Targeted Delivery of Carbaprostacyclin to Ischemic Hindlimbs Enhances Adaptive Remodeling of the Microvascular Network

Xiaoboing Liu,* Toya Terry,* Su Pan, Zhongwei Yang, James T. Willerson, Richard A.F. Dixon, Qi Liu

Abstract—Prostacyclin and its stable analogs play an important vascular protective role by promoting angiogenesis, but their role in arteriolar growth is unclear. Here, we examined the effect of prostacyclin stable analog carbaprostacyclin on arteriolar growth in mouse hindlimb ischemia. Using an osmotic-controlled release system to continuously deliver carbaprostacyclin or saline (control) to ischemic mouse hindlimbs for up to 14 days, we found that blood perfusion was significantly better at 7 and 14 days in carbaprostacyclin-treated mice than in saline-treated mice. Microscopic examination of the microvasculature showed more morphological signs of arteriolar formation in carbaprostacyclin- versus saline-treated legs. A double-blind, quantitative microcomputed tomography analysis indicated that carbaprostacyclin-treated legs had markedly increased vascular volume and small-to-medium-sized vessel numbers that correspond to decreased vessel separation. A proteome profiler antibody array demonstrated that carbaprostacyclin-treated mice had significantly higher amounts of acidic fibroblast growth factor and other chemokines. Conditioned media containing those secreted factors promoted smooth muscle cell growth and migration. Additionally, increased acidic fibroblast growth factor protein levels were detected in smooth muscle cells and skeletal myotubes at different time periods after carbaprostacyclin treatment. Furthermore, the selective peroxisome proliferation-activated receptor β/δ antagonist significantly suppressed carbaprostacyclin-induced acidic fibroblast growth factor protein production. Collectively, our data provide the first morphological and molecular evidence that local delivery of carbaprostacyclin promotes vascular growth in hindlimb ischemia, and that peroxisome proliferation-activated receptor β/δ signaling plays a critical role in inducing acidic fibroblast growth factor expression. (Hypertension. 2013;61:1036-1043.) ● Online Data Supplement

Key Words: acidic fibroblast growth factor ■ arterioles ■ carbaprostacyclin ■ hindlimb ischemia ■ peroxisome proliferator-activated receptor β/δ ■ prostacyclin

Critical limb ischemia (CLI) occurs in response to arterial occlusion, which leads to insufficient blood supply to the lower extremity.1,2 Conventional treatments for CLI are less effective when CLI progresses and causes obstruction of arterioles. In such cases, patients may develop untreated claudication, rest pain, and ulcers that can progress to gangrene and other infections requiring amputation of the lower limb.3,4 New therapeutic approaches are needed to promote arteriolar growth, which potentially could reroute nutrient blood to the ischemic region and reduce peripheral resistance distal to the occlusion.

Prostacyclin (PGI2) has several favorable properties that could be used in CLI therapy. As a vasoactive drug, PGI2 is an important mediator of vascular homeostasis5,6 and also inhibits thrombosis and platelet aggregation. Because PGI2 is chemically unstable and has a short circulating half-life of 1 to 2 minutes, stable analogs have been developed for clinical use.7,8 PGI2 and its stable analogs exert their actions by interacting with the surface PGI2 receptor (IP) or nuclear peroxisome proliferator-activated receptor β/δ (PPARβ/δ).9–11 Although PGI2 limits inward vessel remodeling, such as neointima formation and atherosclerosis, little is known about its action on adaptive outward remodeling such as growth of arterioles. Furthermore, we and others have shown the positive effect of PGI2 and its stable analogs on angiogenesis,12,13 in which the PPARβ/δ signaling pathway plays a critical role. However, the specific role of PGI2 in adaptive arteriolar growth is unknown.

In the current study, we created a hindlimb ischemia model to study the vascular protective effect of the stable PGI2 analog and PPARβ/δ agonist carbaprostacyclin (cPGI2). We tested the hypothesis that cPGI2 promotes arteriolar growth in hindlimb ischemia. To achieve the maximal drug effect
within the target tissue, we used an osmotically controlled release system to selectively deliver cPGI2 or saline (control) to the ischemic leg. We used multiple imaging techniques to assess the vascular effects of cPGI2 compared with saline on the microvascular network of ischemic legs. Our results demonstrated that the constant local release of cPGI2 in ischemic hindlimbs promotes vascular remodeling. In addition, we showed that the cPGI2-PPAR β/δ axis is involved in promoting arteriolar growth.

Methods
Detailed methods are available in the online-only Data Supplement.

Results
cPGI2 Treatment Improves Perfusion and Promotes Arteriolar Growth in Hindlimb Ischemia
To examine whether cPGI2 enhances perfusion, we compared the efficacy of local delivery of cPGI2 or saline in restoring blood flow in ischemic hindlimbs. We measured blood perfusion before and after ligation of the femoral artery, 24 hours after treatment, and up to 14 days thereafter. Figure S1 in the online-only Data Supplement shows the representative color-coded images and quantitative raw data analysis of hindlimb perfusion. At 7 days after surgery, laser Doppler-derived data showed that blood perfusion was significantly better in cPGI2-treated mice than in saline-treated mice (58.80±5.74% versus 40.60±3.14%, respectively; *P<0.05; n=5/group; Figure 1A); this finding also held true at 14 days (88.40±8.71% [cPGI2 group] versus 54.60±6.67% [saline group]; *P<0.01; n=5/group; Figure 1A). There were no significant differences (P>0.05) between groups in systolic pressure, mean arterial pressure, heart rate, or tail blood volume before or during the treatment period (Figure S2).

