Sildenafil Promotes Ischemia-Induced Angiogenesis Through a PKG-Dependent Pathway

Annamalai Senthilkumar, Ray D. Smith, Jayant Khitha, Neeraj Arora, Srikar Veerareddy, Will Langston, John H. Chidlow Jr, Shayne C. Barlow, Xinjun Teng, Rakesh P. Patel, David J. Lefer, Christopher G. Kevil

Background—Peripheral artery disease (PAD) is a prevalent cardiovascular disorder that results in tissue ischemia which can progress to critical limb ischemia. Restoration of tissue perfusion in the setting of chronic ischemia through stimulation of arteriogenesis and angiogenesis remains a key therapeutic target for PAD. However, experimental therapeutics, including growth factor and gene therapy, have had limited clinical success indicating the need for a better understanding of molecular pathways required for therapeutic angiogenesis.

Methods and Results—Here we report that phosphodiesterase-5 inhibition by sildenafil significantly increases vascular perfusion, tissue blood flow, and vascular density during chronic ischemia of the mouse hind limb. Importantly, sildenafil therapy did not alter any of these parameters in nonischemic limbs. Sildenafil increased tissue cGMP levels independently of increases in nitric oxide production, and sildenafil therapy stimulated angiogenesis in ischemic limbs of eNOS−/− and iNOS−/− mice. Lastly, sildenafil-mediated angiogenic activity was blocked by inhibition of protein kinase G using the PKG antagonist DT-3.

Conclusions—These data demonstrate that sildenafil therapy results in increased angiogenic activity through a PKG-dependent pathway that is independent of nitric oxide production or NO activity and identify the angiogenic therapeutic potential of sildenafil for critical limb ischemia. (Arterioscler Thromb Vasc Biol. 2007;27:000-000.)

Key Words: ischemia □ angiogenesis □ nitric oxide □ PKG □ sildenafil □ PDE-5 inhibition

Peripheral artery disease (PAD) affects 8 to 10 million individuals in the United States. The risk of developing PAD is increased 2- to 4-fold in patients with diabetes, hypercholesterolemia, and hypertension, in whom the disease is often extensive and severe leading to chronic ischemia of distally supplied tissue. Promoting angiogenesis and arteriogenesis in patients with PAD and critical limb ischemia may be beneficial of which minimal therapeutic options exist. Recently, the use of nitric oxide (NO) donors and activators of the endothelial NO synthase (eNOS)/NO pathway such as statins or VEGF-A have been reported to promote angiogenesis or arteriogenesis in critical limb ischemia; unfortunately, these agents have not been transitioned into the clinical arena for various reasons.1,2 For example, NO donor therapy in the setting of cardiovascular diseases may have beneficial as well as harmful effects.2,3 In diabetic mice, aberrant oxidant production combined with uncoupling of eNOS results in the formation of superoxide and peroxynitrite which portends to exacerbated tissue damage during ischemia-reperfusion.3 Moreover, NO supplementation via various donors or gene therapy can further exacerbate tissue damage in ischemia-reperfusion settings attributable to increased formation of peroxynitrite.4 Thus, it may be desirable to identify therapeutic strategies that stimulate NO downstream signaling pathways necessary for angiogenesis and vascular growth without the generation of toxic reactive species that could exacerbate tissue pathology.

NO is a well accepted angiogenic regulator that participates in several cellular responses including but not limited to increased endothelial cell proliferation and survival, increased endothelial cell motility, and increased activation of signaling pathways necessary for angiogenic activity.2,4,5 Moreover, NO is known to stimulate soluble guanylate cyclase activity resulting in increased cyclic guanosine monophosphate (cGMP) production which may also participate in angiogenic pathways through poorly defined mechanisms.6–9 Intracellular cGMP levels are not only governed by guanylate cyclase activity, but also by phosphodiesterase conversion of cGMP back to GMP.10 Sildenafil is a well known phosphodiesterase type 5 (PDE-5) antagonist that significantly atten-
ulates catabolism of cGMP thereby enhancing signaling pathways involving cGMP. As such, sildenafil has been shown effective for the treatment of erectile dysfunction, pulmonary hypertension, congestive heart failure, and diabetic neuropathy. Moreover, sildenafil has also shown promise in the management of ischemia-reperfusion injury in the heart and has been useful in the treatment of experimental embolic stroke in the rat. Importantly, all of these pathologies entail a significant ischemic component indicating the effectiveness of sildenafil in resolving these tissue pathologies. However, no cellular or molecular evidence exists regarding the mechanisms by which sildenafil therapy could confer protection during ischemic disease.

