Deletion of AT2 Receptor Prevents SHP-1–Induced VEGF Inhibition and Improves Blood Flow Reperfusion in Diabetic Ischemic Hindlimb

Judith Paquin-Veillette, Farah Lizotte, Stéphanie Robillard, Raphaël Béland, Marc-André Breton, Andréanne Guay, Marc-Antoine Despatis, Pedro Geraldes

Objective—Ischemia caused by narrowing of femoral artery is a major cause of peripheral arterial disease and morbidity affecting patients with diabetes mellitus. We have previously reported that the inhibition of the angiogenic response to VEGF (vascular endothelial growth factor) in diabetic mice was associated with the increased expression of SHP-1 (SH2 domain–containing phosphatase 1), a protein that can be activated by the AT2 (angiotensin II type 2) receptor. Deletion of AT2 receptor has been shown to promote angiogenesis within the ischemic muscle. However, the relative impact of AT2 receptor in diabetic condition remains unknown.

Approach and Results—Nondiabetic and diabetic AT2 null ($Atgr2^{-/Y}$) mice underwent femoral artery ligation after 2 months of diabetes mellitus. Blood perfusion was measured every week ≤4 weeks post-surgery. Expression of the VEGF, SHP-1, and renin–angiotensin pathways was evaluated. Blood flow in the ischemic muscle of diabetic $Atgr2^{-/Y}$ mice recovered faster and ≤80% after 4 weeks compared with 51% recovery in diabetic control littermates. Diabetic $Atgr2^{-/Y}$ had reduced apoptotic endothelial cells and elevated small vessel formation compared with diabetic $Atgr2^{+/Y}$ mice, as well as increased SHP-1 expression and reduced VEGF receptor activity. In endothelial cells, high glucose levels and AT2 agonist treatment did not change SHP-1, VEGF, and VEGF receptor expression. However, the activity of SHP-1 and its association with the VEGF receptors were increased, causing inhibition of the VEGF action in endothelial cell proliferation and migration.

Conclusions—Our results suggest that the deletion of AT2 receptor reduced SHP-1 activity and restored VEGF actions, leading to an increased blood flow reperfusion after ischemia in diabetes mellitus.

Visual Overview—An online visual overview is available for this article. **(*Arterioscler Thromb Vasc Biol*. 2017;37: 2291-2300. DOI: 10.1161/ATVBAHA.117.309977.)**

Key Words: angiotensin receptors ■ diabetes mellitus ■ reperfusion ■ SH2 domain-containing phosphatase 1 ■ vascular endothelial growth factor ■ vascular endothelial growth factor receptor

Peripheral artery disease is a severe and prevalent complication of diabetes mellitus. Among diabetic patients aged over 75 years and suffering from this vascular disease, more than half will die within 5 years.[1] Diabetic patients affected with peripheral artery disease are usually characterized by a decreased blood flow in the lower extremities resulting from occluded arteries. Because of diabetes mellitus–induced insufficient collateral vessel formation, this disease often leads to ulcer formation and eventually amputation.[2] Multiple evidences pointed toward an abnormal angiogenic response to ischemia in diabetes mellitus, which involves different vascular cell types and growth factors.[3]

VEGF (vascular endothelial growth factor) plays a major role in the process known as angiogenesis.[4] By binding to its tyrosine kinase receptors, it permits the activation of several pathways, leading to multiple cellular effects.[5] In particular, VEGF stimulation promotes cell permeability, migration, and proliferation, all of which are essential to collateral vessel formation. The importance of VEGF in ischemia-induced angiogenesis has been demonstrated in several studies in a nondiabetic setting.[6,7] In contrast, the poor collateral vessel formation in diabetes mellitus–induced ischemia is linked to the lack of action of multiple growth factors, including VEGF.[8] In addition to reduced VEGF actions by hyperglycemia, previous studies have demonstrated that diabetes mellitus caused bone marrow–derived progenitor cell dysfunction[9,10] and reduction of extracellular matrix degradation by metalloproteinases,[11] which contributed to poor neovascularization. Despite the presence of oxidative stress, a major regulator of VEGF, VEGF levels are reduced in peripheral muscle of diabetic rodents.[12] Experiments to improve angiogenesis and vascular cell survival by therapeutic agent and system delivery of VEGF by increasing its expression have been reported in nondiabetic animal models.[13,14] However, to date, the clinical

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Nonstandard Abbreviations and Acronyms	
Ang II	angiotensin II
AT1R	angiotensin type I receptor
AT2	angiotensin II type 2
AT2R	angiotensin II type 2 receptor
PKC	protein kinase C
RAS	renin angiotensin system
SH2	Src homology 2
SHP-1	SH2 domain–containing phosphatase 1
VEGF	vascular endothelial growth factor
VEGFR2	VEGF receptor 2

benefits of VEGF administration have been limited.[15,16] Thus, the underlying mechanisms of reduced VEGF expression in diabetic peripheral artery disease remain unresolved. Our group has previously reported that diabetes mellitus caused the activation of the PKC (protein kinase C) delta, leading to the reduction of VEGF receptor activity and lower blood reperfusion in ischemic muscle.[17] The inhibition of the VEGF receptor tyrosine phosphorylation was associated with an elevated expression of SHP-1 (SH2 domain–containing phosphatase 1).