In live mice, we microscopically examined the dynamic changes in the microvascular morphology of the limb region distal to the ligation where cPGI2 was applied. Three days after treatment, more microvascular rearrangement was observed in the cPGI2-treated group than in the saline-treated group (Figure S3). Consistent with our 3-day findings, we noted more distinct structural remodeling at the arteriolar level in the cPGI2 group than in the saline group at 7 days (Figure S4), including tortuosity of arteriolar-to-arteriolar connections (white arrows, Figure S4D versus S4B, respectively), and increased intersecting of adjacent arterioles (blue arrows, Figure S4D versus S4B) in cPGI2-treated legs. Arteriolar networks were identified by their branching out from a large feeder femoral artery and from the saphenous branch of the descending genicular artery.

Because PGI2 and its analogs are short-acting vasodilators,14 we used 2 independent approaches to evaluate whether the different configuration of vascular structures between cPGI2- and saline-treated ischemic legs was a result of cPGI2-induced vasodilation. First, we simultaneously applied cPGI2 to the anterolateral thigh muscle of the left limb and saline to the right limb in the same mouse for 24 hours; ischemia was not induced in either hindlimb. Interestingly, microscopic examination of live mice at 24 hours showed no visible differences in the preexisting microvascular network of cPGI2-treated legs compared with saline-treated contralateral legs (Figure S5A and S5B). Moreover, we did not observe a corkscrew-like pattern of arterioles or well-developed arteriolar connections. In the second approach, we compared the vasculature in the same ischemic anterolateral thigh before and 20 minutes after the constant application of
cPGI2. As seen in the first approach, the acute application of cPGI2 did not enhance the visibility of the vasculature, and the morphology was similar with and without cPGI2 administration (Figure S5C and S5D). Together, our data suggest that arteriolar growth, not vasodilation, accounts for the differences observed in the vascular network in cPGI2-treated ischemic hindlimbs at 3 and 7 days.

**cPGI2 Treatment Positively Affects Remodeling of the Microvascular Network in Ischemic Hindlimbs**

We used high-definition, volumetric, quantitative micro-computed tomography (CT) to assess the overall microvascular geometry of ischemic and contralateral nonischemic legs at 14 days after femoral occlusion and constant local administration of cPGI2 or saline in ischemic legs. Bones were decalcified during sample preparation to eliminate interference during visualization of the microvasculature. We evaluated 4 different morphological properties in a double-blind manner. Supporting the perfusion data presented above, we found that the vascular volume of cPGI2-treated legs was significantly higher than that of saline-treated legs (41.28±2.22 versus 27.11±2.85 mm³; P<0.05; n=5/group; Figure 1B). In addition, cPGI2-treated legs had significantly more blood vessels (0.16±0.014 versus 0.09±0.011/1/mm; P<0.05; n=5/group; Figure 1C) and less vessel separation (distance between vessels, 6.6±0.52 versus 10.1±1.14 mm; P<0.05; n=5/group; Figure 1D) than did saline-treated legs. These findings suggest better development of the vascular system in the cPGI2-treated group than in the saline-treated group. We also found a positive but not significant effect on average connectivity (n=5/group; Figure 1E) in cPGI2 versus saline groups. Similar global morphometric analyses were used to evaluate contralateral nonischemic legs. No significant differences were observed between cPGI2 and saline groups in any of the 4 morphological variables (Figure S6A–S6D).

To further verify the proarteriogenic effect of cPGI2, we generated a quantitative histogram by using micro-CT to illustrate the frequency and distribution of blood vessel size in cPGI2-treated and saline-treated ischemic legs and contralateral nonischemic legs. Compared with saline-treated ischemic legs, cPGI2-treated ischemic legs showed a significant increase in small vessels, with vessel diameter bins ranging from 40 to 60 μm (P<0.05; n=5/group; Figure 1F). These data strongly indicate that cPGI2 improves perfusion, in part, by arteriogenic rather than dilatory effects because dilation would cause an increase in the diameter of all-sized vessels, not just small vessels. Representative micro-CT images of vessel remodeling of cPGI2- and saline-treated limbs showed that vascular remodeling is more prominent in the region of cPGI2 delivery than in the similar anatomic location of saline delivery (Figure 1G). We similarly evaluated contralateral nonischemic legs and found no significant differences in vessel distribution in the cPGI2 and saline groups (Figure S6E). Figure S6F shows representative micro-CT images of the vasculature in contralateral nonischemic limbs of cPGI2- and saline-treated groups. Together, these data indicate that increased vessel formation is an important means by which cPGI2 improves perfusion in ischemic legs.

**Acidic Fibroblast Growth Factor and Other Cytokines Released From cPGI2-Treated Ischemic Hindlimbs Increase Smooth Muscle Cell Growth and Migration**

To help determine how cPGI2 affects arteriolar growth, we performed a proteome profiler array to assess the simultaneous secretion of chemokines and soluble factors from ischemic thigh tissues 3 days after treatment with cPGI2 or saline (Figure S7). Of the 53 factors measured in the array (Figure 2A), acidic fibroblast growth factor (fibroblast growth factor-1, FGF1), insulin-like growth factor binding protein-1, pentraxin-3, and plasminogen activator inhibitor-1 were significantly increased in cPGI2-treated thigh muscles as compared with saline-treated samples (n=3/group; Figure 2B). Collectively, our array results suggest that multiple mediators within the local environment may affect vessel growth.

Because vascular smooth muscle cells (SMCs) are the major cellular component of arterioles, we evaluated the paracrine effect of factors released by cPGI2- and saline-treated ischemic thigh tissue on the proliferation and migration of SMCs. SMCs were cultured for 48 hours in conditioned medium (CM) from cPGI2- or saline-treated ischemic thigh tissues; the number of SMCs was significantly higher after treatment with cPGI2-CM than with saline-CM (Figure 2C). We then used a scratch wound migration assay to determine the effects of CM on SMC migration. Incubation with cPGI2-CM significantly increased SMC migration over that seen with saline-CM (Figure 2D and 2E). Thus, cPGI2 treatment created a favorable microenvironment for SMC growth and migration.

