK 1/2 (p44/42) and (B) MEK 1/2 in rat VSMC. The samples were separated by 10% SDS-PAGE, as described in Materials and Methods. The quantification of the phosphorylation of ERK 1/2 or MEK 1/2 from 3 separate experiments is shown in the each lower panel, and each bar represents mean ± SE; *$P < .05$, **$P < .01$ *v* pioglitazone (−).

and 24.7% ± 6.6% at 100 μmol/L (lower panel of Fig 7). On the other hand, pioglitazone could not affect the level of PPAR-α, PPAR-γ, or CD36 in VSMC (data not shown).

DISCUSSION

In this study, we show that pioglitazone has an inhibitory effect even on the early events of atheromatous formation by the direct suppression of VSMC growth via a MEK-ERK pathway coupled with Pyk2 in a strict dose-dependent manner and that the effect is independent of blood glucose, IRI, and serum lipid levels. In addition, pioglitazone also suppressed PCNA levels dose dependently. These favorable results suggest that pioglitazone could be one of the most promising agents for preventing the progression of vascular complications such as restenosis and possibly atherosclerosis.

First, we examined the in vivo effect of pioglitazone to determine whether it could suppress rat intimal thickening induced by balloon catheterization. As shown in Figs 1 and 2, the I/M ratio in the pioglitazone-treated group was significantly decreased to 57% ($P < .05$), compared with the control group. The lesions were composed mainly of SMA-positive cells (Fig 1), which suggested that pioglitazone has a significant inhibitory effect on the content of VSMC in the neointima and the subsequent intimal thickening. Because blood glucose, IRI, and serum lipid levels were not changed in the 2 groups, these factors can be considered insufficient to influence the formation of intimal thickening. These results were obtained at 10 mg/kg/d of pioglitazone, a much lower dose than that used in another study of troglitazone, another member of thiazolinedinediones, because their daily doses for rats were as much as 4 g/kg.[21] Differences in the ability of the drug to penetrate VSMC could possibly explain its efficacy to inhibit VSMC proliferation.

Based on the above in vivo findings, we next examined the in vitro effect of pioglitazone on the proliferation of rat VSMC. Similar to the in vivo findings, the values of both [^{3}H]thymidine incorporation and total protein content were reduced by treatment with pioglitazone in a dose-dependent fashion (Figs 3 and 4). In addition, the values of LDH in culture media from untreated or pioglitazone-treated cells were not changed, indicating that pioglitazone itself can inhibit VSMC proliferation. In agreement with our findings, Dubey et al[19] have shown that pioglitazone can inhibit the proliferation of renal VSMC in rats

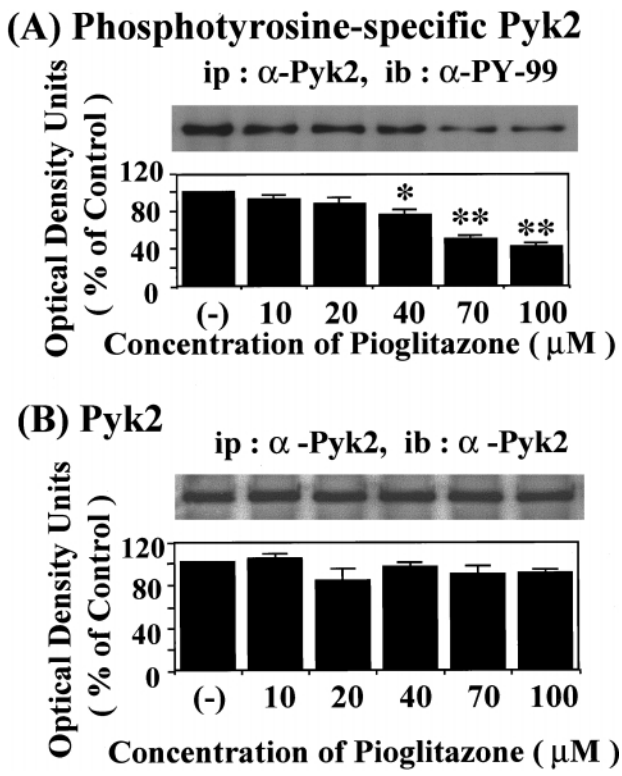

Fig 6. Effect of pioglitazone on the levels of (A) phosphotyrosine-specific and (B) protein of Pyk2 in rat VSMC. The samples were separated by 7.5% SDS-PAGE, as described in Materials and Methods. The levels of phosphotyrosine-specific or protein of Pyk2 were detected by immunoblot analysis using antibody specific for (A) phosphotyrosine (PY99) or (B) Pyk2, respectively. The quantification of the phosphorylation or protein of Pyk2 from 3 separate experiments is shown in each lower panel, and each bar represents mean ± SE; *$P < .05$, **$P < .01$ *v* pioglitazone (−).