We present important and novel evidence that sildenafil therapy augments ischemia-induced angiogenic activity which significantly furthers our understanding by which sildenafil therapy could be protective in tissue ischemia pathologies. We report that sildenafil therapy in the mouse ischemic hind limb model stimulates a significant increase in tissue blood flow that is concomitant with an increase in blood vessel density. Importantly, sildenafil therapy increases tissue cGMP levels and does not require NO synthase activity to stimulate ischemia-induced angiogenesis. Moreover, the effect of sildenafil therapy is dependent on PKG activity demonstrating that sildenafil therapy could confer protection during ischemic disease.

Hind Limb Ischemia Model

Hind limb ischemia was induced in wild-type mice as previously reported. Male wild-type (C57BL/6J) mice weighing 20 to 25 g and age 2 to 3 months were used. The mice were bred and housed at the Association for Assessment and Accreditation of Laboratory Animal Care, International-accredited LSUHSC-Shreveport animal resource facility and maintained according to the National Research Council’s Guide for Care and Use of Laboratory Animals. iNOS−/− mice were originally obtained from Dr Paul Huang at Harvard Medical School (Boston, Mass) and backcrossed onto the C57BL/6J background for 8 generations. C57BL/6J iNOS−/− mice obtained from Jackson Laboratories (Bar Harbor, Mass) were bred in house at the LSUHSC-Shreveport animal resource facility. The PKGI inhibitor peptide, DT-3, was purchased from AXXORA, LLC. All chemicals were obtained from Sigma Chemical. 8-bromo-cGMP was purchased from Biomol.

Methods

Animals and Reagents

Male wild-type (C57BL/6J) mice weighing 20 to 25 g and age 2 to 3 months were used. The mice were bred and housed at the Association for Assessment and Accreditation of Laboratory Animal Care, International-accredited LSUHSC-Shreveport animal resource facility and maintained according to the National Research Council’s Guide for Care and Use of Laboratory Animals. iNOS−/− mice were originally obtained from Dr Paul Huang at Harvard Medical School (Boston, Mass) and backcrossed onto the C57BL/6J background for 8 generations. C57BL/6J iNOS−/− mice obtained from Jackson Laboratories (Bar Harbor, Mass) were bred in house at the LSUHSC-Shreveport animal resource facility. The PKGI inhibitor peptide, DT-3, was purchased from AXXORA, LLC. All chemicals were obtained from Sigma Chemical. 8-bromo-cGMP was purchased from Biomol.

Hind Limb Ischemia Model

Hind limb ischemia was induced in wild-type mice as previously reported by ligating the left common femoral artery proximally to origin of the profunda femoris artery. Mice were anesthetized with intraperitoneal injection of ketamine (100 mg/kg) and xylazine (8 mg/kg). Aseptic surgery was performed to ligate the left common femoral artery as follows. An incision was made in left groin of mice. Blunt dissection was performed and the left common femoral artery was identified by its pale pink color and pulsatile nature. The common femoral vein and femoral nerve were dissected free of the tissue. The incision was sutured in a single layer using the same suture material as for ligation. Mice were noted to be limping and dragging the left hind limb after recovery from anesthesia.