**cPGI2 Induces Expression of FGF1 in Skeletal Myotubes and Vascular SMCs Under Hypoxia**

Because our array showed an increase in FGF1 levels in cPGI2-treated limb tissues and FGF1 seems to promote the growth of arterioles in hindlimb ischemia, we focused our investigation on how cPGI2 regulates FGF1 and on the types of cells that are responsible for increased FGF1 levels. Myofibers are the main component of limb muscles; therefore, we used C2C12 myotubes to study cPGI2-mediated FGF1 expression. To mimic in vivo ischemia, we conducted these experiments in a hypoxic environment (1.5% O₂). At 2 hours after cPGI2 treatment, FGF1 protein levels were similar in cPGI2-treated and untreated cells. However, the expression of FGF1 in untreated cells was transient, peaking at 4 hours, and a rapid decrease was detected at 8 hours. In contrast, FGF1 expression was sustained in cPGI2-treated cells, persisting for up to 8 hours (Figure 3A). We also evaluated the effects of cPGI2 on FGF1 expression in vascular SMCs under similar hypoxic conditions. Treatment of SMCs with cPGI2 resulted in a more acute effect than that seen in myotubes; a marked increase in FGF1 protein levels was observed 2 hours after the addition of cPGI2 as compared with untreated SMCs (Figure 3B). An FGF1-positive lysate was used in parallel during Western blot (Figure S8).

Because dual cellular pathways are involved in cPGI2-induced biological activities, we examined whether cell surface prostacyclin receptor (IP) signaling or nuclear receptor PPARβ/δ signaling is responsible for the increased...
FGF1 expression. Specific receptor antagonists were used to selectively target cPGI2-induced IP or PPARβ/δ signaling in C2C12 myotubes and SMCs. Although the IP receptor antagonist CAY-10441 (1 μmol/L) attenuated FGF1 expression in C2C12 myotubes (P>0.05) 8 hours after cPGI2 (10 μmol/L) treatment, the PPARβ/δ antagonist GSK3787 (1 μmol/L) exerted a more significant suppression on cPGI2-induced FGF1 upregulation (P<0.05; Figure 3C). In SMCs, the IP receptor antagonist CAY-10441 did not affect FGF1 protein levels induced by cPGI2 after 2-hour treatment. However, similar treatment with the PPARβ/δ antagonist GSK3787 significantly decreased FGF1 expression (Figure 3D). Together, our data suggest a novel role of cPGI2-PPARβ/δ signaling in regulating FGF1 protein levels in both skeletal myotubes and SMCs under hypoxia.

cPGI2 Treatment Increases Nascent Vessel Formation in Ischemic Hindlimbs

To convert nascent vascular tubes into functional vasculature, supporting mural cells must be recruited to encircle the newly formed endothelial tube and ensure vessel survival. Mural cells interact with luminal endothelial cells of newly formed microvessels to preserve the integrity of the abluminal barrier and to facilitate cell–cell communication for vessel stabilization and maturation. To understand the role of cPGI2 on mural cell recruitment during vessel growth, we performed immunofluorescence staining to localize endothelial and mural cells within the microvasculature. We used anti–von Willebrand factor antibody to label the abluminal endothelial surface and anti-neuron-glial antigen 2 (NG2) antibody to label mural cells. NG2 is a transmembrane proteoglycan expressed exclusively by mural cells during development of the neovasculature.17 We used laser scanning confocal microscopy to examine NG2+ mural cells around the luminal endothelial layer 7 days after arterial ligation. We found more NG2+ vessels in cross sections of anterior thigh muscles in cPGI2-treated mice than in saline-treated mice (Figure 4A and 4B). Interestingly, most of the NG2+ arterioles were located in subcutaneous tissue and were seen underneath the muscle; these are the areas where cPGI2 was delivered (sustaining high cPGI2 concentrations) and where active vascular remodeling occurred. Quantitative analysis indicated that the number of NG2+ microvessels ranging in size from 15 to 50 μm in diameter was significantly higher in the cPGI2 group than in the saline group (38.00±2.41/high-power field versus 18.69±2.12/high-power field; P<0.01; n=3/group; Figure 4C). To further confirm that the NG2+ cells were mural cells, we performed α-smooth muscle actin (SMA) staining and found that NG2+ vessels >15 μm in diameter coexpressed SMA (Figure S9). Together, our data suggest that the controlled release of cPGI2 in ischemic hindlimbs increased the formation of NG2+ vessels.
Discussion

In the current study, we provide evidence that local cPGI2 delivery facilitates arteriolar growth in hindlimb ischemia. We compared perfusion changes in ischemic legs after vascular occlusion and constant cPGI2 or saline delivery and found that the relative perfusion was significantly better at 7 and 14 days in cPGI2-treated legs than in saline-treated legs. Furthermore, we showed that cPGI2 treatment resulted in a more elaborate microvascular structure at the arteriolar level, distal to the ligation site, than did saline treatment. Improved blood flow in the cPGI2 group coincided with the emergence of arteriolar anastomoses, including the development of arteriolar loops adjoining arterioles and collateral vessels connecting parallel neighboring arterioles. The structural remodeling response to cPGI2 was further confirmed by double-blind micro-CT analyses. Finally, we indicated a positive role of cPGI2-PPAR β/δ signaling in regulating FGF1 protein levels in vascular SMCs and C2C12 myotubes under hypoxia.