Others pathways are known to play a role in ischemia-induced angiogenesis, notably the angiotensin II (Ang II) receptors pathway.[18] Activation of the renin angiotensin system (RAS) has been shown to promote some of the metabolic syndrome risk factors, including insulin resistance and hyperglycemia.[19] Inhibition of RAS (with angiotensin-converting enzyme inhibitors and angiotensin II receptor blockers) is used to treat several conditions such as high blood pressure, heart failure, and kidney damage associated with diabetes mellitus.[20] In the recent years, RAS inhibitors have been proposed to reduce the risk of developing type 2 diabetes mellitus in high-risk populations.[21] Although RAS is typically known to play a role in blood pressure regulation, it is now established that it can act locally in many tissues, leading to diverse effects depending on which Ang II receptor is activated. AT1R (angiotensin type I receptor) is believed to be the major effector after RAS activation. However, opposing actions can be obtained via stimulation of the AT2R (angiotensin II type 2 receptor), which may play an antagonizing role. One of the many biological processes involving Ang II receptors is angiogenesis. Although AT1R seems to promote angiogenesis,[22] AT2R activation has antiangiogenic properties.[23] Silvestre et al[24] demonstrated that the AT2R negatively regulated ischemia-induced angiogenesis. However, to our knowledge, there is no study that investigated the role of AT2R during blood flow recovery after surgery-induced hindlimb ischemia in a context of diabetes mellitus. In addition, previous studies demonstrated the capacity of AT2R to interact with SHP-1 in rat fetal tissues.[25] Therefore, we hypothesized that AT2R activation may play a role in diabetes mellitus–triggered abnormal angiogenic response through activation of SHP-1.

Materials and Methods

Materials and Methods are available in the online-only Data Supplement.

Results

Deletion of AT2 Receptor Enhanced Blood Flow Reperfusion and Collateral Vessel Formation in Response to Ischemia on Diabetic Limbs

Nondiabetic and diabetic male *Atgr2*$^{-/Y}$ mice and control littermates (*Atgr2*$^{+/Y}$) were subjected to unilateral right femoral artery ligation. Body weight and fasting glucose levels were measured at euthanasia (Table II in the online-only Data Supplement). Blood flow reperfusion was evaluated using the Perimed PIMIII laser Doppler imaging system (Figure 1A). As expected, blood flow reperfusion was reduced to 47% in diabetic *Atgr2*$^{+/Y}$ ischemic limb as compared with nondiabetic *Atgr2*$^{+/Y}$ mice at 62% (P=0.0156). In contrast, diabetic *Atgr2*$^{-/Y}$ exhibited a significant 80% improvement in blood reperfusion (P=0.0062) compared with diabetic *Atgr2*$^{+/Y}$ mice even slightly but not significantly better recovery than nondiabetic *Atgr2*$^{+/Y}$ and *Atgr2*$^{-/Y}$ mice 28 days after the ligation (Figure 1B). One potential mechanism of impaired blood flow reperfusion in diabetes mellitus is because of endothelial cell apoptosis during ischemia. Thus, we have performed in vivo TUNEL (terminal deoxynucleotidyl transferase [TdT] dUTP nick-end labeling) staining of endothelial cells (marked by CD31). As shown in Figure 1C and 1D, the number of endothelial apoptotic positive cells was significantly elevated in the diabetic *Atgr2*$^{+/Y}$ as compared with nondiabetic *Atgr2*$^{+/Y}$ and diabetic *Atgr2*$^{-/Y}$ mice (P<0.0001). Because diabetic patients are at high risk of lower limb amputation, we have assessed limb recovery and necrosis. Impairment of blood flow reperfusion in ischemic limbs of diabetic *Atgr2*$^{+/Y}$ mice was associated with elevated tissue necrosis as compared with nondiabetic counterparts and diabetic *Atgr2*$^{+/Y}$ mice (Figure IA in the online-only Data Supplement).

Collateral vessel formation, a normal response to occlusion of a large artery, is seriously impaired in patients with diabetes mellitus, rendering the tissue downstream more prone to critical ischemia. Because the diabetic *Atgr2*$^{-/Y}$ showed greater improvement of lower limb blood flow reperfusion after ischemia, we have assessed vascular density and capillary formation in the ischemic adductor muscles. Histological analysis indicated that adductor muscles of diabetic *Atgr2*$^{+/Y}$ mice exhibited a significant 39% (P=0.0448) vascular density reduction as compared with nondiabetic *Atgr2*$^{+/Y}$ mice, a phenomenon that was not observed in the diabetic *Atgr2*$^{-/Y}$ mice (Figure 2A and 2B). To evaluate the proportion of small vessel density, the diameter of vessels of 50 μm or less was considered as capillaries. Thus, the deterioration of the capillary density in diabetic *Atgr2*$^{+/Y}$ mice was associated with a significant 57% reduction in number of small vessels (Figure 2C and 2D). Interestingly, diabetic *Atgr2*$^{-/Y}$ mice had similar capillary density and number of small vessels (<50 μm) as compared with nondiabetic *Atgr2*$^{+/Y}$ and *Atgr2*$^{-/Y}$ mice (Figure 2B and 2D).

