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ラット大動脈置換モデルにおけるピオグリタゾンの静脈グラフト劣化抑制効果関する実験的検討.

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Pioglitazone preserves vein graft integrity in a rat aortic interposition model

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ABSTRACT

Objective: Improvement of vein graft patency could be highly beneficial in coronary artery bypass grafting, but graft degeneration is considered to be one of the main pathophysiologic causes for vein graft failure. As peroxisome proliferators-activated receptor (PPAR)-γ activator pioglitazone has recently been reported to possess pleiotropic protective effects on various organs and tissues, experiments conducted here tested the hypothesis that pioglitazone could prevent graft degeneration, leading to the preservation of vein graft integrity.

Methods: In a rat aortic interposition model with autologous femoral vein, pioglitazone (3 mg/kg/day) or vehicle (normal saline) was given to rats by gastric gavage once a day beginning 3 days before surgery and ending 8 weeks after surgery. Vein graft degeneration and remodeling were assessed at 24 hours, 7 days, 8 weeks and 6 months after surgery.

Results: At 24 hours, pioglitazone significantly reduced endothelial desquamation, reactive oxygen species generation, myeloperoxidase activity and lipid peroxidation in vein grafts. At 7 days, mRNA expression and gelatinolytic activity of matrix metalloproteinase (MMP)-2 and MMP-9 in vein grafts were significantly suppressed by pioglitazone treatment. Immunofluorescent staining showed that pioglitazone enhanced PPAR-γ expression in vein grafts at 8 weeks, especially in their intimal

side. At 6 months, pioglitazone treatment prevented graft dilation (52.3±3.1 vs. 90.7±9.9%, P=0.0041) and neointimal hyperplasia (14.6±1.3 vs. 29.9±2.9%, P=0.0008), and increased graft flow velocity ratio (0.86±0.03 vs. 0.59±0.04, P<0.0001), compared with vehicle treatment.

Conclusion: Pioglitazone prevents graft degeneration under arterial pressure stress, and can preserve the vein graft integrity in rat aortic interposition model.

Ultramini-Abstract

We evaluated the efficacy of peroxisome proliferators-activated receptor-γ activator pioglitazone on vein grafts under arterial environment in a rat aortic interposition model with autologous femoral vein. Pioglitazone treatment prevented vascular degeneration including graft dilation and neointimal hyperplasia, leading to preserve the vein graft integrity.

Introduction

Although the clinical use of arterial conduits, such as internal mammary artery or radial artery, is a current trend in coronary arterial bypass grafting (CABG) due to its superior long-term patency, saphenous vein is still widely used as the bypass conduit. After grafting, the vein grafts are immediately exposed to arterial pressure and pulsatile blood flow, thereby increasing wall tension and shear forces. These hemodynamic change and physiologic stress lead to graft overdistension, endothelial damage and dysfunction, and involve proliferation and migration of smooth muscle cells (SMCs) into the vein graft. Eventually, the vein grafts could have a vascular remodeling including neointimal hyperplasia, which is considered to be one of the major causes of vein graft failure(1;2).

Thiazolidinediones, which include the presently available drugs pioglitazone, are well established insulin sensitizing agents that act as agonists of the ligand-activated transcriptional factor peroxisome proliferator-activated receptor (PPAR)-γ(3). It has recently been reported that pioglitazone possesses pleiotropic cardiovascular protective actions in experimental settings, including anti-inflammatory and anti-proliferative properties(4;5). Migration and proliferation of vascular SMCs are inhibited by pioglitazone(6), and pioglitazone reduces hypertensive vascular injury and hypertrophy(7;8). However, there is no report to

elucidate an efficacy of pioglitazone on vein grafts in arterial environment yet.

Taken together, the aim of this study was to evaluate whether pioglitazone has a potential to preserve vein graft integrity in arterial environment, associated with preventing graft distension and intimal hyperplasia.

Materials and Methods

Animals

A total of 60 male Sprague-Dawley rats weighing 450 to 550 g (10 weeks of age, CLEA Japan Inc, Tokyo) were used in the present study. The handling of laboratory animals and their use in experiments conformed to the *Guidelines for Animal Experiment at Kobe University Graduate School of Medicine (Permission number: P080120)* and the *Guide for the Care and Use of Laboratory Animals* (www.nap.edu/catalog/5140.html).

Drug treatment

Pioglitazone, provided by Takeda Chemical Industries (Tokyo, Japan), was diluted in normal saline. Pioglitazone (3mg/kg/day(9;10)) or vehicle (normal saline) was given to rats by gastric gavage once a day beginning 3 days before surgery and ending 8 weeks after surgery.

Rat Aortic bypass Model

Rats were placed in a closed chamber containing diethyl ether (Wako, Osaka, Japan). Following loss of consciousness, the rats were weighed and anesthesized with pentobarbiturate (5 mg/kg, intraperitoneally). The anesthetized rats were placed under an operating microscope Leica M651 (Leica microsystems, Heerbrugg, Switzerland) with 6 to 10 times magnification. The right femoral vein was harvested and rinsed in heparinized saline. The infrarenal abdominal aorta was transected and interposed with the autologous femoral vein graft (1.0 cm in length) by end-to-end anastomosis with a continuous running suture of 8-0 polypropylene (PROLENE; Johnson & Johnson Gateway, LLC, NJ). The right femoral vein was not reconstructed. After the anastomosis, graft patency was evaluated by direct visualization before layered surgical closure. The surgeon (Z.C.) was blinded to treatment group in the present study. One-week postoperative prophylactic antibiotic regimen was followed for all rats. Their body weight was measured once a week. For monitoring pioglitazone-related adverse effects, the rats were observed everyday postoperatively.

Study design

The present study design shows in Figure E1. The endpoints of the present study were 24 hours, 7 days, 8 weeks and 6 months after surgery in each group. The experiments of 7-day, 8-week and 6-month endpoints were performed on 6 rats per each group, respectively. The 24-hour experiment was performed in two subsets of each group, which provided samples for Evans blue assay (n=6 in each group) and other assays (n=6 in each group). The 7-day samples were used for matrix metalloproteinases (MMPs) analysis, and the 8-week and 6-month samples were used for physiologic, morphometric and histological analyses.

Macroscopic assessments

Animals were anesthetized, and then the abdominal aorta was exposed and photographed with digital camera Leica IC D (Leica microsystems). Graft diameter was measured under physiologic conditions with an optical micrometer by the person (A.T.) who was blinded to the different treatment groups. The graft diameter was defined as the maximum dimension of the transverse minor axis of the vein graft, and the graft dilation was calculated according to the following formula based on our previous study(11):

Graft dilation ratio (%) = (diameter at harvesting - diameter at implantation)/diameter at implantation $\times 100$

The velocity of blood flow through the vein graft was measured using a Transonic flowmeter (3mm in size of the probe; Transonic System Inc, NY), and recorded with PowerLab/MacLab800 (AD Instruments Japan Inc, Nagoya, Japan). It was measured at proximal aorta, vein graft and distal aorta, and the mean value was taken as the flow velocity. The graft flow velocity ratio was calculated according to the following formula:

Graft flow velocity ratio = graft flow velocity / proximal aorta flow velocity

Histology

Harvested tissues were rinsed in saline and fixed in 10% formalin. The formalin-fixed samples were dehydrated in a graded ethanol bath, cleaned in xylene and embedded in paraffin. Sections (thickness, 5μm) were stained by hematoxylin and eosin (HE) and Elastica von Gieson (EVG). After those images were captured using a microscopic system (BZ-8100, KEYENCE Co., Osaka, Japan), histological analyses of the sections were performed using ImageJ version 1.41 software (National Institute of Health, Bethesda, MD). The neointimal thickness at the anastomosis site was calculated by converting the number of pixels to micrometers using a stage micrometer on the captured images, and the neointimal hyperplasia at the aneurysmal site was calculated by using the following formula(11):

Neointimal hyperplasia (%) = neointimal area / area bounded by the media × 100

Immunohistochemical staining

Immunohistochemical staining was performed on the paraffin-embedded section by using ENVISION kit/HRP (DAKO, Kyoto, Japan) with primary antibody, anti-human α-smooth muscle actin (αSMA)/HRP (DAKO). Diaminobenzidine substrate (DAKO) was used as a chromogen, and cell nuclei were counterstained with hematoxylin. The αSMA-positive area in a cross-section was semi-quantified using the ImageJ (National Institute of Health), and was calculated by dividing the αSMA-positive area by the cross-sectional vein graft area.