Because cPGI2 is a vessel dilator, we evaluated its dilatory effects on microvascular morphology. The hemodynamic response to prostacyclin has been shown to be measurable after a 24-hour continuous infusion of PGI2; therefore, we believe that our time point of measuring after 24 hours of cPGI2 exposure should be sufficient to view the vasodilatory effects of cPGI2 but would allow only minimal vascular growth. Interestingly, our microscopic observations showed that cPGI2 did not induce visible vasodilation; we found a similar network of microvascular structures in both cPGI2- and saline-treated legs. This finding indicates that cPGI2-induced dilatory effects cannot be seen by en face observation. Moreover, to minimize the effect of inherited morphological microvascular discrepancies between mice, we compared the microvascular morphology of left and right legs from the same mouse after local delivery of either cPGI2 or saline. Again, the application of cPGI2 in the ischemic anterolateral thigh for 20 minutes did not induce a change in the vasculature or lead to enhanced microvascular emergence when compared with the same vascular area before cPGI2 application. To our knowledge, no published studies have shown that cPGI2 can produce profound vessel relaxation resulting in a morphological

Figure 3. cPGI2-induced acidic fibroblast growth factor (FGF1) protein expression was dependent primarily on peroxisome proliferator-activated receptor β/δ (PPAR β/δ) signaling under hypoxia. A, A time course study indicates that cPGI2 induces a more sustained expression of FGF1 in C2C12 myotubes compared with untreated cells. B, cPGI2 stimulates a rapid increase in FGF1 protein expression in smooth muscle cells (SMCs). C, Schematic representation of the experimental procedure for treating C2C12 myotubes with the prostacyclin receptor antagonist CAY10441 or the PPAR β/δ antagonist GSK3787 and representative Western blots indicating the effects of CAY10441 or GSK3787 on FGF1 protein expression in C2C12 myotubes after 8 hours of cPGI2 treatment. D, Schematic representation of the experimental procedure for treating SMCs with CAY10441 or GSK3787 and representative Western blots showing that PPAR β/δ antagonist GSK3787 blocks FGF1 protein expression in SMCs after 2 hours of cPGI2 treatment. For each treatment, n=3 independent experiments, *P<0.05.
change detectable via light microscopy. The vessel dilatory effect of cPGI2 has been previously observed by measuring the arteriolar response at 80 mm Hg intravascular pressure ex vivo in a vessel chamber administered with cPGI2. In support of the arteriogenic role of cPGI2 shown by live animal imaging, quantitative micro-CT demonstrated that cPGI2 delivery resulted in a significant increase in the number of vessels with diameters ranging from 40 to 60 µm. This finding is the first to demonstrate cPGI2-induced arteriolar remodeling that resulted in improved perfusion in ischemic legs.

In the current report, we limited our study of cPGI2 to an acute ischemia model. An animal model that mimics the effects of the chronic ischemic disease that occurs in patients would enable us to better elucidate the therapeutic effects of cPGI2. However, creating a mouse model with chronic ischemic limbs is challenging. In the presence of arterial occlusion, angiogenesis and arteriogenesis occurs to compensate for perfusion loss. Although we created severe ischemia after double ligation of the common femoral artery proximal to the origin of the femoral bifurcation, the average perfusion without drug treatment reached 54.6% at day 14 after surgery (saline-treated group, Figures 1A and S1). This compensatory blood recovery prevents us from examining drug function in a chronic limb ischemia model. To provide a prolonged window of time to investigate the therapeutic effects of cPGI2, we treated mice with cPGI2 immediately after surgery when minimal perfusion is observed. Using mice with a diminished ability of vascularization may enable us to establish a stable chronic ischemia model for testing drug effects.

To better understand how cPGI2 promotes microvascular remodeling in ischemic limbs, we performed a proteome profiler antibody array to screen for the expression of arteriogenic factors. Our results indicated that cPGI2 upregulates the local release of FGF1 from ischemic muscles at 3 days after femoral artery ligation. We further validated the array results by examining the effects of cPGI2 exposure on FGF1 protein levels in C2C12 myotubes and SMCs under hypoxia. We detected the presence of higher amounts of FGF1 protein in both cell types at different times after cPGI2 treatment. Overexpression of FGF1 has been shown to promote the growth of arterioles in mice by increasing the number and density of branches. In a hamster model of peripheral arterial disease, FGF1 gene transfer promoted arteriogenesis in ischemic hindlimbs. However, FGF1 has low thermal stability in the absence of heparin, is easily degraded by proteases, and has a short half-life in vivo. Thus, the instability of FGF1 limits its biological action in therapeutic applications. Our data suggest that administration of cPGI2 may help maintain a constant FGF1 concentration for an extended period, which may compensate for FGF1’s instability and increase its efficacy during the critical window of vascular remodeling.

We examined the signaling pathway involved in cPGI2-induced FGF1 protein expression under hypoxia. Our results indicate a novel role for PPARβ/δ signaling in promoting arteriolar growth. PPARβ/δ is a ligand-activated transcriptional factor that modulates target gene expression. Evidence has emerged suggesting a potential role for PPARβ/δ in prostacyclin-induced vascular protection. Although the proangiogenic property of stable PGI2 analogs is attributed to PPARβ/δ axis on the production of arteriogenic factors, little is known about how the PGI2-PPARβ/δ axis affects the growth or remodeling of preexisting arteriolar networks. cPGI2 is ideal for studying this issue because it functions as a stable PGI2 analog and is known to be an effective PPARβ/δ agonist. Here, we first used multiple quantitative imaging techniques to illustrate the positive effects of cPGI2 on arteriolar growth. We then found that a combination of soluble factors produced in the local tissue microenvironment of cPGI2-treated legs promotes SMC migration and growth. Furthermore, using a selective and irreversible PPARβ/δ inhibitor GSK3787, we demonstrated the positive effects of cPGI2-PPARβ/δ axis on the production of arteriogenic factor FGF1. Our results indicate a role for PPARβ/δ in adaptive vascular remodeling and suggest that PPARβ/δ may be a new therapeutic target for treating CLI.