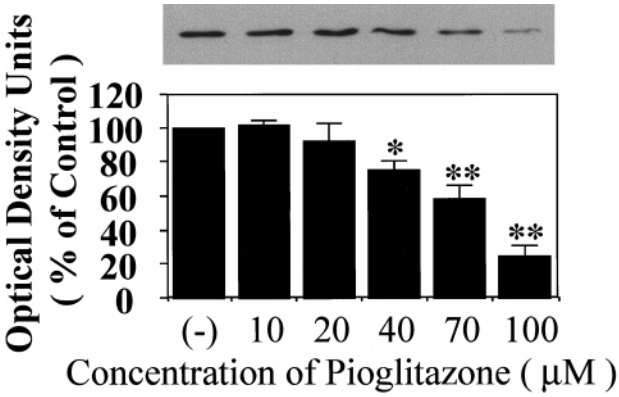

Fig 7. Effect of pioglitazone on the levels of PCNA in rat VSMC. The samples were separated by 12% SDS-PAGE, as described in Materials and Methods. The quantification of the level of PCNA from 3 separate experiments is shown in the lower panel, and each bar represents mean ± SE; *$P < .05$, **$P < .01$ *v* pioglitazone (−).

and that these effects were reversible and not caused by cytotoxity, even with 100 μmol/L pioglitazone in the presence of 0.4% FCS.

Recently, it was revealed that the MAP kinase superfamily plays a crucial role in cell growth, differentiation, or even programmed cell death in response to a diverse of extracellular stimuli in eukaryotic cells.[32] Extensive studies have clarified that a variety of growth factors and hormones can activate ERK 1/2 signal-transduction pathways through GTPase-activating protein of Ras (Ras-GTP), leading to cellular proliferation and differentiation by stimulating transcription factors that induce the expression of *c-fos* and other growth-responsive genes.[35,36] As shown in Fig 5A, pioglitazone clearly inhibited the levels of phosphorylated ERK 1/2 in a dose-dependent fashion and significantly reduced the levels at more than 40 μmol/L. In addition, levels of phosphorylated MEK 1/2, the upstream of ERK 1/2, were also inhibited by the agent in the same fashion as ERK 1/2 (Fig 5B), suggesting that pioglitazone may have an effect on the change(s) in signal transduction through Ras-GTP to a MEK-ERK pathway. In addition, because Eguchi et al[37] have recently reported that the p70 S6 kinase, which regulates protein synthesis, is activated through Ras and the subsequent ERK in VSMC, the decrease in total cellular protein contents by pioglitazone might be considered the consequence of reducing the phosphorylation of p70 S6 kinase through the Ras-MEK-ERK pathway.

Although the precise molecular mechanism(s) by which pioglitazone can affect the MEK-ERK pathway remain unclear, one likely contributor is Pyk2, a focal adhesion kinase, because Pyk2 is activated by various extracellular signals that increase intracellular calcium concentration and subsequently activate the signaling pathway from Ras-GTP to MAP kinase.[33,34,38] In addition, several investigators have shown that the changes in intracellular calcium concentration have an important role in regulating ERK activation in the various types of cells[39,40] and that thiazolidinediones directly inhibit the calcium channel functions in VSMC, resulting in decreased vascular contractility.[23-25] Our results show that the levels of phosphorylated Pyk2 were decreased dose dependently by treatment with pioglitazone and also significantly suppressed at more than 40 μmol/L (Fig 6A), clearly demonstrating that pioglitazone itself can suppress the proliferation of VSMC via a MEK-ERK pathway coupled with Pyk2.

As for the blood pressure-lowering effect by pioglitazone, Nakamura et al,[24] using the patch-clamp method, have reported that pioglitazone could inhibit the 77 mmol/L K^+-induced contraction at more than 10 μmol/L pioglitazone in VSMC. In addition, several investigators have recently shown that in VSMC, insulin can enhance angiotensin II type 1 (AT_1) receptor gene expression and the subsequent angiotensin II (Ang II) signaling[41] and that the Ang II signaling through AT_1 receptor uses calcium-dependent Pyk2 for the activation of tyrosine kinases in epidermal growth factor receptor and of the downstream signals,[42] suggesting that the up-regulation of Ang II signaling further elevates blood pressures and promotes the hypertrophy and hyperplasia of VSMC in the insulin-resistant state. In contrast, Takeda et al,[43] have revealed that the thiazolidinedione derivatives down-regulate AT_1 receptor expression and the calcium response to Ang II in VSMC, indicating that the thiazolidinedione derivatives directly influence the activity of VSMC in the vascular wall and decrease blood pressures. Thus, it is reasonable to assume that the above effect of pioglitazone may contribute to inhibition of VSMC growth in our balloon-injured model.

It is generally accepted that PCNA is synthesized in the early G_1 and S phases of the cell cycle and behaves as a marker for proliferating cells.[12,44] In this study, we showed that pioglitazone can suppress the level of PCNA in VSMC (Fig 7) in dose-dependent fashions, suggesting that pioglitazone can attenuate VSMC growth even at the nuclear level by inducing G_1 arrest. In contrast, pioglitazone was not found to affect the level of PPAR-α, PPAR-γ, or CD36 in VSMC (data not shown). Especially, PPAR-γ has been revealed as a promoter of monocyte/macrophage differentiation and the subsequent secretion of CD36[45] and as a molecular target for the adipogenic effects of thiazolidinediones in CV-1 cells.[18] It is plausible to assume that the discrepancy is based on the difference in cell types.

In conclusion, in this study, we have established that pioglitazone has an inhibitory effect on intimal thickening by attenuating intimal VSMC proliferation via a MEK-ERK pathway coupling with Pyk2, suggesting that intracellular calcium kinetics play an important role in the pathogenesis of atherosclerosis. Furthermore, the agent consequently resulted in G1 arrest in VSMC, proving that pioglitazone could be effective to prevent the progression of atherosclerotic plaquelike restenosis after percutaneous transluminal coronary angioplasty. These results provide a new insight into the potential cellular and molecular mechanisms whereby pioglitazone treatment may have clinical benefits and contribute to the prevention of atherosclerosis.

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