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Pioglitazone Attenuates Injury-Induced Neointima Formation in Mouse Femoral Artery Partially through the Activation of AMP-Activated Protein Kinase

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Abstract

Background/Aims—Pioglitazone (PIO), an antidiabetic drug, has been shown to attenuate vascular smooth muscle cell (VSMC) proliferation, which is a major event in atherosclerosis and restenosis after angioplasty. Till date, the likely contributory role of AMP-activated protein kinase (AMPK) toward PIO inhibition of VSMC proliferation has not been examined in vivo. This study is aimed at determining whether pharmacological inhibition of AMPK would prevent the inhibitory effect of PIO on neointima formation in a mouse model of arterial injury.

Methods—Male C57BL/6J mice were subjected to femoral artery injury using guide wire. PIO (20 mg/kg/day) was administered orally 1 day before surgery and for 3 weeks until sacrifice in the absence or presence of compound C (an AMPK inhibitor). Injured femoral arteries were used for morphometric analysis of neointima formation. Aortic tissue lysates were used for immunoblot analysis of phosphorylated AMPK.

Results—PIO treatment resulted in a significant decrease in intima-to-media ratio by ~50.3% ($p < 0.05$, compared with vehicle control; $n = 6$), which was accompanied by enhanced phosphorylation of AMPK by ~85% in the vessel wall. Compound C treatment led to a marked reduction in PIO-mediated inhibition of neointima formation.

Conclusion—PIO attenuates injury-induced neo-intima formation, in part, through the activation of AMPK.

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Disclosure Statement

The authors declare no conflicts of interest.

Keywords

Arterial injury; Neointima formation; Pioglitazone; AMP-activated protein kinase

Introduction

Atherosclerosis and restenosis after angioplasty are characterized by the exaggerated proliferation of vascular smooth muscle cells (VSMCs) [1–3]. It is well accepted that thiazolidinediones (TZDs; e.g., pioglitazone [PIO] and rosiglitazone) exhibit vasoprotective effects through the inhibition of neointima formation/VSMC proliferation [4–6] beyond their role as insulin sensitizers in patients with type 2 diabetes [7]. For instance, TZDs attenuate neointima formation after coronary stenting in patients with type 2 diabetes [8–11] and in nondiabetic patients with metabolic syndrome [12]. In addition, TZDs prevent neointima formation after arterial injury in experimental animal models including normal rats [13–15], insulin-resistant Zucker fatty rats [16], stroke-prone spontaneously hypertensive rats [4], and hypercholesterolemic rabbits [17]. Furthermore, TZD treatment diminishes mitogen-induced VSMC proliferation in vitro [18–20]. However, the molecular mechanisms by which TZDs inhibit neointima formation are not fully understood.

Although TZDs are considered classical agonists for peroxisome proliferator-activated receptor- γ (PPAR γ), studies by several investigators suggest that TZD inhibition of VSMC proliferation occurs by PPAR γ -dependent [21, 22] and PPAR γ -independent mechanisms [20, 23]. Of importance, in vivo transfer of PPAR γ gene inhibits smooth muscle proliferation and reduces neointima formation after balloon injury in rat carotid artery [21]. In contrast, treatment with TZD (e.g., rosiglitazone) inhibits injury-induced neointima formation in rat carotid artery through the activation of glycogen synthase kinase-3 β independent of PPAR γ [23]. Our recent study demonstrates that TZD (e.g., PIO) inhibits VSMC proliferation under in vitro conditions, in part, through the activation of AMP-activated protein kinase (AMPK) [20]. The objective of the present study is to determine whether the pharmacological inhibition of AMPK would prevent the inhibitory effect of PIO on neo-intima formation in a mouse model of arterial injury.

Materials and Methods

Materials

PIO and compound C were purchased from Cayman Chemical Company (Ann Arbor, MI, USA). VITROS DT-slides were purchased from Ortho Clinical Diagnostics (Rochester, NY, USA). All surgical tools were purchased from Roboz Surgical Instrument (Gaithersburg, MD). The primary antibodies for phospho-AMPK α ^{Thr172} (2535) and pan-AMPK α (2532) were purchased from Cell Signaling Technology (Danvers, MA, USA). The primary antibody for SM α -actin (ab5694) was purchased from Abcam (Cambridge, MA, USA). Goat anti-Rabbit IgG secondary antibody Alexa Fluor[®] 594 conjugate (A-11037) and prolong gold anti-fade mountant with DAPI (P36931) were purchased from Life Technologies (Carlsbad, CA, USA). All other chemicals were from Fisher Scientific (Fair Lawn, NJ, USA) or Sigma Chemical (St. Louis, MO, USA).