Immunofluorescent staining

Immunofluorescent staining was performed on the paraffin-embedded section with primary antibody, mouse monoclonal anti-PPAR-γ antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Fluorescein-linked sheep anti-mouse antibody was used as secondary antibody (Amersham Biosciences, Buckinghamshire, UK). The cell nuclei were counterstained with 4′,6-diamidino-2-phenylindole (Chemicon International, Temecula, CA).

Evans Blue Staining

To evaluate the endothelial desquamation in the whole area of the vein graft at 24 hours after surgery, Evans blue staining was performed as we described previously with some modifications(11;12). Briefly, Evans blue (10 mg/kg; Sigma-Aldrich, St. Louis, MO) was injected intravenously 2 hours before vein graft harvesting. After perfusion-fixation with 100% methanol, vein grafts including both proximal and distal anastomotic sites were harvested and opened longitudinally for macroscopic examination. After the vein graft was separated from aorta, its uptake of Evans blue was quantified by formamide extraction (55°C for 2 hours) measuring absorbance at 595nm. The concentration was determined by the calibration curve, and the data were standardized to the protein concentration of each sample as determined by a micro BCA protein assay kit (Pierce, Rockford, IL).

Myeloperoxydase assay

Myeloperoxidase (MPO) activity in vein grafts at 24 hours after surgery was assessed as previously described(12). MPO values were standardized to the protein concentration of each sample. Data are expressed as the change in absorbance at 450 nm/min/mg of total protein.

Lipid peroxidation assay

Lipid peroxidation in vein grafts at 24 hours after surgery was assessed using spectrophotometric assay for malondialdehyde (BIOXYTECH MDA-586; OXIS International Inc., Foster City, CA), according to the manufacturer's instructions. Data are standardized by protein concentration.

Quantitative Real-Time PCR analysis

Total RNA was isolated from graft samples using an RNeasy fibrous tissue mini-kit (QIAGEN) according to the manufacturer's instructions. The RNA was transcribed and amplified to cDNA using a High capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA). Quantitative real-time polymerase chain reaction (PCR) analysis for mRNA of MMP-2, MMP-9, tissue inhibitors of MMP (TIMP)-1 and TIPM-2 was performed using ABI Prism 7500 sequence detector system (Applied Biosystems) with TaqMan universal PCR master mix and TaqMan real-time PCR primers (Applied Biosystems). The expression level of each mRNA was divided by mRNA level of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Gelatin zymography

Proteins of graft samples were extracted using a buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, 0.2% SDS, and 1 mM EDTA, supplemented with protease inhibitors (20 μg/ml aprotinin, 10 μg/ml leupeptin and 1 mM phenylmethylsulfonyl fluoride). To determine gelatinolytic activities of MMP-2 and MMP-9 in vein grafts, Gelatin-zymography kit (Primary Cell Co., Hokkaido, Japan) was used according to the manufacturer's instructions. After the protein concentration was standardized, 20 μg of protein was applied in each lane to the electropheresis. Densitometric analysis of lytic bands for MMP-2 and MMP-9 was performed by the ImageJ Software, Version 1.41 (National Institute of Health).

Statistics

Database management and statistical analysis were performed with Statview Software Version 5.0 (SAS institute Inc., Cary, NC). All values are expressed as means ± SEM. Statistical analyses of data were performed by unpaired Student *t* test or one-way analysis of variance (ANOVA) by the Bonferroni post hoc test, as appropriate. Values of $P<0.05$ were considered statistically significant.

Results

The surgical ischemic times during an end-to-end interposition of the vein graft were

approximate 20 minutes in both pioglitazone and control groups. There was no significant difference of the ischemic time between the two groups. There was neither technical failure nor death intraoperatively, and all animals have survived until each end-point uneventfully. No thrombus formation in vein grafts was observed postoperatively. During the postoperative observation, there were no significant differences of body weight change or histological findings of heart, lung, liver and kidney between the pioglitazone and the control group postoperatively.

Preservation of endothelial function

To evaluate endothelial desquamation of vein grafts due to its exposure to arterial environment, the grafts were stained with Evans blue dye, which is believed to penetrate intimal areas that was not covered by endothelium(11). At 24 hours after surgery, vein grafts in the control treatment were stained more deeply with Evans blue than those in pioglitazone treatment. Quantitatively, the uptake of Evans blue in vein grafts was significantly less in the pioglitazone group, compared with the control group (29.1±2.5 vs. 45.6±2.8 μg/mg protein, P=0.0003; **Fig. 1A**). Because the arterial pressure stretch of vein grafts would induce a generation of reactive oxygen species (ROS) and oxidative injury(13;14), we next evaluated oxidative stress in vein grafts at 24 hours. The intensity of red oxidative fluorescence in the

pioglitazone group was apparently lower than that in the control group (**Fig. 1B**). The graft oxidative damage, quantified by MDA levels in vein grafts, was significantly reduced by pioglitazone treatment, compared with the control treatment (25±13 vs. 85±14 pmol/mg protein, P=0.0104; **Fig. 1C**). MPO activity in the pioglitazone group was also significantly less than that in the control group (0.06±0.01 vs. 0.29±0.05 ΔAb/min/mg protein, P=0.0009; **Fig. 1D**).

Suppression of matrix metalloproteinases

The arterial pressure stretch of vein grafts also enhances the activity of MMPs, which are especially important regulators of vein graft architecture because of their specificity for elastin(15). At 7 days after surgery in the present study, the MMP-2 and MMP-9 mRNA expressions were significantly downregulated by pioglitazone treatment, compared with control treatment (MMP-2, 0.97±0.12 vs. 1.53±0.18, P=0.0254; MMP-9, 0.46±0.06 vs. 0.83±0.05, P=0.0004; **Fig. 2A**). Gelatinolytic activities of MMP-2 and MMP-9 proteins were also significantly lower in the pioglitazone group than those in the control group (MMP-2, 47.4±2.6 vs. 74.6±8.4, P=0.0113; MMP-9, 20.9±1.7 vs. 33.0±2.5, P=0.0023; **Figs. 2B**). Because the MMPs activities are tightly regulated by TIMPs(15), we also evaluated mRNA expression of TIMP-1 and TIMP-2 in vein grafts. However, there were no significant difference of

these mRNA expressions between the pioglitazone and the control group (**Fig. 2A**).