Vascular Density Measurements of Tissue Blood Flow

Determination of the vascular density of muscle tissue was performed as we have previously reported. Mice were euthanized at 7 and 21 days by cervical dislocation after anesthesia. The gastrocnemius muscles from the ischemic (left) and nonischemic (right) limbs were dissected and embedded in OCT freezing medium and frozen, and 8 μm sections cut. Slides were fixed at 20°C in 95% ethanol/5% glacial acetic acid for 1 hour. Slides were washed 3 to 4 times in cold PBS with 1% percent horse serum (5 minutes per wash) and blocked overnight with 5% horse serum in PBS at 4°C. Primary antibody against CD31 (platelet/endothelial cell adhesion molecule-1 [PECAM-1]) was added at 1:200 dilution (in PBS with 0.05% horse serum) and incubated at 37°C for 1 hour. Slides were then washed 3 times with 1% horse serum/PBS. Cy3 conjugated anti-rat secondary antibody was added at 1:250 dilution (in PBS with 0.05% horse serum) and incubated at room temperature for 1 hour. Slides were washed again and mounted using Vectashield DAPI (4',6-Diamidine-2'-phenylindole dihydrochloride) mounting medium. 4 slides per hind limb with 3 sections per slide were made for vascular labeling and analysis. At least 3 fields were acquired per section of muscle. Pictures were taken with a Hamamatsu digital camera using a Nikon TE-2000 epifluorescent microscope (Nikon Corporation, Japan) under TRITC and DAPI illumination at 200× magnification for PECAM-1 and DAPI staining, respectively. Simple PCI software version 6.0 (Compix Inc, Sewickly, PA) was used to quantitate the area of PECAM-1 and DAPI staining, respectively.
vided by DAPI staining. Image acquisition and vascular density measurements were accumulated, analyzed, and calculated in a double blinded fashion before identity of the data were used for statistical analysis and graph generation.

Evaluation of Endothelial Cell Proliferation

Immunofluorescent staining of frozen tissue sections were performed as described above by dual staining with anti-Ki67 (1:350 dilution; Abcam, Cambridge Mass) cell proliferation marker and anti-CD31 (1:200 dilution) endothelial cell marker for 1 hour at 37°C. Sections were washed and stained with DTAF anti-rabbit secondary (1:150 dilution) and Cy3 anti-rat secondary (1:250 dilution) antibodies. Sections were washed again and mounted using Vectashield DAPI (4',6-Diamidine-2'-phenylindole dihydrochloride) mounting medium. Colocalization images were acquired with a Hamamatsu digital camera using a Nikon TE-2000 epifluorescent microscope and Simple PCI software.

Measurement of Tissue Nitrite Levels and cGMP

To evaluate NO production, tissue nitrite levels were measured using chemiluminescence techniques as we have previously reported. Gastrocnemius muscle tissue cGMP levels were determined using the cGMP ELISA from Cayman Chemical (Ann Arbor, Mich) according to the manufacturer’s directions.

Statistical Analysis

All mice were randomly assigned to experimental groups in a double-blinded manner and remained so until experimental procedures were complete. Student t test (unpaired) was used to analyze the differences between vehicle control and sildenafil treatment groups, and a P<0.05 was considered significant. Statistics were done with GraphPad Prism 4.0 software. The number of mice used per experiment is designated in figure legends. The authors had full access to the data and take responsibility for its integrity. All authors have read and agree to the manuscript as written.

Results

Sildenafil Treatment Enhanced Vascular Perfusion by Angiography

Chronic tissue ischemia is associated with arterial dysfunction and decreased microvascular density resulting in poor tissue perfusion. As such, there is a great clinical interest in enhancing arterial function and tissue perfusion during PAD. Therefore, we examined the effect of sildenafil therapy on ischemic hind limb vascular perfusion using angiography. Figure 1A and 1B shows representative hind limb angiograms at day 7 in vehicle control and sildenafil-treated mice, respectively. Likewise, Figure 1C and 1D illustrates hind limb angiograms at day 7 in vehicle control and sildenafil-treated mice, respectively. Sildenafil treatment enhanced perfusion of ischemic hind limbs in a time-dependent manner compared with vehicle treatment, indicating that inhibition of PDE-5 serves to restore vascular perfusion of ischemic tissue.

Sildenafil Treatment Improves Blood Flow in Ischemic Hind Limbs

We next performed blood flow measurements using a deep tissue penetrating laser doppler technique to corroborate angiography findings and to measure the effect of sildenafil on vascular function. Figure 2 reports hind limb blood flow as measured by laser doppler (ml/min/100g) in vehicle control and sildenafil-treated mice at days 7 and 21. Blood flow was significantly increased in ischemic limbs of sildenafil-treated mice versus vehicle control mice at 7 days, which was maintained at 21 days (panel 2A). Importantly, blood flow in the nonischemic right hind limbs were similar to the blood flow in the left hind limbs before ligation, and serial blood flow
measurements of nonischemic limbs at 7 and 21 days did not show any significant difference between sildenafil and vehicle control mice (panel 2B).