Animals

All animal experiments were performed in accordance with the Charlie Norwood Veterans Affairs Medical Center Institutional Animal Care and Use Committee guidelines and were approved by the committee. Male C57BL/6J mice (12 weeks of age, Jackson Laboratories, Bar Harbor, ME, USA) were maintained in a room at a controlled temperature of 23°C with a 12: 12-h dark-light cycle and fed regular chow. For all surgical procedures, mice were anesthetized with isoflurane via inhalation (1–4% in oxygen).

Measurement of Blood Glucose, Plasma Triglycerides, and Total Cholesterol

On the day of sacrifice, mice were allowed to fast overnight for 14 h. Blood samples were then collected from the tail vein for determining blood glucose using Contour glucometer (Bayer Health-Care, Mishawaka, IN, USA). In addition, blood samples were drawn from the retro-orbital plexus of mice using heparinized microhematocrit tubes. Blood samples were centrifuged at 800 *g* for 10 min at 4°C. Plasma was carefully transferred to new tubes and kept at –80°C until further analysis. Plasma triglycerides and total cholesterol were measured by an enzymatic colorimetric method using VITROS DT60 II Chemistry System from Ortho-Clinical Diagnostics, Inc. (Rochester, NY, USA).

Femoral Artery Guidewire Injury and Treatments

Guidewire injury was performed in C57BL/6J mice as previously described [24, 25]. The injury was induced by inserting a straight spring wire (0.38 mm in diameter; Cook, Bloomington, IN, USA) from an exposed muscular branch artery into the left femoral artery for >5 mm toward the iliac artery. The wire was left in place for 1 min to denude and dilate the femoral artery. Then, the wire was removed and the proximal portion of the muscular branch artery was secured with silk sutures and blood flow was restored in the left femoral artery. The right femoral artery was not subjected to guidewire injury and therefore served as sham-operated control. PIO was prepared as a suspension in 0.5% carboxymethyl cellulose (CMC). PIO (20 mg/kg/day) or vehicle (0.5% CMC) treatments were initiated 1 day before surgery and were administered daily by oral gavage for 3 weeks until sacrifice. In select experiments, compound C (20 mg/kg, every other day) dissolved in DMSO or vehicle (DMSO) treatment was initiated 1 day before surgery and was administered every other day by intraperitoneal injection for 3 weeks until sacrifice [26]. Three weeks after arterial injury, mice were euthanized with isoflurane via inhalation and perfused at a constant pressure via the left ventricle with 0.9% NaCl solution, followed by perfusion fixation in 4% paraformaldehyde (PFA) in PBS, pH 7.4. The femoral artery was carefully excised, further fixed in 4% PFA overnight at 4°C, then washed in deionized water, and kept in 30% sucrose for 24 h at 4°C before cryosection.

Morphometric Analysis of Femoral Artery

Cross-sections (5 µm) of femoral artery were stained with haematoxylin and eosin (H&E) for examination of overall morphology or elastica van Gieson's (EVG) to depict the internal elastic lamina (IEL) and the external elastic lamina (EEL), and the images were captured by AxioCam high-resolution camera attached to an Observer Z1 microscope (Carl Zeiss Microimaging, Inc., Thornwood, NY, USA). Sections were analyzed for intima-to-media

ratios using image analysis software (Axiovision, release 4.8.2 SP3). The intimal area was determined by subtraction of the luminal area from the IEL area (i.e., intimal area = IEL area – luminal area). The medial area was determined by subtraction of the IEL area from the EEL area (i.e., medial area = EEL area – IEL area) [25]. The cryosectioning, H&E staining, and EVG staining of the injured femoral arteries (from control and treated conditions) were performed in a blinded manner as described earlier [27].

Immunofluorescence Analysis of Femoral Artery

Cross-sections (5 μ m) of femoral artery were fixed in 4% PFA and then blocked by incubation with 5% normal goat serum/0.3% Triton X-100 in PBS for 1 h. Subsequently, sections were exposed to the primary antibody specific for SM α -actin (1:200) for 1 h at room temperature, washed 3 times with PBS, and incubated with the secondary antibody (goat anti-rabbit IgG conjugated to Alexa fluor 594) for 1 h at room temperature. Sections were mounted using prolong antifade with DAPI and the images were captured using a confocal microscope (Zeiss, Thornwood, NY, USA) as described [25].