Different mechanisms may be involved in cPGI2-induced FGF1 expression in different cell types. In SMCs, increased
amounts of FGF1 were detected at 2 hours after cPGI2 treatment. This acute response depended on PPARβ/δ signaling because GSK3787 blocked the increase; this finding indicates the possibility that PPARβ/δ directly regulates the FGF1 gene. A recent study showed that the FGF1 gene promoter region contains a conserved PPAR response element. Thus, PPARβ/δ might act through transcriptional activation of FGF1. Interestingly, in C2C12 myotubes, a prolonged period of time (8 hours) was required for cPGI2 to exert a positive effect on FGF1 protein expression. Moreover, treatment of myotubes with the PPARβ/δ antagonist GSK3787 did not completely block the increase in FGF1 levels. Considering that similar treatment with the IP antagonist CAY 10441 also decreased cPGI2-induced FGF1 protein levels (although statistically not significant), it is possible that IP signaling is, in part, responsible for increased FGF1 production. Therefore, a more complex mechanism involving interaction between IP and PPARβ/δ signaling may affect the upregulation of FGF1 in skeletal myotubes. In the current study, we demonstrated a novel association between cPGI2-PPARβ/δ-FGF1 signaling and arteriolar growth in ischemic hindlimbs. However, we did not rule out additional mechanisms (eg, nitric oxide pathway) that may affect cPGI2-induced arteriolar growth.

Given the finding that the secreted arteriogenic factor FGF1 is persistently present in skeletal myotubes treated with cPGI2, the paracrine action of skeletal myofibers in promoting the formation of vasculature is noteworthy. Cytokines and growth factors released from inflammatory and vascular cells stimulate vascular growth, but the role of myofibers is poorly understood. Because myofibers are a major cell type surrounding arterioles in skeletal muscles, their signaling to neighboring vascular cells (eg, endothelial cells and SMCs) may affect arteriolar growth. Such signaling may not only reestablish a sufficient vascular network to provide fuel for myofibers but also reduce myofiber necrosis during ischemia. We postulated that the paracrine action of skeletal muscles triggered by therapeutic agents in ischemic conditions is an efficient strategy to potentiate arteriolar growth and to rapidly compensate for insufficient blood supply by recruiting preexisting arteriolar networks. Supporting this idea, our findings indicate that myotubes serve as a critical effector of cPGI2 signaling and as a hub for secreting the arteriogenic factor FGF1.

In conclusion, we present comprehensive evidence that cPGI2 positively affects adaptive vascular remodeling in hindlimb ischemia. We also provide evidence that cPGI2 promotes FGF1 protein expression in SMCs and skeletal myotubes via PPARβ/δ signaling. Because PG2 is known to inhibit vascular dysfunction (eg, thrombosis and atherosclerosis), we believe our findings will be useful in developing novel treatment strategies for atherosclerotic diseases such as CLI.

**Perspectives**

CLI is the severe obstruction of blood flow to the lower extremities and is caused by adverse cardiovascular conditions such as atherosclerosis, hypertension, and hypercholesterolemia. Revascularization is the best treatment strategy for CLI. Prostacyclin and stable analogs are clinically recommended agents for treating CLI and have shown beneficial effects. In the current study, we used a local drug delivery system and demonstrated a novel function of the stable prostacyclin analog carbaprostacyclin (cPGI2) in promoting revascularization in hindlimb ischemia. Using multiple imaging techniques, we showed that cPGI2 attenuates hindlimb ischemia by promoting arteriolar growth. Moreover, we uncover a novel mechanism for cPGI2-enhanced vascular remodeling. We demonstrate cPGI2-induced nuclear receptor PPARβ/δ signaling positively affects FGF1 protein levels in vascular SMCs and skeletal myotubes. Our data suggest that cPGI2 may improve the paracrine action of ischemic skeletal myofibers to enhance arteriolar growth. Because ischemia results in blood loss at the microvascular level and arterioles directly control blood distribution throughout the hindlimbs, the cPGI2-PPAR β/δ axis may serve as an effective target for inducing arteriolar growth and increasing blood flow to ischemic tissue beds. Together, the current findings will advance our understanding of the prostacyclin-mediated PPARβ/δ signaling that controls adaptive vascular remodeling in hindlimb ischemia.

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**Disclosures**

None.

**References**


Novelty and Significance

What Is New?

- cPGI2 administered directly to the ischemic tissue bed improved perfusion by inducing arteriolar growth and remodeling and promoted the release of acidic fibroblast growth factor.
- Nuclear receptor peroxisome proliferator-activated receptor β/δ signaling may be the primary pathway involved in arteriolar remodeling.

What Is Relevant?

- Because of our aging population, critical limb ischemia is a major therapeutic challenge. Prostacyclin and its stable analogs are a therapeutic choice for treating critical limb ischemia, but the role of prostacyclin analogs in arteriolar growth is unknown.
- The current study facilitates further understanding of the cPGI2-peroxisome proliferator-activated receptor β/δ axis in promoting arteriolar growth and helps to formulate a new strategy for the development of therapeutic agents.

Summary

Prostacyclin and stable analogs have shown beneficial effects in treating patients with critical limb ischemia. By using multiple imaging techniques, we have shown here that a stable prostacyclin analog, carbaprostacyclin (cPGI2), improves local perfusion by enhancing the growth of arteriolar networks. Our results provide the first evidence that the cPGI2-peroxisome proliferator-activated receptor β/δ axis positively affects acidic fibroblast growth factor protein expression in skeletal myotubes and smooth muscle cells. Together, the data suggest local delivery of cPGI2 exerts its therapeutic effects at the level of the arteriolar circulation.
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Mouse Ischemic Hindlimb Model