Deletion of AT2 Receptor Promoted VEGF Receptor Activation

VEGF activation plays a critical role in the induction of the angiogenesis process. Expression of VEGF-A (Figure 3A), VEGFR2 (VEGF receptor-2 or Flk-1/KDR [fetal liver kinase-1/

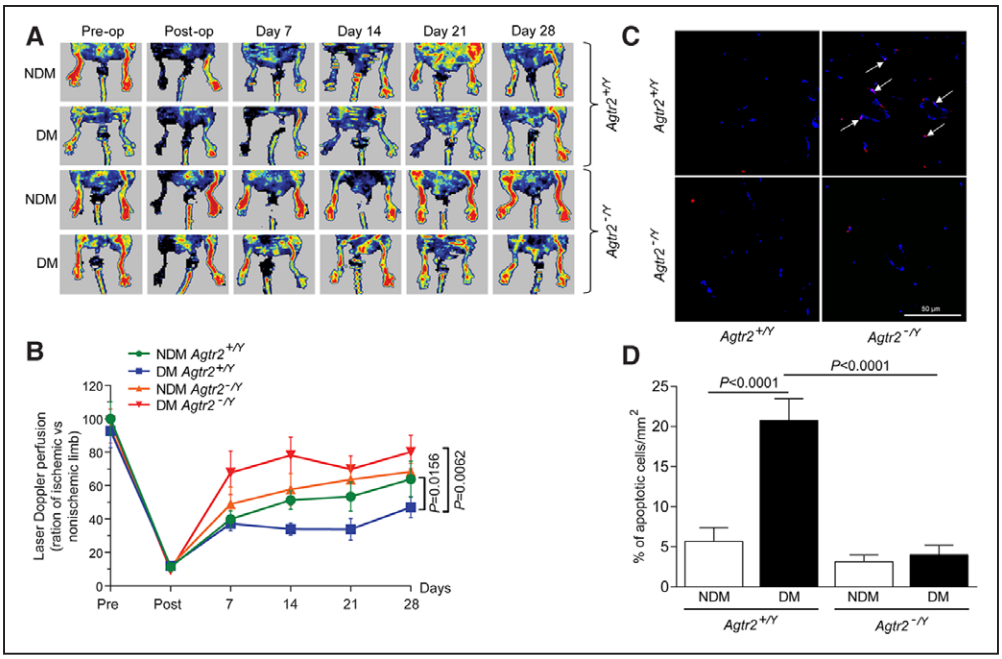

Figure 1. Blood flow reperfusion and recovery limb from ischemia. **A**, Laser Doppler imaging and (**B**) reperfusion analysis of nondiabetic (NDM) and diabetic (DM) *Atgr2*$^{+/Y}$ and *Atgr2*$^{-/Y}$ mice. **C**, Immunofluorescence images of endothelial cells (CD31; blue) and apoptotic positive cells (red). **D**, Quantification of the number of apoptotic positive endothelial cells in the ischemic adductor muscles of NDM (white bars) and DM (black bars) *Atgr2*$^{+/Y}$ and *Atgr2*$^{-/Y}$ mice. Results are shown as mean±SEM of 10 to 12 mice per group (**A** and **B**) and 7 to 8 mice per group (**C** and **D**). *P*=0.0156 vs NDM *Atgr2*$^{+/Y}$, *P*=0.0062 vs DM *Atgr2*$^{-/Y}$.

kinase insert domain receptor]; Figure 3B) and HIF-1α (hypoxia-inducible factor-1 alpha; Figure IB in the online-only Data Supplement) at the mRNA and protein (Figure 3C) levels were decreased by diabetes mellitus in *Atgr2*$^{+/Y}$ and were not prevented by the absence of the AT2R in diabetic *Atgr2*$^{-/Y}$ mice (Figure 3A through 3C; Figure IA in the online-only Data Supplement). These data suggest that the AT2 receptors did not influence the expression of VEGF and its receptor in diabetes mellitus. We have previously shown that the tyrosine phosphorylation of the VEGFR2 was reduced in diabetic mice.[17] We have replicated our previous finding showing that the phosphorylation of VEGFR2 was significantly decreased in diabetic *Atgr2*$^{+/Y}$ mice, an effect that was completely restored in diabetic *Atgr2*$^{-/Y}$ mice (Figure 3D), suggesting that the activation of AT2 receptor participated in VEGFR2 inhibition in diabetes mellitus. One of the downstream target of VEGFR2 is phosphorylation of Akt (protein kinase B). Our data demonstrated that the phosphorylation of Akt was bunted in diabetic *Atgr2*$^{+/Y}$ mice but not in diabetic *Atgr2*$^{-/Y}$ mice. Interestingly, despite reduction of phosphorylation of Akt, phosphorylation of eNOS (endothelial nitric oxide synthase), a downstream target of VEGF-stimulated Akt, was not affected (Figure IC in the online-only Data Supplement).