Extraction and Quantification of Proteins in Aortic Tissues

Mice were euthanized with isoflurane via inhalation and perfused at a constant pressure via the left ventricle with 0.9% NaCl solution. The aortic tissues were then carefully excised and cleared from the fat and connective tissue, rinsed in ice-cold fresh phosphate-buffered saline, blotted to dryness, snap-frozen in liquid nitrogen, and stored at -80°C until analysis. Aortic tissues were thawed in 100 μ L of ice-cold RIPA lysis buffer containing protease and phosphatase inhibitors (Thermo Scientific, Rockford, IL, USA) for 2 min, followed by homogenization using TissueLyser LT (Qiagen, Valencia, CA, USA) at a setting of 50 Hz for 5 min with samples being placed on ice intermittently. The homogenates were incubated at 4°C for 1 h on a rotator and centrifuged at 1,000 g for 10 min at 4°C to remove tissue debris. The supernatants were mixed with $2\times$ Laemmli sample buffer at a ratio of 1:1 followed by heating at 67.5°C for 10 min. Proteins were quantified using Bio-Rad DC assay kit (Bio-Rad, Hercules, CA, USA).

Immunoblot Analysis

Immunoblot analysis was performed as described [20]. Mouse aortic tissues (20 μ g protein per lane) were subjected to electrophoresis using pre cast 4–12% Nu Page mini-gels (Life Technologies). The resolved proteins were then transferred to PVDF membranes (EMD Millipore, Billerica, MA, USA). Subsequently, the membranes were blocked in 5% nonfat milk and probed with the respective primary antibodies. The immunoreactivity was detected using HRP-conjugated goat anti-rabbit secondary antibody (7074; Cell Signaling) followed by enhanced chemiluminescence (ECL; Thermo Scientific, Wilmington, DE, USA). The immunoblots for ECL detection of AMPK and its phosphorylated form were run in parallel. The protein bands were quantified by densitometric analysis using ImageJ.

Statistical Analysis

Results are expressed as the means $\pm$ SEM. Statistical analyses of the data were performed using one-way analysis of variance followed by Bonferroni t test for data involving more

than 2 groups, or unpaired 2-tailed *t* test for data involving 2 groups only. Values of $p < 0.05$ were considered statistically significant.

Results

PIO Treatment Does Not Affect Fasting Blood Glucose Levels, Plasma Triglycerides, or Plasma Total Cholesterol in CJ57BL/6J Mice

As shown in Table 1, PIO treatment for 3 weeks did not result in significant changes in the levels of fasting blood glucose, plasma triglycerides, and plasma total cholesterol, compared with vehicle-treated control mice. In addition, there was no significant difference in body weight during the 3-week treatment period with PIO, compared with control mice (data not shown).

PIO Inhibits Neointima Formation in CJ57BL/6J Mice

Previous studies have shown that TZDs attenuate neo-intima formation after coronary stenting in diabetic and nondiabetic patients [8–12]. In addition, TZDs prevent neointima formation after arterial injury in different experimental animal models [4, 13–17]. In this study, we examined the effects of PIO on neointima formation in CJ57BL/6J mice. As shown in Figure 1a–c, femoral artery injury in CJ57BL/6J mice resulted in extensive neointima formation. In addition, most cells in the intimal area were positive for SM α -actin, a marker for VSMCs (Fig. 1d). PIO treatment led to significant decreases in the intimal area and neointima/media ratio by ~48.1 and ~50.3%, respectively (Fig. 1a–d). The medial area was not significantly different between all groups (data not shown).

PIO Enhances the Phosphorylation of AMPK in Mouse Aorta

Next, we examined the extent to which PIO regulates AMPK activation in the vessel wall. As shown in Figure 2, PIO administration for 3 weeks led to enhanced phosphorylation of AMPK in the aorta by ~85%.