All animal procedures were conducted according to the University of Texas Health Science Center Animal Welfare Committee guidelines and in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. C57 Black mice (C57BL/6J, 8-12 weeks old) were anesthetized by isoflurane inhalation (2-4% isoflurane in oxygen) for the surgical procedure. Hindlimb ischemia was created by unilateral surgical ligation with the use of 2 adjacent sutures to interrupt the left femoral artery and vein proximal to the origin of the femoral bifurcation; this ligation site was chosen to induce severe ischemia in C57BL/6J mice. The osmotic pump (ALZET, Durect Corporation, Cupertino, CA, USA) was implanted subcutaneously on the mouse’s back. A P50 catheter (outside diameter=0.045 in) attached to the osmotic pump was tunnelled into the left anterolateral thigh to deliver either cPGI2 (Cayman Chemical, Ann Arbor, MI, 100ng/min/kg) or saline (control) into the distal area of the ligation site. The catheter tip was secured by stitching to superficial tissue to prevent migration during substance delivery, and the delivery rate was 0.25 µl/hour. The concentration of cPGI2 was determined based on the previous study. Equal numbers of mice were randomly divided to receive either cPGI2 or saline. Continuous delivery of cPGI2 or saline into ischemic hindlimbs was sustained for 3, 7, or 14 days. The physical daily activities of cPGI2-treated and saline-treated mice were similar during the study. To examine whether cPGI2 induces vessel dilation, 2 osmotic pumps loaded with saline or cPGI2 were simultaneously implanted and delivered to the right (saline) and left (cPGI2) anterolateral thigh of the same mouse for 24 hour. No hindlimb ischemia was created. We used a total of 42 mice during the study.

Laser Doppler Perfusion Imaging

Serial measurements of perfusion were taken with a laser Doppler image device (Perimed AB, Germany) before and immediate after surgery, 1 day, 7 days, and 14 days after cPGI2 or saline treatment (5 mice/group). Perfusion was expressed as the blood perfusion ratio in the ischemic compared to the contralateral, nonmanipulated legs.

Blood Pressure, Heart Rate, and Tail Blood Volume

A non-invasive tail cuff method was used to monitor blood pressure in experimental mice before and 1 day, 7 days, and 14 days after cPGI2 or saline treatment (5 mice/group). We recorded 4 physiologic parameters (systolic pressure, mean arterial pressure, heart rate, and tail blood volume) by using a volume pressure recording (VPR) sensor and a tail occlusion cuff (Coda 6; Kent Scientific Corp., Torrington, CT). The mice were first placed on a warming platform (37°C) and then in a conical restrainer with a darkened nose cone fitted over the mouse’s head to reduce stress and mobility. Mice were also subjected to several acclimation cycles before the reading to reduce variability in blood pressure recordings. The detailed procedure has been described previously.

Microscopic Examination

At 3 and 7 days after constant cPGI2 or saline delivery, C57BL/6J mice were anesthetized by isoflurane inhalation (2-4% isoflurane in oxygen) and transferred to the microscope stage. An incision was made to expose the thigh and calf muscles for visualization of the vascular structures. Mice were immobilized with laboratory tapes to reduce motion during imaging. All images were taken with the use of a Nikon digital camera (Coolpix 5700) attached to a dissecting microscope (Zeiss Stemi 2000-C, Carl Zeiss Microimaging, Göttingen, Germany).

Micro-Computed Tomography (Micro-CT) Analysis
Mice (5 mice/group) were euthanized, and the thoracic cavity was opened. The right atrium was cut, and the lower abdominal aorta was ligated; the vasculature, distal to the ligation site, was flushed with 0.9% normal saline containing heparin (1000 IU/L) for 5 minutes with a syringe pump. Then, 10% neutral buffered formalin was pressure perfused for an additional 5 minutes. A contrast agent, radiopaque silicone rubber compound (Microfil, MV-112, Flow Tech Inc. Carver, MA), was injected into the abdominal aorta distal to the ligation site for 5 minutes. The mice were immediately placed into 10% neutral buffered formalin and maintained at 4°C overnight for proper polymerization of the agent. After 24 hours, the skin was removed, and the lower portion was dissected (distal to the lower abdominal aorta ligation) and placed in 10% formalin for an additional 4 days. The contralateral and ischemic hindlimbs were then dissected and placed in formic acid (Cal Ex II, Fisher Scientific, Pittsburgh, PA) for 48 hours to facilitate decalcification of bones. The hindlimbs were washed thoroughly under running water for 1 hour and then placed in 10% formalin and immediately imaged by micro-CT.

The vasculature in the hindlimb was imaged by using a high-resolution (10-28 µm isotropic voxel size) micro-CT imaging system (Explore Locus SP MicroCT, GE Healthcare; Little Chalfont, UK). The scanner was positioned to a voltage of 80 kVp and a current of 80 µA. The entire width of the field of view, imaged at a medium resolution, created an initial thumbnail reconstruction with a 458 x 458 pixel image matrix and a 70.3 µm isotropic voxel size. This thumbnail image was cropped around the hindlimb resulting in a rectangular volume that was reconstructed at 28 µm and then at 14 µm isotropic voxel size. Serial tomograms were recreated from raw data by using a cone beam filtered back projection algorithm. The tomograms were globally thresholded with a HU value of 200 and used to produce binarized 3D images of the microvasculature in the hindlimb partitioned from the surrounding tissues.

The morphologic vascular network in the 3D images of the ischemic (left) and non-ischemic (right) hindlimbs was assessed for several parameters. The mouse hindlimbs were scanned and reconstructed at a 14-µm voxel size, and an isosurface threshold of 200 was chosen to be consistent with the 2-D tomograms rendered in Microview 2.0.29 (GE Healthcare). Vascular volume, number, separation, and connectivity were evaluated. These parameters were chosen on the basis of the standard outputs rendered by using a trabecular bone thickness analysis tool (BoneJ, http://bonej.org), and those values were applied to the microvascular network of the hindlimbs. A volume of interest (VOI) was reconstructed in the upper and lower hindlimb. The vascular volume was calculated by using the generated voxel size and the number of segmented voxels in the 3D images. Vascular number and separation were computed by using the BoneJ thickness analysis tool. Connectivity was generated by using the Euler characteristic method. Additionally, vessel diameter was assessed and represented the total number of vessel of a specified diameter and output in a 16-bit 3D image with corresponding grayscale values. For each hindlimb, histograms were generated with a consolidated number of bins (which correspond to vessel diameter in µm) evenly spaced from 0 to the maximum measured thickness of every point in the leg and the number of voxels that were associated with that particular bin diameter.