Expression of SHP-1 Is Prevented in Absence of the AT2 Receptor

The phosphorylation of specific tyrosine residues within the receptor tyrosine kinase, such as VEGFR2, creates binding sites for phosphotyrosine-binding domain– and SH2 (Src homology 2) domain–containing protein. These domains can recruit specific proteins that either activate downstream signals (Src and phospholipase C) or downregulate receptor tyrosine kinase, including the protein tyrosine phosphatase. We have previously showed that diabetes mellitus–induced inhibition of VEGFR2 in ischemic muscles was associated with an increased expression of SHP-1.[17] These observations were confirmed in our study demonstrating that SHP-1 mRNA (Figure 4A) and protein (Figure 4B) expression were upregulated in ischemic adductor muscles of diabetic *Atgr2*$^{+/Y}$ mice, without affecting SHP-2 or VEPTP (vascular endothelial protein tyrosine phosphatase) expression (Figure 4B and 4C). In contrast, diabetic *Atgr2*$^{+/Y}$ mice did not exhibit elevated SHP-1 mRNA and protein levels, suggesting that enhanced expression of SHP-1 can be regulated by the activation of the AT2 receptor in diabetes mellitus. Furthermore, the increased expression of SHP-1 in diabetic ischemic muscle enhanced its interaction with the VEGFR2 and reduced VEGFR2 tyrosine phosphorylation, which was not observed in diabetic *Atgr2*$^{-/Y}$ mice (Figure 4E). These data suggest that AT2R regulates SHP-1 activity and interaction with VEGFR2 in diabetic ischemic muscle.

High Glucose Exposure and AT2 Activation Lead to Endothelial Cell Proliferation and Migration Inhibition

To explain how diabetes mellitus and activation of the AT2 receptor reduced proangiogenenic actions of VEGF on endothelial cell capacity to proliferate and migrate, we have performed in vitro cell count and migration scratch assays using the AT2 receptor agonist CGP42112A and antagonist PD123319. As expected, stimulation of endothelial cells with VEGF-A (25 ng/mL) for 24 hours promoted cell proliferation in normal glucose levels (Figure 5A). Cells exposed to

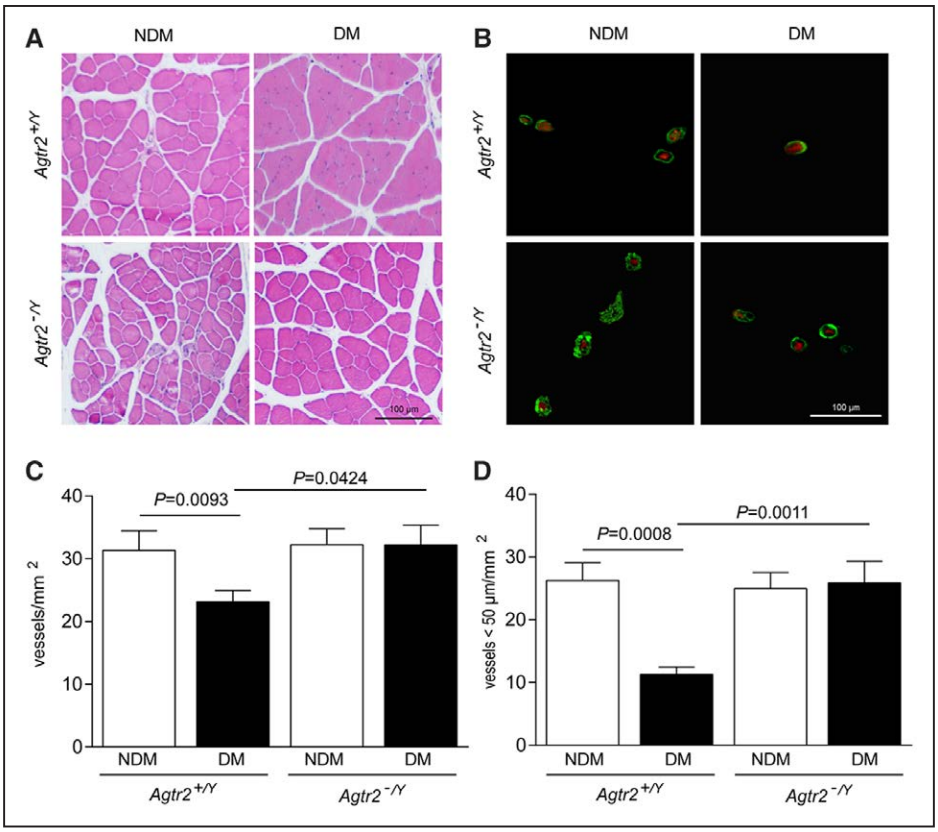

Figure 2. Histological and vascular density analysis. **A**, Structural analysis of the ischemic muscles stained with hematoxylin and eosin (H&E) and (**B**) immunofluorescence of endothelial cells (red) and α–smooth muscle actin (green) labeling in the ischemic adductor muscles of nondiabetic (NDM; white bars) and diabetic (DM; black bars) *Atgr2*$^{+/Y}$ and *Atgr2*$^{-/Y}$ mice. **C**, Quantification of the number of vessel per mm^2 and (**D**) the number of vessels smaller than 50 µm. Results are shown as mean±SEM of 3 sections of 7 mice per group.