Maintenance of graft blood flow velocity

There was no significant difference in mean graft blood flow velocity immediately after surgery between the both groups. Although the graft flow velocity ratio gradually decreased after surgery, it kept significantly higher in the pioglitzone group than in the control group at both 8 weeks and 6 months after surgery (8 weeks, 0.87±0.02 vs. 0.66±0.05; 6 months, 0.86±0.03 vs. 0.59±0.04, $P<0.0001$ by ANOVA; **Fig.3**).

Prevention of graft dilation

Graft dilation was observed under physiologic conditions at 8 weeks and 6 months. In both 8 weeks and 6 months, the graft dilation ratios were significantly lower in the pioglitazone group than those in the control group (8 weeks, 36.3±2.2 vs. 61.4±3.9%, $P=0.0002$; 6 months, 52.3±3.1 vs. 90.7±9.9%, $P=0.0041$; **Fig. 4A**)

Enhancement of PPAR-γ expression

Immunofluorescent staining has shown that pioglitazone treatment upregulates PPAR-γ expression in vein grafts at 8 weeks after surgery, compared with the control

(**Fig. 4B**). Especially, there was a stronger enhancement of PPAR-γ expression in the intimal side of grafts than in the adventitial side by pioglitazone.

Reduction of neointimal hyperplasia

At 6 months, neointimal hyperplasia and SMC proliferation in vein grafts was assessed by EVG staining and αSMA immunostaining, respectively (**Fig. 5A and 5B).** Pioglitazone treatment reduced both neointimal hyperplasia and αSMA-positive area significantly as compared with the control group (neointimal hyperplasia: 14.6±1.3 vs. 29.9±2.9%, P=0.0008; αSMA-positive area: 27.7±2.0 vs. 47.6±3.6%, P=0.0007). At the anastomosis site of vein grafts, neointimal thickness was also significantly thinner in the pioglitazone group than that in the control group (67.6±2.1 vs. 134.6±19.6 μm, P=0.0069; **Fig. 5C**).

Discussion

It is well recognized that inevitable exposure of vein grafts to the arterial system with high pressure and pulsatility contributes to their circumferential stretch. It leads to structural and functional changes in the graft wall, which is known as vascular remodeling. Here we have first described that pioglitazone can regulate the distended vascular remodeling of vein graft likely due to PPAR-γ up-regulation. The

main findings of this study are that pioglitazone treatment can bring (i) prevention of acute graft damage caused by both mechanical and oxidative stresses, (ii) regulation of the ECM integrity by suppressing MMP-2 and MMP-9 activities, (iii) inhibition of the graft dilation and maintenance of the graft flow velocity, and (iv) reduction of neointimal hyperplasia with less SMC proliferation in vein grafts.

It is not surprising that pioglitazone enhances PPAR-γ expression in vein grafts. Previous experimental studies have demonstrated that PPAR-γ activation with thiazolidinediones has protective effects against hypertensive vascular injury and hypertrophy(7;8). Interestingly, treatment with PPAR-γ agonists, such as pioglitazone and rosiglitazone, can reduce aortic aneurysmal formation in mice(16;17). Therefore, there would be obvious potential benefits of vein graft protection induced by PPAR-γ activation with pioglitazone. In the present study, the enhancement of PPAR-γ expression was clearly observed in the intimal side of vein grafts, possibly due to a direct pharmacologic effect of pioglitazone carried through the bloodstream. Although pioglitazone has reported some clinical drawbacks, such as causing fluid retention and congestive heart failure, we firmly believe that pioglitazone has numerous advantages as long as the dose is optimal. In accordance with the previous report(9;10), we administered the same dose (3 mg/kg/day) of pioglitazone in this study without those adverse effects.

Circumferential pressure stretch of the vein graft is believed to contribute to the degradation of its endothelium. The average circumferential pressure stress in the vein graft can be increased by 140 times or higher compared with that in a native vein(2). Liu et al. (18) reported that vein grafts exposed to arterial pressure lost up to 60% of the endothelial cells and SMCs within 12 hours after surgery. Previously, our study also demonstrated that the endothelium of vein grafts was desquamated and the media disrupted at 24 hours after surgery(11). In the present study, the endothelial desquamation of vein graft, which was evaluated with Evan's blue staining, was significantly reduced by pioglitazone treatment. The arterial pressure stretch with endothelial damage in vein grafts induced a generation of reactive oxygen species (ROS) and an activation of early inflammatory(19). ROS were produced by all vascular cells, including endothelial cells, SMCs and infiltrated leukocytes. There was a numerous superoxide in vein grafts as we observed with dihydroethidium staining in the present study. Pioglitazone treatment effectively attenuated the superoxide generation. Moreover, both MPO activity as a maker of leukocytic activity, and lipid peroxidation as an indicator of oxidative lipid damage in vein grafts were also significantly suppressed by pioglitazone. Because oxidative stress including lipid peroxidation has been implicated in the initiation and progression of vein graft failure(13), those results could suggest the subsequent vein graft failure.

The activities of extracellular matrix (ECM)-degrading MMPs, which are important regulators of vein graft architecture(15), have a strong mechanistic link to ROS generation in vascular aneurysm formation(20). In addition, the upregulation of MMPs, especially MMP-2 and MMP-9, have been shown to induce SMC proliferation and migration leading to neointimal formation in vein grafts(15). The present study demonstrated that pioglitazone treatment downregulated MMP-2 and MMP-9 expressions and activities at 7 days after surgery. Although the MMPs activities are regulated by TIMPs(15), pioglitazone did not affect significant changes of TIMP-1 and TIMP-2 mRNA expression in vein grafts. These results suggest that pioglitazone can regulate the ECM integrity by suppressing MMP-2 and MMP-9, eventually resulting in the regulation of vein graft remodeling such as dilation and neointimal formation.

Poiseuille's law defines that increasing diameter in vessels induces a decrease of flow velocity with low shear stress. In fact, the present study has shown distinct circumferential dilation with low flow velocity after vein grafting. Pioglitazone treatment significantly suppressed the graft dilation, and maintained the graft flow velocity possibly with good shear stress. In addition, both high circumferential pressure stress and low shear stress are the main triggers for the development of neointimal hyperplasia in vein grafts, associated with subsequent

SMC proliferation(2;21). The present study has shown that pioglitaone treatment significantly suppressed the SMC proliferation and the neointimal hyperplasia at the aneurysmal site and the anastomosis site of vein grafts. These results suggest that pioglitazone could preserve the vein graft integrity and regulate its remodeling in the chronic phase. Although the mechanism of preservation of the vein graft integrity was not able to elucidate in the present study, Duan et al. (22) has currently reviewed the effects of PPAR-γ in the vasculature, and described that PPAR-γ activation inhibits SMC proliferation and migration, as well as endothelial inflammation. They have introduced several mechanisms of the inhibitory effect on SMC proliferation in the review, and one of the interesting mechanisms is that the suppression of ROS production via PPAR-γ activation can contribute the inhibition of SMC proliferation and migration through an inhibition on telomerase activity and/or MMP-9 activity. Further studies should be conducted to elucidate the mechanism of preservation of the vein graft integrity by pioglitazone.