Compound C, an AMPK Inhibitor, Reduces PIO-Mediated Inhibition of Neointima Formation

To examine the likely contributory role of AMPK toward PIO inhibition of neointima formation, CJ57BL/6J mice were treated with compound C (an AMPK inhibitor) during the oral administration of PIO. As shown in Figure 3a–c, Compound C by itself led a marked increase in neointima formation by ~32.9%. This increase in neointima formation did not reach statistical significance ($p = 0.058$), owing to an *n* value of 6 used in the present study. Notably, in the presence of compound C, PIO treatment led to significant decreases in intimal area and neointima/media ratio by ~29.5 and ~29%, respectively. However, in the absence of compound C, PIO treatment alone resulted in significant decreases in intimal area and neointima/media ratio by ~49.1 and ~55.7%, respectively. Thus, compound C treatment led to a marked reduction in PIO-mediated inhibition of neointima formation in the injured femoral artery.

Discussion

The present findings reveal that PIO (an antidiabetic drug) inhibits neointima formation after arterial injury, in part, through the activation of AMPK. Previously, several pharmacological agents and adipokines have been shown to inhibit neointima formation via AMPK-dependent mechanism. For instance, metformin (an antidiabetic drug) [28], 5-aminoimidazole-4-carboxamide-1-beta-d-ribofuranoside (an AMPK activator) [29], and adiponectin [30] inhibit injury-induced neointima formation through the AMPK activation. As a corollary, genetic ablation of AMPK enhances neointima formation in vivo [31, 32]. In addition, a previous study [33] and the present findings show that treatment with compound C (an AMPK inhibitor) alone tends to induce an increase in the intima/media ratio in the injured arteries of control mice. Of importance, compound C partially counters the PIO-mediated decrease in neointima formation in the injured femoral artery. Together, antidiabetic drugs (e.g., PIO and metformin) and adiponectin have the potential to attenuate restenosis after angioplasty by targeting AMPK in vulnerable vessels.

From a mechanistic perspective, PIO activation of AMPK in the vessel wall may occur through indirect and direct effects. The indirect effect is attributed to its insulin-sensitizing action via PPAR γ activation in the adipose tissue. In this regard, PIO-mediated PPAR γ activation in adipocytes results in enhanced synthesis and release of adiponectin into the systemic circulation [34]. An increase in the circulating concentration of adiponectin may thus activate AMPK in VSMCs [35, 36]. Our recent in vitro study demonstrates that PIO activates AMPK through its direct effect in VSMCs independent of PPAR γ activation [20]. This study shows that PIO activates AMPK in the aorta under in vivo conditions. Notably, PIO also enhances the phosphorylation of AMPK in rabbit vein grafts [37]. Thus, PIO inhibition of neointima formation may occur indirectly through adiponectin-mediated AMPK activation in VSMCs and also through its direct effect in VSMCs in vulnerable vessels.

Recently, we have reported that PIO inhibits PDGF-induced VSMC proliferation via AMPK-dependent inhibition of mTOR signaling and AMPK-independent inhibition of ERK signaling [20]. In addition to the well-documented role of AMPK toward the inhibition of mTOR/ p70S6K signaling leading to cell cycle arrest [38], several other mechanisms have been proposed to explain the inhibitory effect of AMPK on VSMC proliferation. For instance, AMPK upregulates cell cycle inhibitors (p21^{Cip1} and p27^{Kip1}) that negatively regulate the cell cycle [39]. In addition, AMPK activation results in the inhibition of many biosynthetic pathways (e.g., protein synthesis, fatty acid synthesis, and cholesterol synthesis), which provide adequate macromolecules before cells can commit to mitotic division [40]. It is noteworthy that AMPK activation is associated with other salutary effects in the vessel wall in addition to the inhibition of VSMC proliferation. For instance, AMPK activators inhibit VSMC migration [41], preserve endothelial cell function [42, 43], exhibit anti-inflammatory effects [44], and exhibit endothelial cell-independent vasorelaxant effects [45, 46], which subserve their role as viable pharmacological tools to ameliorate vascular dysfunction.