**Sildenafil Treatment Increases Vascular Density in Ischemic Hind Limbs**

We next examined the effect of sildenafil therapy on angiogenic activity in ischemic hind limbs using CD31 immunofluorescent quantification of tissue vascularity. Figure 3A and 3B shows representative CD31 staining of gastrocnemius muscle from ischemic hind limbs in vehicle control and sildenafil-treated mice at 7 days, respectively. Figure 3C shows the quantitative measurement of ischemic hind limbs from vehicle control and sildenafil-treated mice at days 7 and 21. Sildenafil therapy significantly increased the vascular density of ischemic limbs in a time-dependent manner. Importantly, Figure 3D illustrates that the vascular densities of nonischemic limbs from sildenafil-treated and vehicle control mice were not significantly different.

**Sildenafil Therapy Augments Endothelial Cell Proliferation During Hind Limb Ischemia**

We next examined whether sildenafil therapy altered endothelial cell proliferation, which is an essential feature of angiogenesis. Supplemental Figure I (available online at http://atvb.ahajournals.org) shows representative immunohistochemical staining for the cell proliferation marker Ki67 and CD31 in either control or sildenafil-treated tissues. Sildenafil robustly increased Ki67 staining during ischemia compared with control tissue. Importantly, Ki67 staining was largely colocalized with CD31 staining indicating that the majority of proliferating cells were endothelium. These data, together with the vascular density measurements, strongly demonstrate that sildenafil therapy augments endothelial cell proliferation and angiogenesis during ischemia.

**Sildenafil Treatment Increases Tissue cGMP but not Nitrite in Ischemic Hind Limbs**

We next examined the effects of sildenafil therapy on tissue cGMP and nitrite levels during hind limb ischemia. Figure 4A shows nitrite and cGMP levels at day 7 in vehicle control and sildenafil-treated mice, whereas Figure 4B reports nitrite and cGMP levels at day 21. Sildenafil treatment significantly increased tissue cGMP levels at 21 days in both ischemic and nonischemic hind limbs. Comparison across the 4 experimental groups revealed that the cGMP levels in sildenafil-treated limbs were significantly higher than the vehicle control limbs. Conversely, tissue nitrite levels were not significantly different at day 7 between vehicle control and sildenafil-treated mice. Similarly, tissue nitrite levels, a stable decomposition product of NO, were not significantly altered in either sildenafil-treated or vehicle control mice at day 7. However, tissue nitrite levels were significantly elevated at day 21 in the ischemic hind limb of vehicle control mice but not in sildenafil-treated hind limbs. Together, these data indicate that sildenafil therapy augments ischemic tissue cGMP accumulation without an increase in NO production.

**Sildenafil Enhances Ischemia-induced Angiogenesis Independent of eNOS or iNOS Activity**

It has been reported that sildenafil can increase eNOS or iNOS expression, which may be an important molecular mechanism of sildenafil therapy. Moreover, ischemia-induced angiogenic activity is directly proportional to the amount of eNOS expression and activity. Therefore, we examined whether sildenafil could enhance ischemia-induced angiogenesis in eNOS−/− or iNOS−/− mice in the hind limb ischemia model. Supplemental Figure II shows the vascular density of wild-type, eNOS−/−, and iNOS−/− mice at Day 21. Interestingly, the vascular density of sildenafil-treated eNOS−/− mice were significantly greater than vehicle control–treated eNOS−/− mice (supplemental Figure IIA). Similarly in iNOS−/− mice, the vascular density was also significantly greater on sildenafil treatment versus vehicle control iNOS−/− mice (supplemental Figure IIB). Importantly, the vascular density of nonischemic limbs of sildenafil-treated eNOS−/− and iNOS−/− mice was similar to vehicle control eNOS−/− and iNOS−/− mice nonischemic limbs, respectively (data not shown). The favorable effect of sildenafil treatment in the above knockout mice, especially eNOS−/− mice, strongly suggests that the proangiogenic effect of sildenafil is independent of NO synthase activity and NO production.
Sildenafil-induced Angiogenesis Is Abolished by PKG Inhibition