This study presents some limitations that deserve attention. The first limitation is that there are some discrepancy between the rat aortic interposition model and clinical coronary arterial bypass grafting (CABG), with regards to species, heterotopic grafting, grafting technique and physiological hemodynamics. The second limitation is that the present study has not established the dose-response

relation of pioglitazone to a preservation of the vein graft integrity. Although other doses or dosing regimens of pioglitazone have not been applied in the present study, previous studies demonstrated that plasma pioglitazone concentration in the animals treated at the dose of 3 mg/kg/day are within the range achieved in humans after oral administration of its clinical dosage (30 mg/day)(9;23). The current studies have shown that pioglitazone could prevents reactive hypoglycemia in patients with an impaired glucose tolerance(24), and that pioglitazone could improve an coronary endothelial function without any changes of blood glucose, insulin or HbA1c levels in non-diabetic patients with coronary artery disease(25). Although any adverse effects of pioglitazone was not observed in the present study, the third limitation was that the use of pioglitazone in the CABG patients, particularly those with congestive heart failure and cardiomyopathies, might be the high incidence of fluid retention associated with its use. Further studies of pioglitazone for the vein graft integrity preservation, including clinical trials, should be needed.

In conclusions, pioglitazone treatment prevents graft dilation and neointimal hyperplasia, which leads to preservation of the vein graft integrity in rat aortic interposition model. Pioglitazone has a potential use for post-CABG regimens by providing an improvement of the long-term patency of the vein grafts. Further investigation will be required to determine if this will hold true in a clinical setting.

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Figure legends

Figure 1. Effects of pioglitazone on endothelial desquamation and oxidative stress in vein grafts at 24 hours. (**A**) *In vivo* Evans blue staining of vein grafts. Arrows, anastomotic sites. Bar=5 mm. (**B**) *In situ* detection of superoxide generation (red fluorescence). Bar=100 μm. (**C**) Malondialdehyde (MDA) levels as an indicator of lipid peroxidation. (**D**) Myeloperoxidase (MPO) activity. ΔAbs, change in absorbance. **P*<0.05. All data are expressed as mean±SEM for n=6 per group.

Figure 2. Protease activity and mRNA expression in vein grafts at 7 days (**A**) mRNA expression of MMP-2, MMP-9, TIMP-1 and TIMP-2. (**B**) Gelatinolytic activities of MMP-2 and MMP-9 by gelatin zymography, and their densitometric analysis. **P*<0.05. Pio, pioglitazone. NS, not significant. All data are expressed as mean±SEM for n=6 per group.

Figure 3. Graft flow velocity in vein grafts. (**A**) Blood flow waveforms in proximal aorta, vein graft, and distal Ao at 6 months. Ao, aorta. (**B**) Graft flow velocity ratio at 8 weeks and 6 months. **P*<0.05 by ANOVA. All data are expressed as mean±SEM for n=6 per group.

Figure 4. (**A**) Graft dilation ratio at 8 weeks and 6 months. *P<0.05. All data are expressed as mean±SEM for n=6 per group. (**B**) Immunofluorescent staining of PPAR-γ in vein grafts at 8 weeks. Intracellular expression of PPAR-γ is identified as the green emission. Cell nuclei are identified by the blue emission. Bar=200 μm.

Figure 5. Histological findings in vein grafts at 6 months (**A**) Elastin von Gieson (EVG) staining and αSMA immunostaining. Bar=500 μm. (**B**) Area of neointimal hyperplasia and αSMA-positive area. (**C**) Neointimal thickness at anastomosis site (HE staining). Bar=200 μm. *P<0.05. All data are expressed as mean±SEM for n=6 per group.

Figure E1. Schematic representation of the present study design. Drug treatment (pioglitzone or vehicle) was provided to rats by gastric gavage once a day beginning 3 days before operation (-3d) and ending 8 weeks after operation (8w). The endpoints of the present study were 24 hours (24h), 7 days (7d), 8 weeks (8w) and 6 months (6m) after operation. Op., operation.

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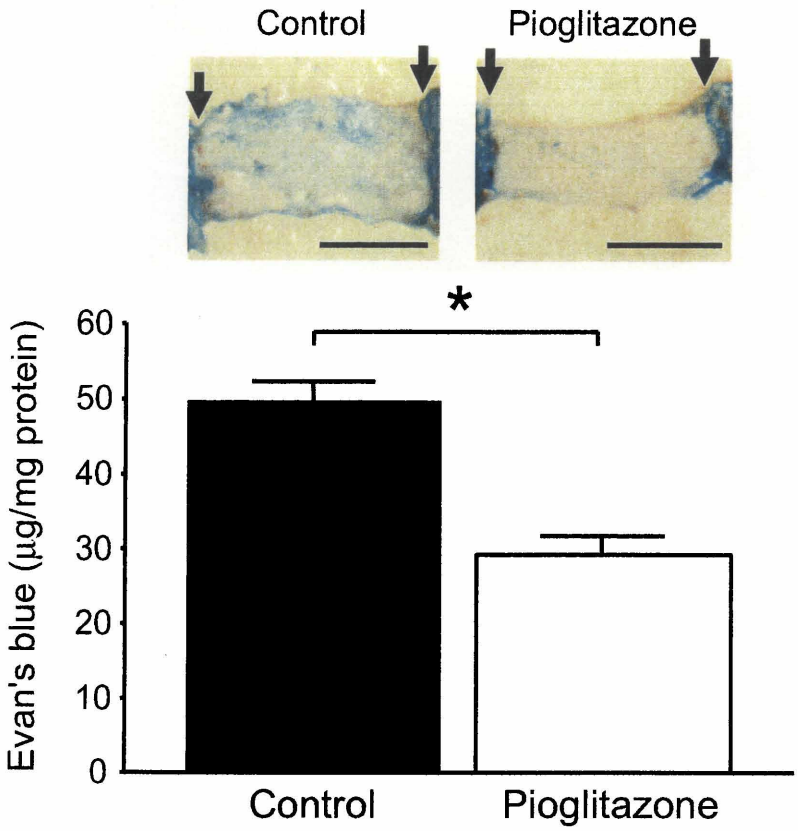
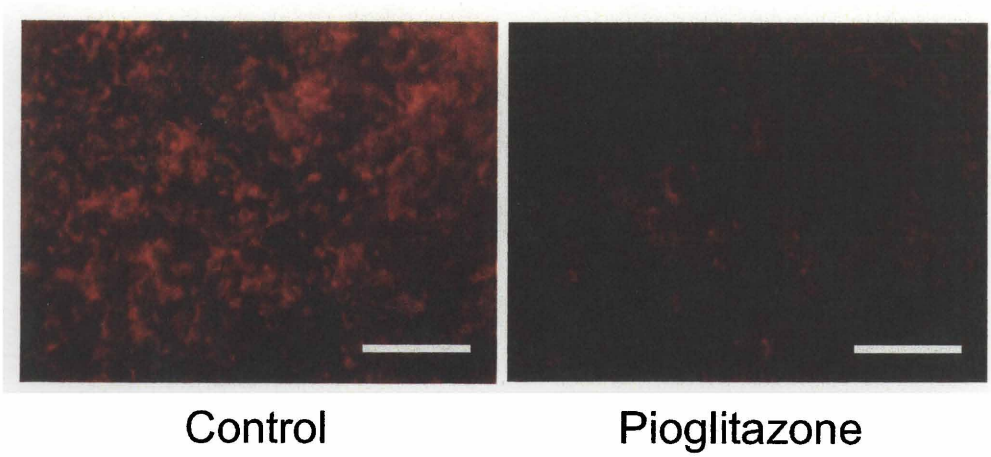
Figure 1