A limitation of this study was that the size of the injured femoral artery was very small and so we could not obtain sufficient amounts of tissue for western blot analysis to quantify AMPK phosphorylation state. We therefore used aorta (a large vessel) to quantify PIO-mediated increase in arterial AMPK phosphorylation. To determine the contributory role of AMPK toward PIO inhibition of neointima formation in mouse femoral artery, we used an ATP-competitive inhibitor of AMPK, compound C [47]. Of importance, compound C counters the inhibition of neointima formation by PIO (present study), an adipokine (e.g., omentin) [33], and a calcium channel blocker (e.g., nifedipine) [48]. Although these findings with compound C suggest a potential role for activated AMPK to inhibit neointima formation, compound C has also been shown to exhibit AMPK-independent effects [49]. While perivascular delivery of compound C attenuates neointima formation in balloon-injured rat carotid artery via AMPK-independent mechanism [49], intra-peritoneal administration of compound C tends to increase neointima formation in guidewire-injured mouse femoral artery (Uemura et al. [33]; and present study). Since compound C treatment alone tends to increase neo-intima formation likely through proliferative signaling pathways independent of AMPK, this may also play a role in countering PIO-mediated inhibition of neointima formation. Together, AMPK-dependent and AMPK-independent effects of compound C toward altered VSMC phenotype may be attributable to several factors, including differences in the species, route of administration/delivery, drug dose/concentration, and vascular beds. Future studies should determine the effects of PIO on injury-induced neointima formation in mice that are deficient in VSMC-specific AMPK α .

A recent meta-analysis of relevant randomized clinical trials suggests that TZD therapy is an effective strategy to prevent instent restenosis in both diabetic and nondiabetic patients undergoing coronary stenting [50]. Yet, TZD treatment is associated with several adverse effects, including weight gain, fluid retention, and congestive heart failure, thus raising concerns about their cardiovascular safety [51]. These unfavorable effects are attributed, in part, to systemic activation of PPAR γ [52, 53]. This study reveals that PIO inhibits neointima formation in normal CJ57BL/6J mice without affecting the basal metabolic parameters such as blood glucose levels, triglycerides, and total cholesterol. These findings suggest that PIO inhibits neointima formation via direct effects in the vessel wall (through activation of AMPK) independent of its antidiabetic properties. From a therapeutic standpoint, the local delivery of PIO at the lesion site may provide a realistic approach to limit restenosis after angioplasty without inducing PPAR γ -mediated systemic adverse effects.

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Fig. 1.

Effects of PIO treatment on neointima formation after femoral artery injury in C57BL/6J mice. PIO was administered orally by gavage (20 mg/kg/day) starting a day before femoral artery injury and for 3 weeks until sacrifice. **a** EVG and H&E staining of injured femoral arteries from control and treatment groups; arrows indicate internal and external elastic laminae. **b, c** Morphometric analyses of intimal area and intima/media ratio in the injured femoral arteries; $*p < 0.05$ compared with vehicle control; $n = 6$. **d** Confocal immunofluorescence analysis of smooth muscle (SM) α -actin (red), elastin autofluorescence (laminae, green), nuclei (DAPI, blue), and merged images in the injured femoral arteries. Representative images from control and treatment groups are shown.

Fig. 2.

Effects of PIO treatment on aortic AMPK phosphorylation in CJ57BL/6J mice. PIO was administered orally by gavage (20 mg/ kg/day) starting a day before surgery and for 3 weeks until sacrifice. Aortic tissue lysates were subjected to immunoblot analysis using primary antibodies specific for pAMPK and AMPK. β-Actin was used as a loading control. * $p < 0.05$ compared with vehicle control; $n = 6$.

Fig. 3.

Effects of compound C, an AMPK inhibitor, on PIO-mediated inhibition of neointima formation in C57BL/6J mice. Compound C (Comp C; 20 mg/kg, every other day) or vehicle control was injected intraperitoneally a day before surgery and for 3 weeks until sacrifice. PIO was administered orally by gavage (20 mg/kg/day) starting a day before surgery and for 3 weeks until sacrifice. **a** EVG and H&E staining of injured femoral arteries from the respective treatment groups; arrows indicate internal and external elastic laminae. **b, c** Morphometric analyses of intimal area and intima/media ratio in the injured femoral arteries; * $p < 0.05$ compared with vehicle control; $n = 6$.

Table 1

Effects of PIO treatment on metabolic parameters in C57BL/6J mice

	Control	PIO treatment
Fasting blood glucose, mg/dL	102.6±8.4	106.4±11.9
Plasma triglycerides, mg/dL	56.2±5.3	51.4±3.9
Plasma total cholesterol, mg/dL	105.1±6.8	103±4.9

Blood samples were collected after a 14-h fast on the day of sacrifice.

Data are expressed as means ± SEM.