**Conditioned Medium Collection and Proteome Profiler Array**

The mice were euthanized at 3 days after constant cPGI2 or saline delivery (3 mice/group). The thigh muscle (10-11 g) was dissected and cut into small pieces (1 mm x 1 mm per piece). Muscle pieces were incubated in 1 mL of serum-free Dulbecco's Modified Eagle Medium (DMEM, Invitrogen) supplemented with 0.1% bovine serum albumin (BSA) and 1% penicillin-streptomycin for 2 hours at 37°C. Tissue mixtures were then centrifuged for 5 minutes to eliminate tissue clumps. Conditioned medium (CM) was collected from the saline-treated group
(Saline-CM) and the cPGI2-treated group (cPGI2-CM) and stored at -80°C. A proteome profiler array was performed according to the manufacturer’s instructions (R&D system, Minneapolis, MN). In short, after a 1-hour membrane-blocking step, the pre-incubated CM-biotinylated antibody mixture was added, and the membrane was incubated overnight at 4°C. After a series of washes, the membrane was incubated with horseradish peroxidase-conjugated streptavidin, and the signal was detected by using ECL-Hyperfilm. Average protein expression levels were measured by using ImageJ software (rsbweb.nih.gov/ij), based on 3 independent arrays.

**Smooth Muscle Proliferation and Scratch Wound Migration Assays**

Mouse SMCs (MOVAS, ATCC) were seeded onto 96-well plates (2 x 10³/well) and cultured with DMEM medium supplemented with 10% FBS and 1% penicillin-streptomycin for 24 hours in a 5% CO₂ incubator at 37°C. Cells were starved with serum-free DMEM medium containing 0.1% BSA and 1% penicillin-streptomycin for an additional 24 hours. Culture medium was then replaced with control medium (serum-free DMEM+0.1% BSA), cPGI2-CM, or saline-CM supplemented with 1% FBS and 1% penicillin-streptomycin, and cells were grown for 48 hours in a 5% CO₂ incubator at 37°C. To measure cell proliferation, cells were treated with trypsin, and Trypan blue exclusion was used to obtain a direct count of viable cells under every culture condition. The average cell number was calculated on the basis of 3 independent experiments.

Scratch wound migration was performed in laminin-coated 96-well plates (BD Biosciences). Mouse SMCs (MOVAS, 6 x 10⁴) were seeded onto each well with DMEM containing 10% FBS. After forming a confluent layer, cells were serum-starved (DMEM + 0.1% BSA) for 18 hours. Scratches were then made with a p200 pipette tip, and cell debris was removed by washing each well once with medium (DMEM + 0.1% BSA). Afterward, 200 µl of serum-free DMEM (control), saline-CM, or cPGI2-CM, all supplemented with 1% FBS and 1% penicillin-streptomycin, was added to individual wells. The plate was placed in the incubator at 37°C and monitored for cell migration for up to 24 hours. Photographs were taken at 0 and 24 hours in the same field of view for all cell conditions. The images were quantitatively assessed with the use of Image Pro-Plus software by comparing the distances from the 0- to the 24-hour image and measuring the scratch closure difference in several areas in the well. Three independent assays were performed.

**C2C12 Cell Culture**

Six-well tissue culture plates were pre-coated with rat tail collagen overnight at 4°C (Sigma-Aldrich, St. Louis, MO). Approximately 4 x 10⁵ mouse C2C12 myoblasts (ATCC) were then seeded in each well and cultured with DMEM medium containing 10% FBS and 1% penicillin-streptomycin in a 5% CO₂ incubator at 37°C. To induce differentiation into myotubes, cells were switched into DMEM medium supplemented with 2% horse serum and grown for an additional 3 days.

**Western Blot Analysis**

C2C12 myotubes and SMCs were starved with serum-free DMEM medium containing 0.1% BSA overnight, the cells were switched into a serum-free DMEM with or without 10μm carboxaprostacyclin (Cayman Chemical) in a hypoxia chamber (1.5% O₂, INVIVO2 400, Ruskinn, UK) and incubated for 2, 4, or 8 hour at 37°C. To test whether IP or PPARβ/δ signaling was involved in cPGI2-induced FGF-1 protein expression, C2C12 myotubes and SMCs were starved with serum-free DMEM medium containing 0.1% BSA overnight. Cells were then pretreated with CAY10441 (1μM, Cayman Chemical) or GSK 3787 (1μM, Sigma-Aldrich) at 37°C for 30 minutes. Afterward, 10μm carboxaprostacyclin was added, and the cells were incubated for an additional 2 hours (SMCs) or 8 hours (myotubes) in a hypoxia chamber (1.5% O₂).
At individual time points, cells were washed with PBS and lysed in an ice-cold lysis buffer (10mM Tris-HCl, pH 7.6, 3 mM MgCl₂, 40 mM KCl, 2mM DTT, 5% glycerol, 0.5% NP40) containing a protease inhibitor cocktail (Roche, Switzerland) on ice. Lysates were sonicated (Bioruptor 300, Diagenode, Belgium) and centrifuged at 12,000 g at 4°C for 10 min. Supernatants were collected, and the proteins were quantified (Bio-Rad DC Protein Assay Reagents). A total 30 µg of protein from each sample was fractionated by SDS–PAGE (4–20% gradient gel, Bio-Rad, Hercules, CA) and transferred onto a PVDF membrane (Bio-Rad). Membranes were incubated in a TBS-Tween solution containing 5% non-fat dry milk for 1 hour at room temperature. The blots were then incubated overnight at 4°C with FGF-1 antibody (Abcam, Cambridge, MA), followed by incubation with HRP-conjugated anti-rabbit secondary antibody (Sigma-Aldrich) for 45 minutes at room temperature. Protein signals were detected by using the ECL system (Thermo Scientific). Mouse kidney tissue lysate was used as a positive control for FGF1 in the Western blot analysis. To verify equal loading of each protein sample, membranes were stripped and reprobed with antibody for β-actin (Sigma-Aldrich). Average protein expression levels were measured by using ImageJ software based on 3 independent western blots.