A



B



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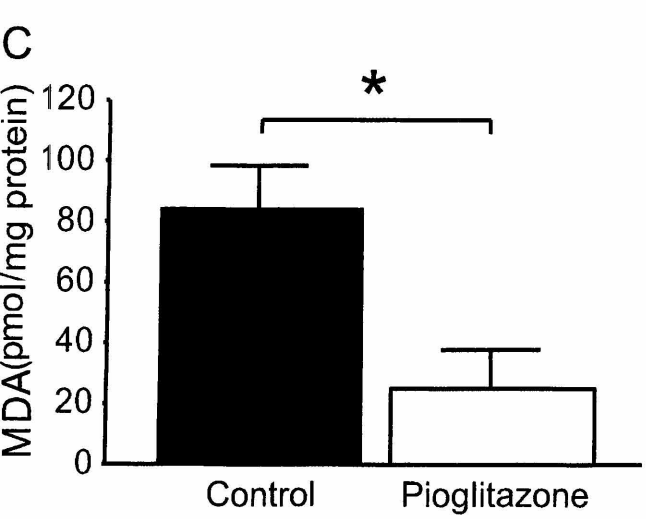

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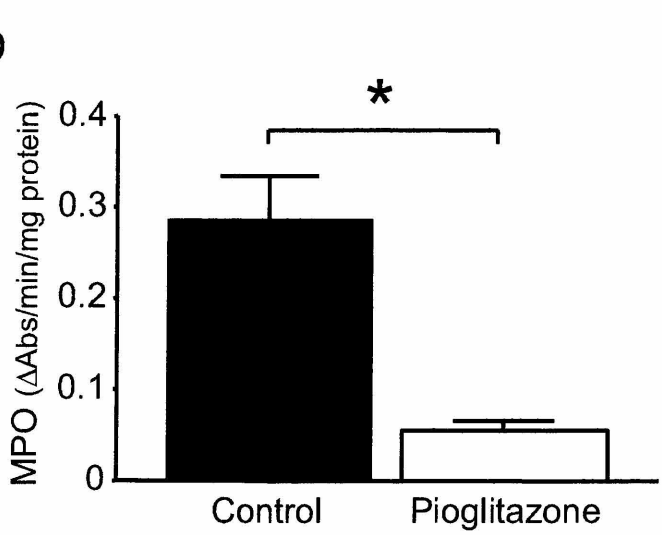
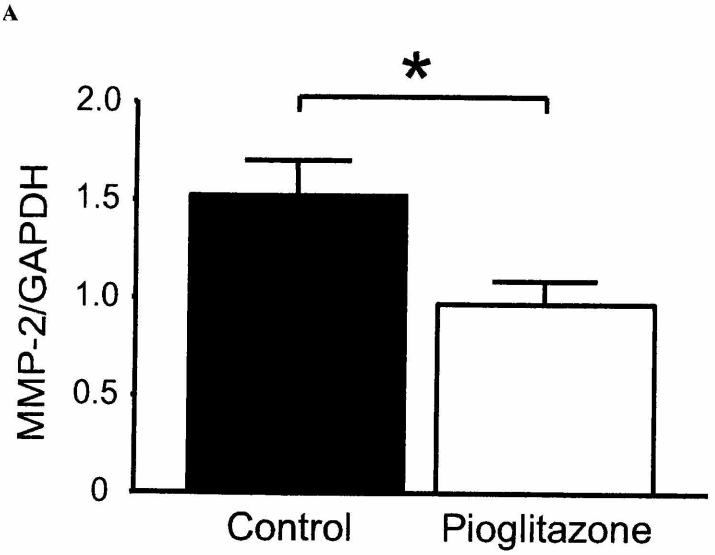
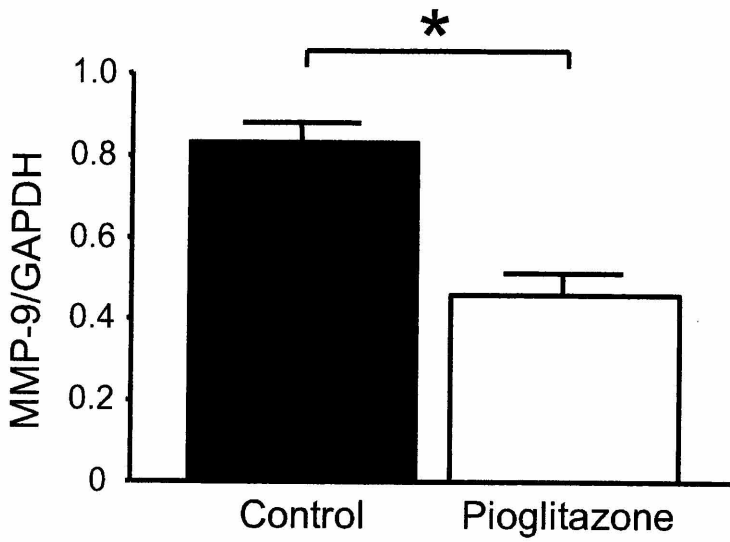
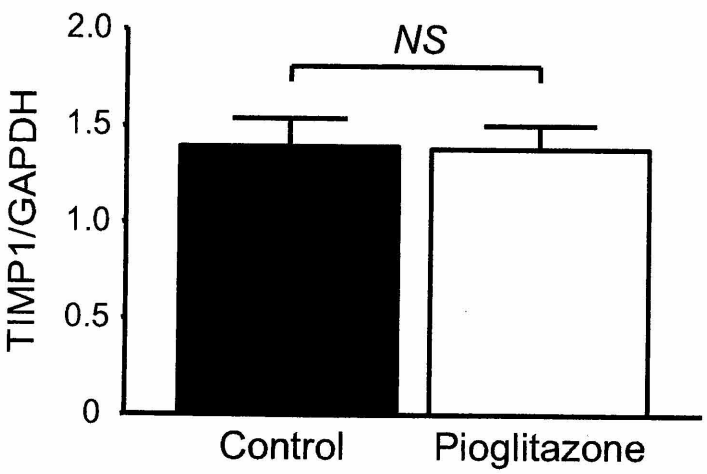