Elevated intracellular cGMP levels, either through increased production or decreased catabolism, can facilitate increased protein kinase G activity. Moreover, PKG activity is an important mediator of ischemia-induced angiogenesis as PKG/− mice show defective angiogenic responses in the ischemic hind limb model. Therefore, we determined whether sildenafil-induced angiogenic activity occurs through PKG pathways using the specific PKGI inhibitor DT-3. Figure 5A shows ischemic hind limb vascular densities at day 7 between ischemia, ischemia plus sildenafil, and ischemia plus sildenafil plus DT-3. Inhibition of PKGI activity with DT-3 completely abolished the ability of sildenafil-mediated increases in vascular density during ischemia. Importantly, vascular densities of nonischemic limbs were not different among the various groups (data not shown). Together, these data demonstrate that sildenafil enhances ischemia-induced angiogenesis through a PKGI-dependent pathway. By way of comparison, we also examined whether cGMP analogue therapy altered ischemia hind limb angiogenesis. Figure 5B demonstrates that daily tail vein injection of 100 μg/kg 8-bromo-cGMP augments ischemia-induced angiogenesis at day 7, which was not altered in nonischemic limbs (data not shown). Together, these data demonstrate that regulation of cGMP levels critically affects ischemia-induced angiogenesis.

Discussion

Therapeutic arteriogenesis and angiogenesis remain an important clinical goal for PAD patients with the goal of avoiding critical limb ischemia and further tissue dysfunction. As such, several experimental approaches have been explored such as VEGF, bFGF, eNOS, NO, and other therapies. However, successful clinical results are limited, thus highlighting the need for continued investigation of alternative approaches to restore vascular function during tissue ischemia. In this study, we provide novel information that sildenafil therapy significantly enhances ischemic tissue per-
fusion through an increase in arterial function and angiogenesis. Our results show that sildenafil increased vascular perfusion and tissue blood flow in a time-dependent manner. We also determined that angiogenesis was enhanced in a time-dependent manner in ischemic hind limbs of mice treated with sildenafil. A few studies have reported that sildenafil can stimulate cerebral vascular angiogenesis after experimental stroke, yet the molecular mechanisms involved in this response are not well understood. Moreover, no studies have been performed examining the effects of PDE-5 blockade in models of PAD or chronic ischemia of other tissues. Thus, our study provides important new information regarding the effect of PDE-5 inhibition on peripheral tissue angiogenesis and reveals novel mechanisms necessary for this response.

Figure 4. Sildenafil increases tissue cGMP but not nitrite levels. Gastrocnemius tissue was obtained from nonischemic and ischemic hind limbs and analyzed for cGMP and nitrite content. Panel A illustrates tissue cGMP and nitrite levels at day 7, whereas Panel B reports tissue cGMP and nitrite levels at day 21. n=10 per experimental group.

Figure 5. Sildenafil-induced angiogenesis is PKG-dependent. The hind limb ischemia model was performed using DT-3, a specific antagonist of PKG, to determine the importance of this kinase for sildenafil-induced angiogenesis. DT-3 (500 μg/kg) was administered daily via tail vein injection. Gastrocnemius tissue was obtained at day 7 and used for vascular density measurements. Panel A illustrates that combined treatment of sildenafil plus DT-3 revealed no increase in angiogenic activity. Likewise, administration of the PKG agonist, 8-bromo cGMP (100 μg/kg), was administered daily by tail vein injection to determine whether an alternative method of PKG activation affected ischemic angiogenesis. Panel B shows that 8-bromo cGMP significantly augments ischemia-induced angiogenesis. n=10 per experimental group.
Interestingly, we found that sildenafil therapy resulted in a significant increase in both nonischemic and ischemic tissue cGMP levels but that increases in tissue blood flow or vascular density were observed only in ischemic limbs. This observation suggests that sildenafil treatment could make ischemic tissues more sensitive to endogenous angiogenic or arteriogenic stimuli during ischemia versus general stimulation of angiogenic activity as recently suggested. It is also possible that sildenafil therapy could augment the production of angiogenic growth factors which would facilitate ischemia-induced angiogenesis over nonischemic tissues. Although we are further investigating these and other proangiogenic mechanisms of sildenafil, the current findings clearly indicate that sildenafil therapy preferentially alters ischemic tissue responses without nonspecific effects on nonischemic tissue.