**Immunofluorescence Staining**

Groups of mice were euthanized by CO₂ inhalation at 7 days after surgery and constant cPGI2 or saline delivery. Thigh muscles were excised and fixed in 4% paraformaldehyde (USB Corporation, Cleveland, OH) at 4°C overnight followed by incubation in 30% (w/v) sucrose solution at 4°C overnight. The muscles were embedded in TISSUE TEK OCT compound and stored at -80°C freezer. Cross sections of muscle tissue (6 µm) were incubated with the following primary antibodies individually or in combination overnight at 4ºC: anti-α-SMA (FITC conjugated, GeneTex, Irvine, CA), anti-vWF (FITC conjugated, GeneTex), or anti-NG2 (Millipore, Billerica, MA). Sections were then incubated with Alexa Fluor-647 donkey anti-rabbit IgG. Nuclei were counterstained with DAPI. Fluorescence images of stained sections were taken with a confocal laser scanning microscope (Leica TCS SP5II, Buffalo Grove, IL). Image processing and quantitative analysis were performed by using Image-Pro Plus software (Media Cybernetics, Bethesda, MD). To quantify NG2⁺ blood vessels, a total of 71 HPFs were analyzed per treatment group (3 mice/group).

**Statistical Analysis**

Data were expressed as mean ± standard error of mean (SEM). The nonparametric Mann-Whitney post hoc test was used to determine statistical significance between cPGI2 and saline groups in all in vivo assays. Student’s t test was used to determine statistical significance between groups in all ex vivo and in vitro assays (Graph Pad Prism 5). *P*<0.05 was considered statistically significant.
**Supplemental References**


Figure S1. Blood perfusion of mouse ischemic limbs treated with cPGI2. 

A, Representative laser Doppler color-coded images of saline- and cPGI2-treated limbs taken at the indicated intervals. 

B, Quantitative raw data analysis of hindlimb perfusion as measured by laser Doppler perfusion imager. The perfusion ratio (ischemic:non-ischemic leg ratio) was significantly higher in the cPGI2-treated group than in the saline-treated group at 7 and 14 days. n=5/group, *P<0.05. 

NI, nonischemic legs; Isch, ischemic legs. Pre-S, before surgery; Post-S, after surgery.
Figure S2. Carboprostacyclin treatment did not significantly alter mouse blood pressure parameters. A, Systolic blood pressure. B, Mean arterial pressure (MAP). C, Heart rate. D, Tail blood volume. n=5/group, P>0.05; cPG12 vs. saline treatment at the indicated intervals.
**Figure S3.** Representative microscopic images demonstrate vascular remodeling in ischemic legs treated with saline or cPGI2 as compared to contralateral non-ischemic legs at 3 days. **A,** Contralateral (contral) nonischemic leg of a saline-treated mouse. **B,** Saline-treated ischemic leg. **C,** Contralateral nonischemic leg of a cPGI2-treated mouse. **D,** cPGI2-treated ischemic leg. Vascular growth was more evident in cPGI2-treated limbs than in saline-treated limbs. Blue arrows, arteriolar-to-arteriolar connections. n=3/group. NI, nonischemic legs; Isch, ischemic legs.
Figure S4. Representative photomicrographs showing vascular anastomosis in contralateral nonischemic and ischemic legs treated with saline (A and B) or cPGI2 (C and D) at 7 days. The boxed areas in A and C are enlarged in B and D, respectively. More intrarterolar connections (blue arrows) and curkocrew extensions of arteriules (white arrows) developed in the cPGI2- versus the saline-treated group. NI, nonischemic legs; Isch, ischemic legs; Control indicates the nonischemic legs of saline or cPGI2 groups. n=3/group.
Figure S5. Caraprostacyclin-induced vessel dilation was not apparent by en-face microscopy A and B. Representative photomicrographs show no noticeable enlargement of vessel diameters in non-ischemic hindlimbs of the same mouse treated with saline (right leg) compared to cPGI2 (left leg) for 24 hours. C and D, Representative photomicrographs show that the vascular morphology in the anterolateral thigh muscle of the ischemic limb is similar before [cPGI2 (-)] and after local cPGI2 [cPGI2 (+)] administration. n=2/treatment.
Figure S6. Quantitative micro-CT analysis of vascular morphology shows similar vascular structures at 14 days in the contralateral nonischemic legs of cPGI2- and saline-treated mice. A, Average vascular volume. B, Average vessel number. C, Average vessel separation. D, Average connectivity. E, Histogram of mean blood vessel size distribution. F, Representative micro-CT images of the microvascular network of cPGI2- and saline-treated contralateral nonischemic legs. The red dashed circles indicate the similar vasculature in the thigh muscle. For all panels, n=5/group.
Figure S8. cPGI2 (carbaprostacyclin) increases FGF1 protein level in C2C12 myotubes and smooth muscle cells. A, Representative Western blot indicates a higher level of FGF1 protein in C2C12 myotubes at 8 hours after cPGI2 treatment. B, Representative Western blot indicates a higher FGF1 protein level in SMCs at 2 hours after cPGI2 treatment. Mouse kidney lysate was used as FGF1 positive control.
Figure S9. Colocalization of NG2⁺ with α-SMA⁺ vessels at 7 days in ischemic legs treated with cPGI2 or saline. Representative confocal images show coexpression of NG2 with α-SMA in saline-treated (A) and cPGI2-treated (B) ischemic legs. n=3/group.