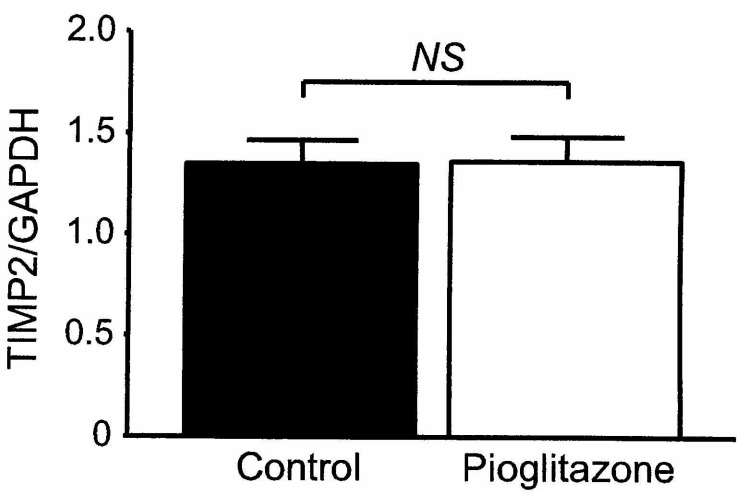
Figure 2

A

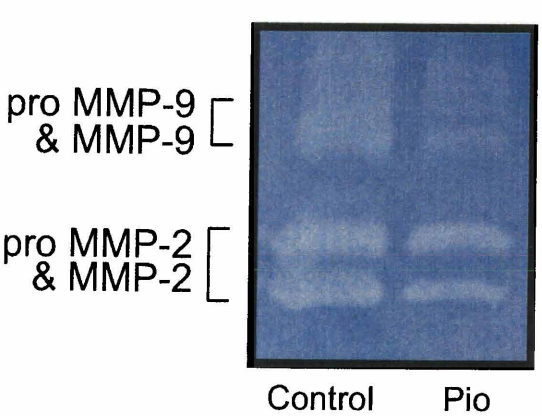

*
2.0
1.5
1.0
0.5
0
MMP-2/GAPDH
Control
Pioglitazone


*
1.0
0.8
0.6
0.4
0.2
0
MMP-9/GAPDH
Control
Pioglitazone


NS
2.0
1.5
1.0
0.5
0
TIMP1/GAPDH
Control
Pioglitazone


NS
2.0
1.5
1.0
0.5
0
TIMP2/GAPDH
Control
Pioglitazone

B


pro MMP-9
& MMP-9
pro MMP-2
& MMP-2
Control
Pio

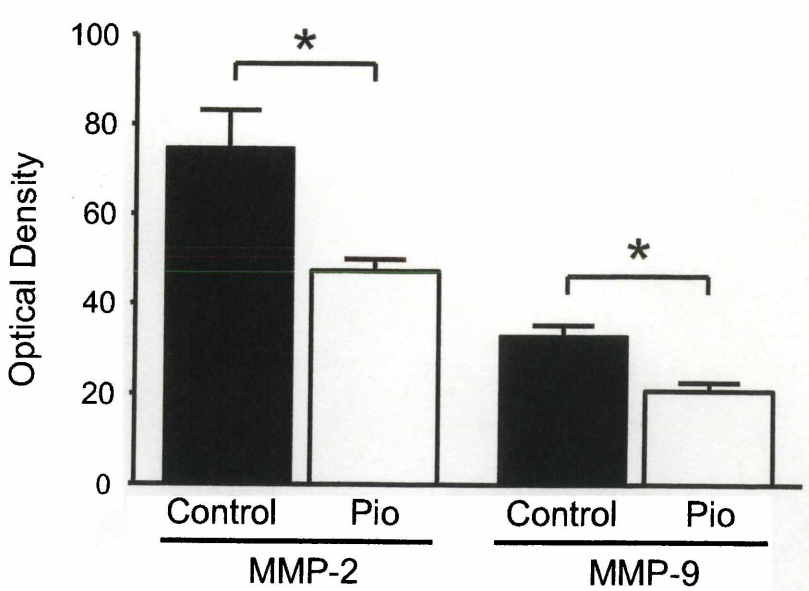
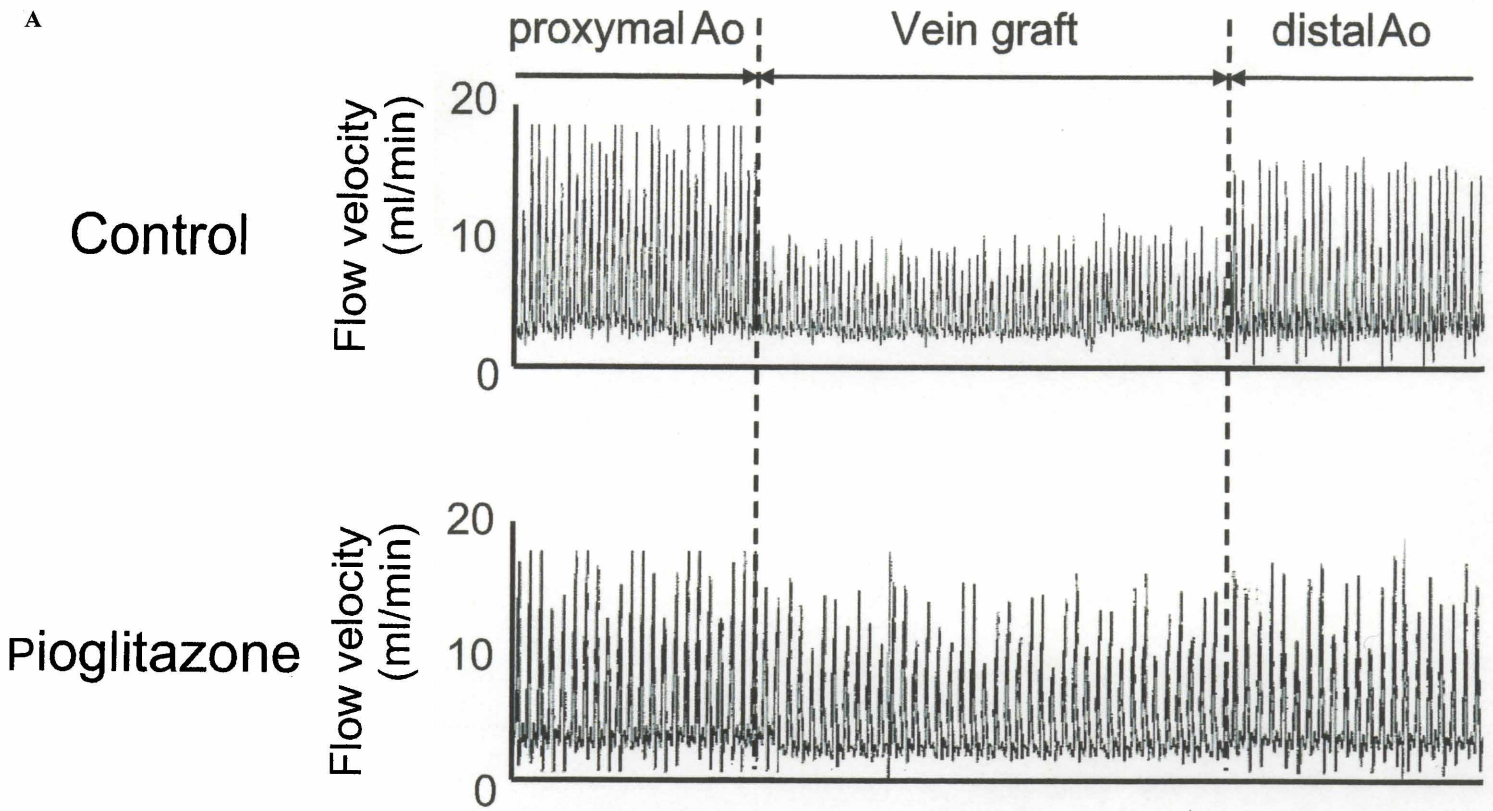

*
*
100
80
60
40
20
0
Optical Density
Control
Pio
Control
Pio
MMP-2
MMP-9

Figure 3

A


proxymal Ao
Vein graft
distal Ao
Control
Flow velocity (ml/min)
20
10
0
Pioglitazone
Flow velocity (ml/min)
20
10
0