high glucose levels (25 mmol/L) or treated with CGP42112A (10 nmol/L) alone completely prevented VEGF-induced endothelial cell proliferation (Figure 5A). In contrast, treating endothelial cells with PD123319 (10 umol/L) during high glucose exposure restored VEGF-induced cell proliferation (Figure 5A). Migration is another important process involved in angiogenesis. Using Ibidi's insert to create equal separation between cells (500 µm) in each experiment, the treatment with VEGF in normal glucose concentration promoted endothelial cell migration from 25% of the surface occupied by the endothelial cells to 57% of the surface area (Figure 5B and 5C). Exposing cells to high glucose concentrations for 24 hours reduced VEGF effect on cell migration with only 35% of the surface area. Interestingly, the AT2 agonist was as potent as high glucose level treatment of decreasing VEGF action on endothelial cell migration (Figure 5B and 5C). No additive effect was noted when cells were exposed to high glucose levels in presence of the CGP42112A.

High Glucose and AT2 Agonist Reduced VEGF Signaling Pathway Through SHP-1 Activity on the VEGF Receptor

VEGF stimulation induces Akt phosphorylation as the main signaling pathway of VEGF-induced cell migration and survival. Therefore, we have evaluated the Akt signaling pathway on VEGF stimulation. As shown in Figure 6A, VEGF raised Akt phosphorylation by more than 3-fold, an effect that was reduced by 45% and 64% in endothelial cells exposed to CGP42112A alone or high glucose levels for 24 hours, respectively. The combined treatment (high glucose concentrations and CGP42112A) completely blunted VEGF-stimulated Akt phosphorylation (Figure 6A). In contrast, treating endothelial cells with the AT2R antagonist enhanced VEGF-induced Y1059 and Y1175 of VEGFR2, as well as Akt phosphorylation in both normal and high glucose concentrations (Figure 6B). Since our in vivo data demonstrated that diabetes mellitus increased SHP-1 expression in ischemic muscle, we have assessed the expression of SHP-1 in endothelial cells after exposure to high glucose concentrations and AT2 activation. Surprisingly, neither high glucose level exposure and CGP42112A treatment influenced the expression of SHP-1 and SHP-2 in endothelial cells (Figure 6C). However, the measurement of the phosphatase activity of SHP-1 was significantly increased by 2-fold and 2.5-fold in endothelial cells treated with the CGP42112A and exposed to high glucose levels, respectively (Figure 6D). Interestingly, elevated SHP-1 activity by either CGP42112A and HG levels was associated to increase interaction of SHP-1 with the VEGFR2 receptor in coimmunoprecipitation assays (Figure 6E). In contrast, increased interaction of SHP-1 with VEGFR2 in endothelial cells exposed to high glucose levels was abrogated with the AT2R antagonist treatment (Figure 6F).