Nitric oxide is a critical angiogenic mediator regulating several aspects of vascular cell function including proliferation, migration, and maturation. Moreover, ischemia induces increased NO production, which facilitates subsequent angiogenic responses. Surprisingly, sildenafil therapy significantly blunted ischemia-induced NO production, suggesting that sildenafil-mediated angiogenesis is independent of NO production during ischemia. Moreover, recent reports have shown that sildenafil augments endothelial and inducible NO synthase expression, which may contribute to the beneficial effects of the drug. These findings, together with the importance of NO for ischemia-induced angiogenesis, prompted us to further investigate the angiogenic potential of sildenafil therapy in eNOS and iNOS–/– mice. Consistent with our tissue nitrite data, sildenafil increased ischemia-induced angiogenesis in both eNOS–/– and iNOS–/– mice. These data are quite striking given the profound defect of ischemia-induced angiogenesis seen in eNOS–/– mice which often leads to hind limb degeneration which we did not observe with sildenafil therapy. This could possibly be attributable to the fact that sildenafil quickly augments ischemia-induced angiogenesis, thereby diminishing the duration and severity of tissue ischemia necessary for NOS induction and NO production attributable to increased cGMP levels or PKG activity. Future studies will more closely examine this unique relationship between NO induction versus cGMP production in relation to angiogenic stimulation. Together, our data suggest that sildenafil exerts its protective effects independent of NO synthase activity, which is consistent with recent reports documenting NOS independent mechanisms of sildenafil tissue protection.

Sildenafil is a potent antagonist of tissue phosphodiesterase 5 activity and results in the intracellular accumulation of cGMP. Our data indicate that sildenafil significantly increased ischemic tissue cGMP at day 21 but not at day 7. The reason for this is not clear, but could involve changes in tissue consumption of cGMP relative to its production (i.e., a greater need and consumption during early periods of ischemia which diminish over time). Nonetheless, we determined if sildenafil enhanced angiogenic activity involved PKG activity, a downstream signaling target of cGMP. Mice were treated with the selective PKGI antagonist DT-3 which blocks cGMP-dependent activation of the kinase. Inhibition of PKGI activity by DT-3 completely abolished the ability of sildenafil to augment ischemia-induced angiogenesis which is consistent with the hypothesis that sildenafil stimulates angiogenesis by increasing cGMP levels and downstream PKGI activity. Our findings are also corroborated by previous reports demonstrating that PKG–/– mice show defective hind limb ischemia-induced angiogenesis similar to that of eNOS–/– mice. Importantly, we demonstrate that cGMP analogue therapy significantly augments ischemic tissue vascular density further solidifying the importance of this pathway for ischemia-induced angiogenesis. Thus, our experimental data demonstrate that sildenafil circumvents the need for NO to induce angiogenesis in the setting of prolonged ischemia.

In summary, we provide evidence that sildenafil enhances angiogenesis in response to tissue ischemia, which is mediated by increased cGMP levels and PKG activity. Moreover, this increase in angiogenic activity is independent of NO production and eNOS or iNOS activity. Thus, therapeutic use of PDE-5 inhibitors may extend to the treatment of chronic tissue ischemic states such as critical limb and PAD.

**Sources of Funding**

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**Disclosures**

None.

**References**

Preliminary studies have shown that the PDE-5 inhibitor sildenafil can improve endothelial function and reduce inflammation in animals with diabetes, providing potential therapeutic benefits for diabetic patients. In a study published in the *Journal of Diabetes and Its Complications*, research indicated that sildenafil significantly lowered blood glucose levels and improved insulin sensitivity in diabetic mice compared to control groups. These findings suggest that sildenafil may offer additional therapeutic options for the management of diabetic complications.

Moreover, a recent study published in *Diabetes Research and Clinical Practice* examined the effects of sildenafil on diabetic nephropathy. The study demonstrated that sildenafil treatment reduced proteinuria and renal fibrosis, indicating its potential for mitigating renal damage associated with diabetes.

These findings, along with other recent research, support the notion that sildenafil may offer new avenues for therapeutic treatment in diabetes, particularly in conditions where traditional methods have limited efficacy. Further clinical trials are necessary to fully elucidate the role of sildenafil in diabetes management and to determine its optimal dosing and administration strategies for different patient populations.
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