B

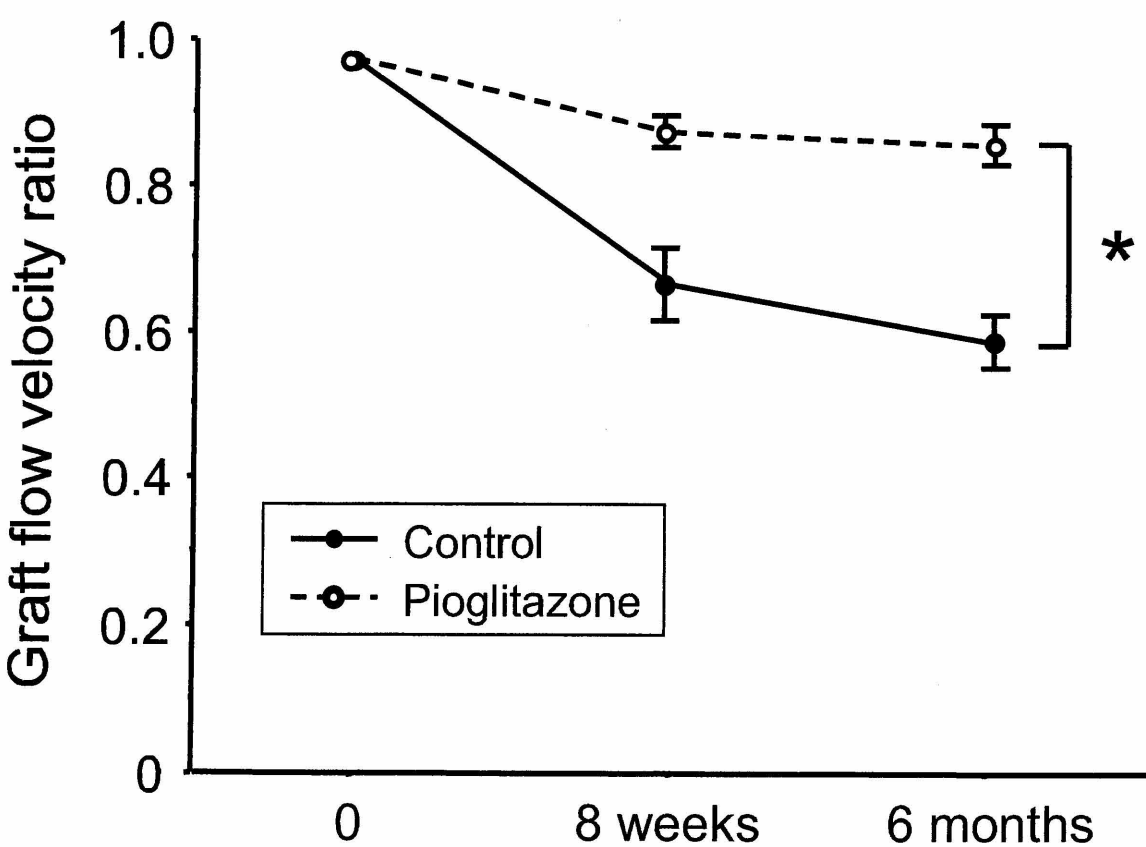
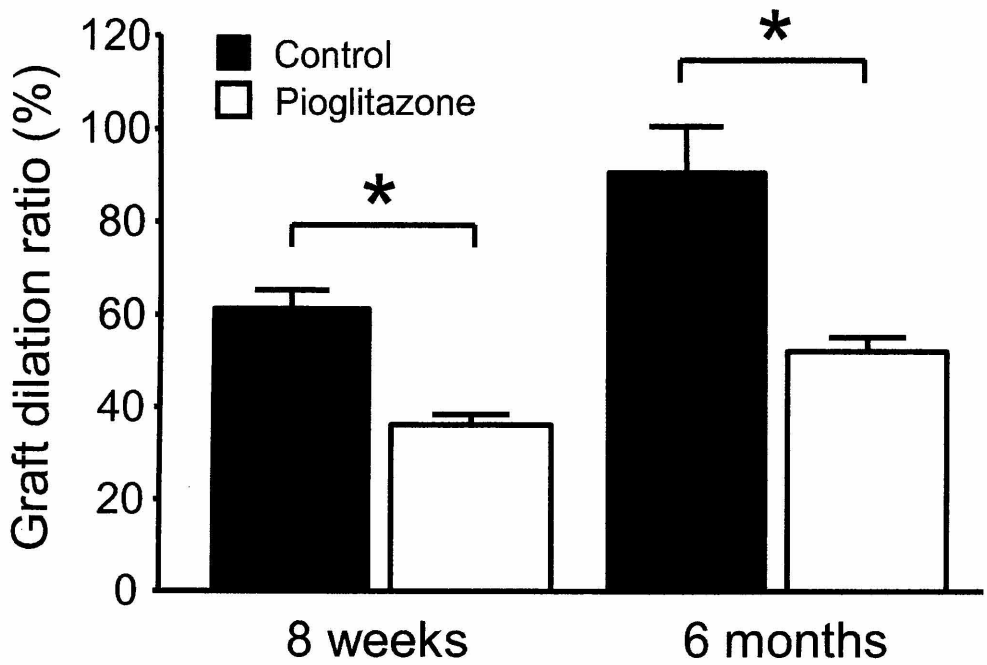

Graft flow velocity ratio
1.0
0.8
0.6
0.4
0.2
0
*
Control
Pioglitazone
0
8 weeks
6 months

Figure 4

A


Graft dilation ratio (%)
120
100
80
60
40
20
0
Control
Pioglitazone
*
*
8 weeks
6 months