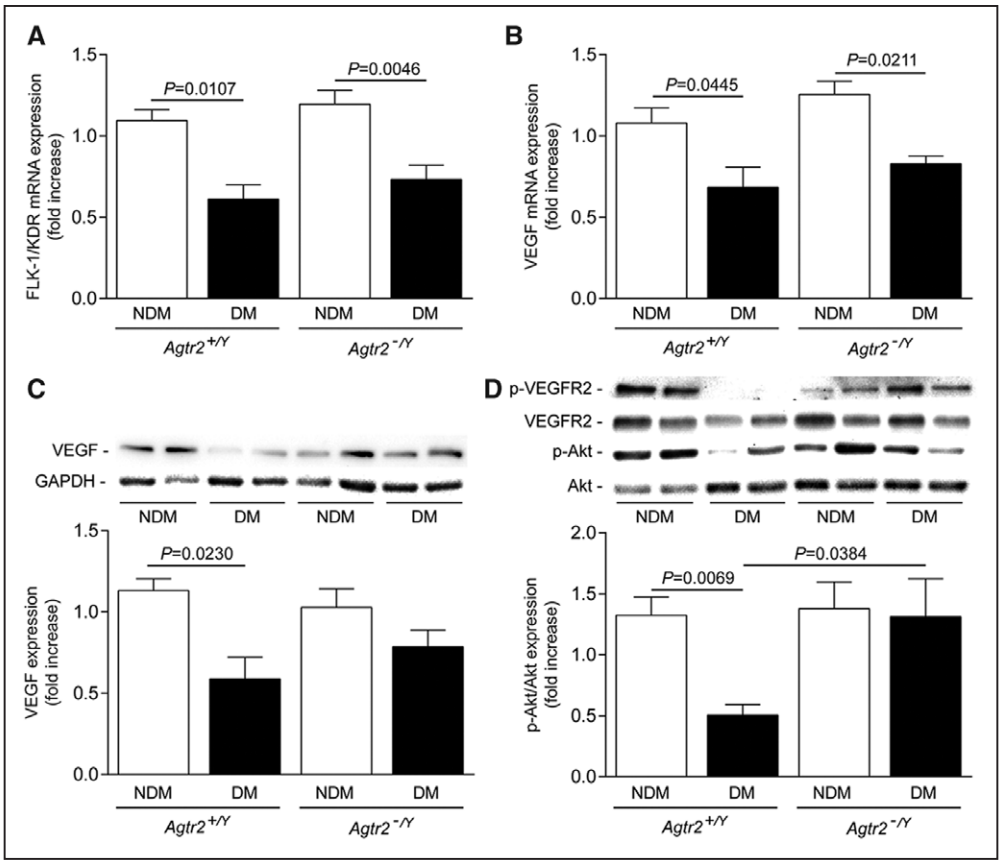

Figure 3. Expression and signaling activity of the VEGF (vascular endothelial growth factor) pathway. Quantitative real-time polymerase chain reaction (PCR) of (**A**) Flk-1/KDR (fetal liver kinase-1/kinase insert domain receptor) and (**B**) VEGF. **C**, Protein expression of VEGF and (**D**) phosphorylation of VEGFR2 and Akt (protein kinase B) in ischemic adductor muscles of nondiabetic (NDM; white bars) and diabetic (DM; black bars) *Atgr2*$^{+/Y}$ and *Atgr2*$^{-/Y}$ mice. The densitometry quantification was measured. Results are shown as mean±SEM of 6 mice per group.

Discussion

The activation of the RAS is recognized to be an important contributing factor of the development and progression of vascular pathology in diabetes mellitus. Inhibition of the activity of Ang II, the main mediator of the RAS, with pharmacological inhibitors such as angiotensin receptor blockers and angiotensin-converting enzyme inhibitors are the main treatment for patients with diabetes mellitus and renal/cardiac disease.[20] Of the 2 major receptors of the Ang II, AT2R is widely and abundantly expressed in fetal tissue,[26] declining after birth. Despite some functional evidence that AT2R is present in adult vasculature, the majority of studies have focused on AT2R during pathophysiological conditions.[27] Our study provided evidence that AT2R activation is involved in diabetes mellitus–induced poor collateral vessel formation after ischemia. Activation of ATR2 resulted in the reduction of endothelial cell proliferation and migration because of the enhanced phosphatase activity of SHP-1 that reduced the proangiogenic actions of VEGF in endothelial cells.

A previous study reported that AT2R induced antiangiogenic effects in mice after hindlimb ischemia with or without Ang II infusion.[24] Our results in nondiabetic *Atgr2*$^{-/Y}$ mice corroborated these findings showing that the absence of AT2R favored neovascularization and blood flow reperfusion. Dysfunction of vessel regeneration during ischemia is a characteristic of people with diabetes mellitus, which can ultimately affect the development, severity, and outcomes of foot ulcerations, including infection, and other common comorbidities. Interestingly, diabetes mellitus–induced poor collateral vessel formation was completely abolished in diabetic *Atgr2*$^{-/Y}$ mice, indicating that AT2R activation could play an important role on high glucose level inhibitory effects on angiogenesis. The absence of the AT2R in diabetic mice promoted the formation of new small vessel (<50 μm) as compared with the diabetic *Atgr2*$^{+/Y}$ mice. It is also interesting to note that diabetes mellitus raised AT1R expression, known to favor angiogenesis, but not the AT2R, angiotensin-converting enzyme 1, and angiotensin-converting enzyme 2 in ischemic adductor muscle that was prevented in diabetic *Atgr2*$^{-/Y}$ mice (Figure IIA through IID in the online-only Data Supplement). Although 4 weeks evaluation of blood flow reperfusion is commonly used in mouse model of hindlimb ischemia, acute limb ischemia, defined as any sudden reduction in leg perfusion because of thrombosis and embolism, is a clinical threat to limb viability. Therefore, additional experiments will be required to determine whether AT2 receptor activation can be beneficial during acute limb ischemia.

Probably, the most important player in new vessel formation are the blood vessel lining endothelial cells. It is well