B

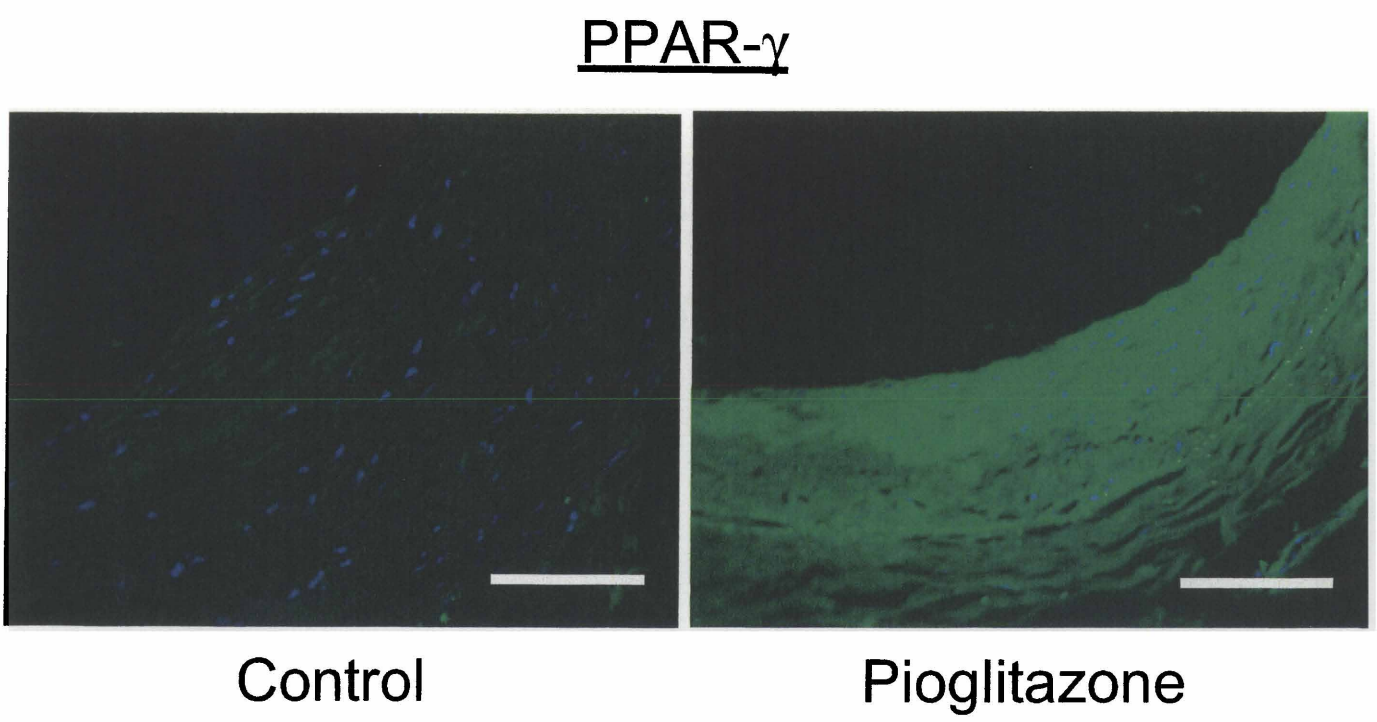

PPAR-γ
Control
Pioglitazone

Figure 5

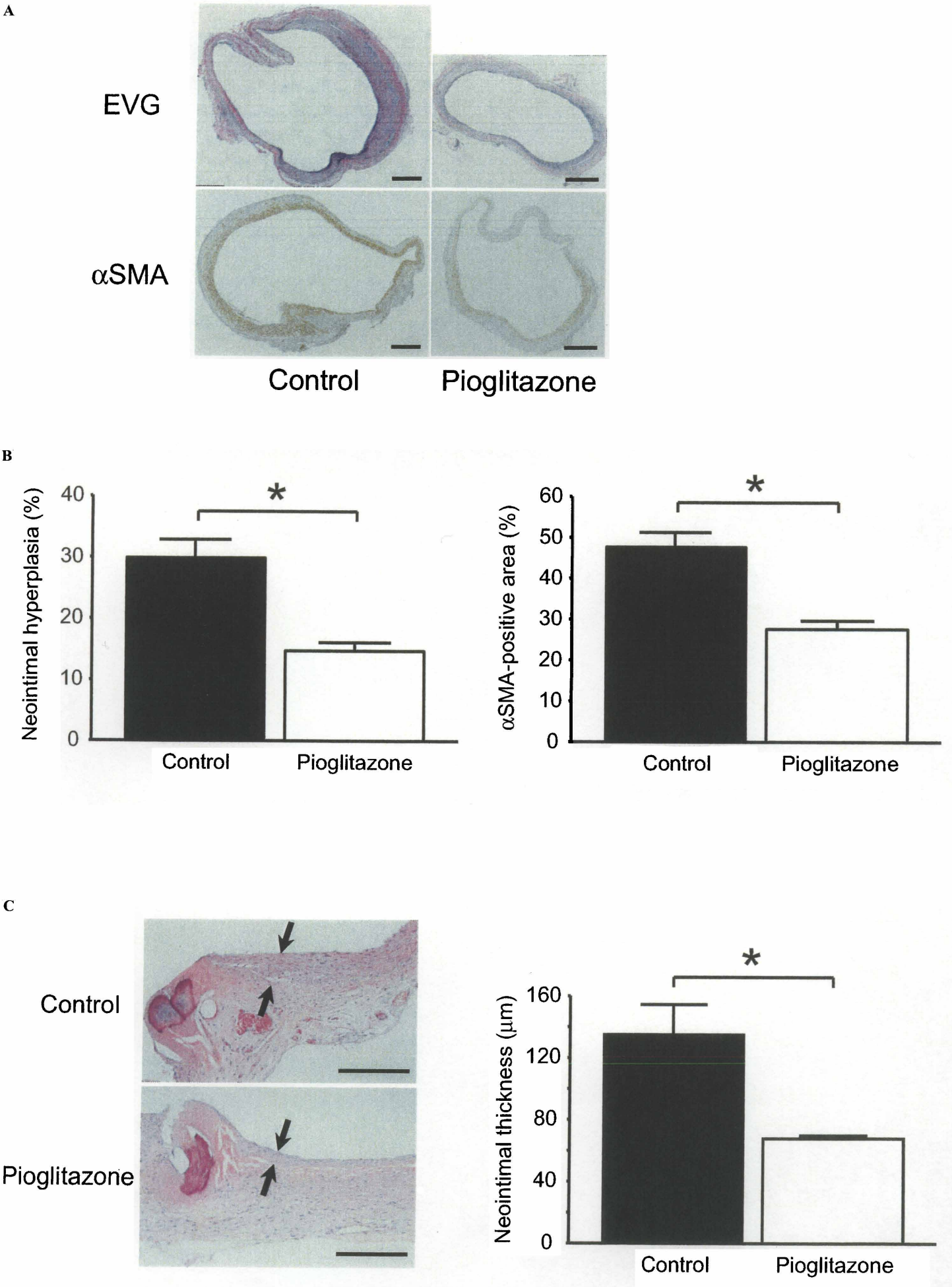
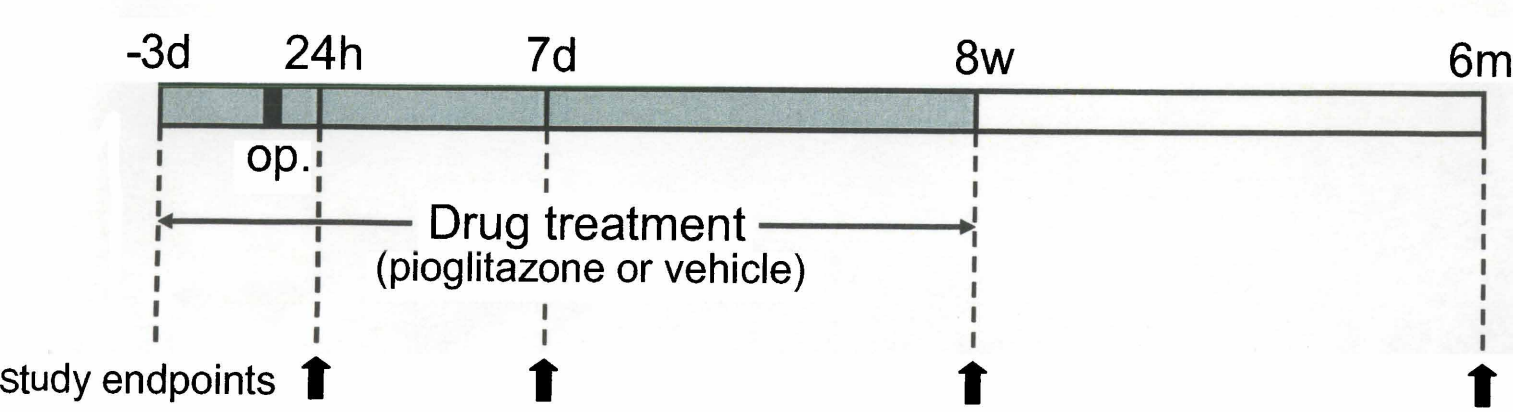

A
EVG
αSMA
Control
Pioglitazone
B
Neointimal hyperplasia (%)
40
30
20
10
0
*
Control
Pioglitazone
αSMA-positive area (%)
60
50
40
30
20
10
0
*
Control
Pioglitazone
C
Control
Pioglitazone
Neointimal thickness (μm)
160
120
80
40
0
*
Control
Pioglitazone

Figure E1


-3d
24h
7d
8w
6m
op.
Drug treatment
(pioglitazone or vehicle)
Study endpoints