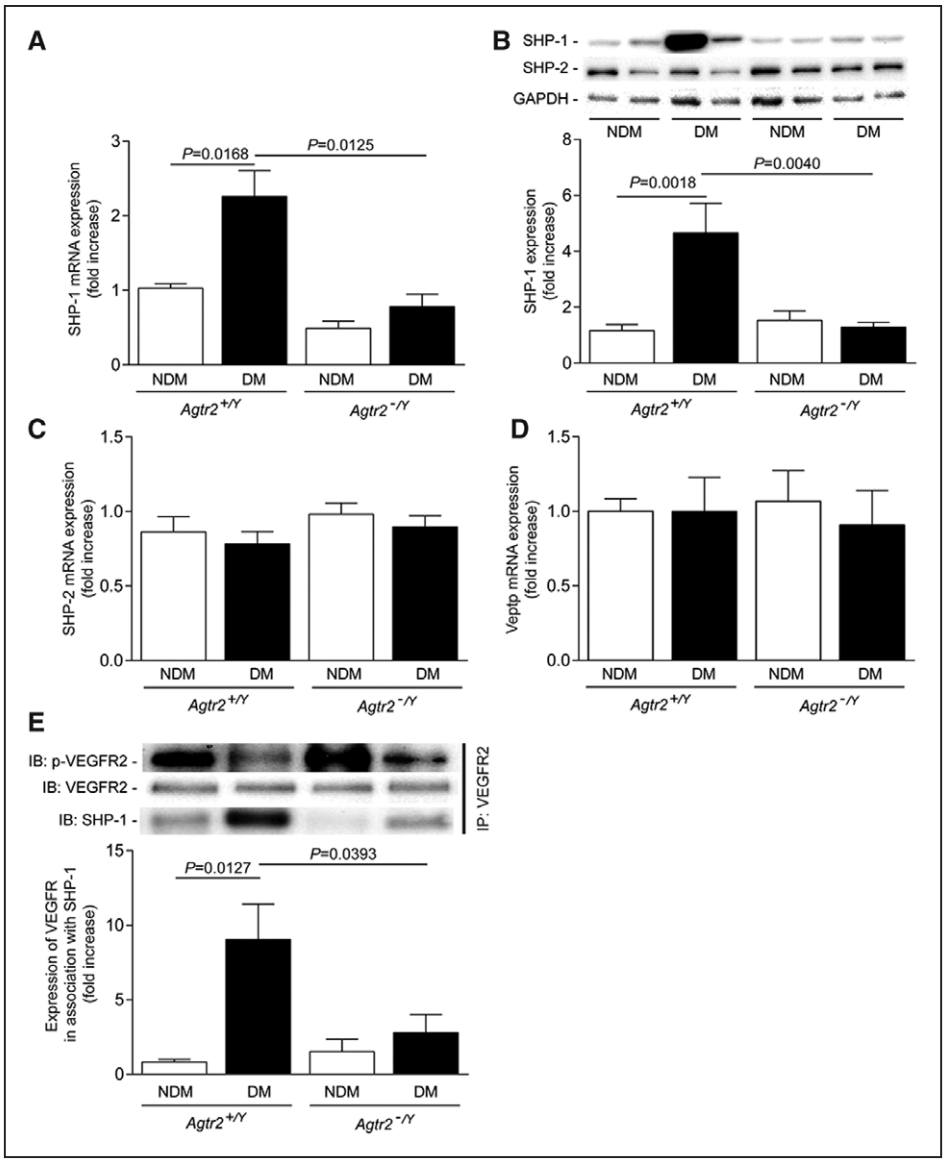

Figure 4. Increased expression of SHP-1 (SH2 domain–containing phosphatase 1) in muscles of diabetic mice. SHP-1, SHP-2, and VEPTP (vascular endothelial protein tyrosine phosphatase; **A**, **C**, and **D**) mRNA and (**B**) protein expression, as well as (**E**) coimmunoprecipitation assay of SHP-1, VEGFR2 (vascular endothelial growth factor receptor 2), and phospho-tyrosine of VEGFR2 were measured in ischemic adductors muscles of nondiabetic (NDM; white bars) and diabetic (DM; black bars) *Atgr2*$^{+/Y}$ and *Atgr2*$^{-/Y}$ mice. The densitometry quantification was evaluated. Results are shown as mean±SEM of 5 (**B** and **E**) and 6 (**A**, **C**, and **D**) mice per group.

established that high glucose levels drastically change endothelial cell metabolism and cause its dysfunction. AT2R is also capable of regulating a class of genes involved in endothelial cell migration and growth-inhibiting factors.[28] Under hypoxia, Ang II promoted endothelial sprout formation, a response that was blocked by AT2R antagonist PD123319 but not the losartan, an AT1R inhibitor.[29] However, previous publications provided conflicting data for the role of AT2R in endothelial cell proliferation and apoptosis. Benndorf et al[30] demonstrated that the activation of the AT2R caused inhibition of VEGF-induced endothelial cell migration and tubule formation. Another study showed that AT2R activation reduced endothelial cell migration by enhancing integrin β like-1 expression that has been associated with the attachment of endothelial cell to the extracellular matrix.[31] In contrast, in a retinal angiogenesis, blockage of AT2R using the PD123319 reduced retinal endothelial cell growth.[32] Furthermore, AT2R activation with C21, a selective agonist, induced the upregulatin of VEGF through the stimulation of the Akt/mTOR (mammalian target of rapamycin) signaling pathway in ischemic neurones.[33] In our study, the absence of AT2R reduced endothelial cell undergo apoptosis after ischemia in a context of diabetes mellitus. Furthermore, both high glucose level exposure and treatment of AT2R agonist prevented VEGF-induced VEGFR2 and Akt phosphorylation, as well as endothelial cell proliferation and migration, whereas treatment with PD123319 enhanced VEGF actions. Overall, these data suggest that the activation of AT2R may generate diverse responses depending on the endothelial cell localization and milieu. Interestingly, a recent elegant

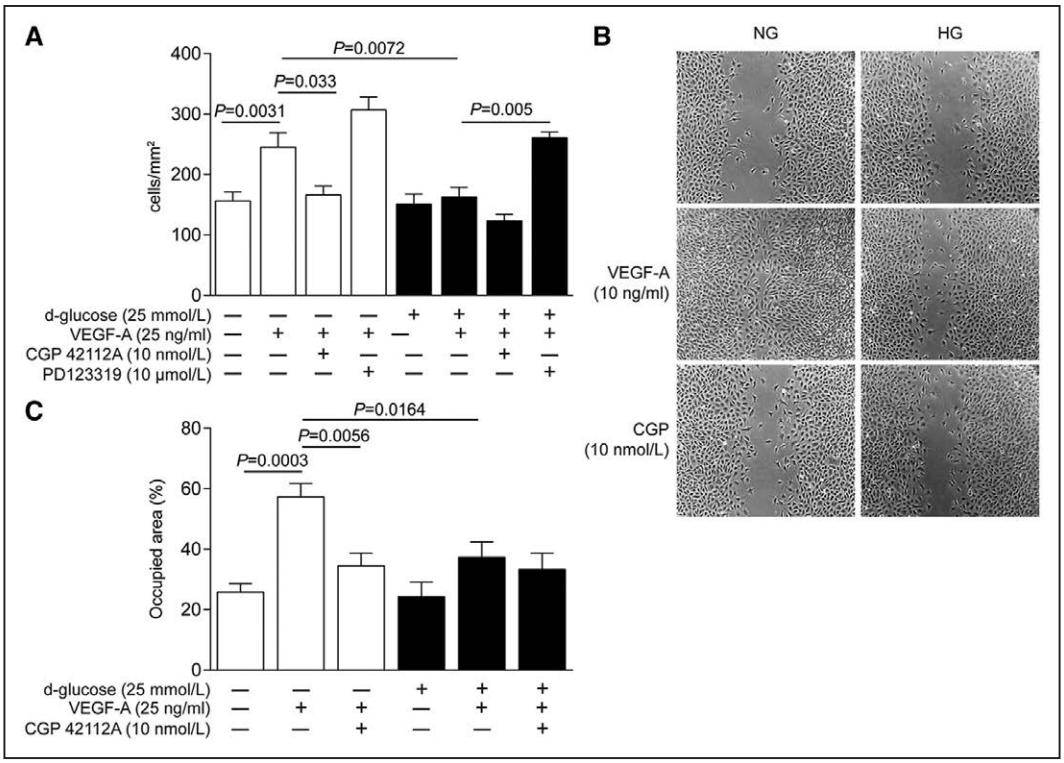

Figure 5. High glucose level and AT2 (angiotensin II receptor type 2) agonist exposure reduced VEGF (vascular endothelial growth factor)–induced cell proliferation and migration. Endothelial cells were incubated with normal glucose (NG; 5.6+19.4 mmol/L of mannitol; white bars) and high glucose (HG; 25 mmol/L; black bars) for 24 h and then stimulated with CGP42112A, PD123319, and VEGF for another 24 h. **A**, Cells were fixed and stained with DAPI (4',6-diamidino-2-phenylindole). Number of cells were counted using the NIS-Elements software of Nikon eclipse Ti microscope. **B**, Representative images of the cell migration assay using the Ibidi's insert. **C**, The percentage of the surface area occupied was quantified. Results are shown as mean±SEM of 4 to 6 independent experiments.

study using the crystal structure of the human AT2R showed that the noncanonical position of the helix VIII prevented the recruitment of G-couple proteins or β-arrestins, providing new insight on designing better selective ligands of the angiotensin receptors.[34]

The activation of both SH2-domain protein phosphatase SHP-1 and SHP-2 is considered to be a critical downstream effect of Ang II stimulation.[35,36] It has been reported that Ang II stimulation generated opposite effects through the activation of AT1R and AT2R. Previous studies have demonstrated in vascular smooth muscle cells that Ang II stimulation activated ERK and Pyk kinase through AT1R-promoting cell survival, whereas AT2R/SHP-1 phosphatase activity attenuated this process.[37,38] Sohn et al[39] indicated that the stimulation of AT2 increased tyrosine phosphatase activity in human umbilical vein endothelial cells. Our data suggest that both high glucose exposure and activation of AT2R induced SHP-1 phosphatase activity and interaction with VEGFR2 that caused the inhibition of VEGF proangiogenic effects on cell proliferation and migration. Interestingly, in vivo data showed that diabetes mellitus–increased binding of SHP-1 with VEGFR2 reduced VEGFR2 phosphorylation in ischemic muscle that was partially prevented in diabetic *Atgr2*$^{-/Y}$ mice, suggesting that AT2R activation is not the solo pathway that decreased VEGFR2 activity in diabetes mellitus. Another study reported that SHP-2 phosphatase activity is necessary for Ang II–induced degradation of Bcl-xl, causing cell apoptosis.[40] However, in our study, we did not observe change in SHP-2 expression, phosphatase activity, and interaction with VEGFR2.

The focus of this article was on AT2R actions on endothelial cell function. Interestingly, in contrast to our in vivo findings, cultured cell data indicated that hyperglycemia and AT2R activation did not change the expression of SHP-1. Therefore, we cannot exclude the possibility that the absence of AT2R in smooth muscle cells may have contributed to the blood flow recovery in diabetic *Atgr2*$^{-/Y}$ mice. Although no consensus has been reached on the biological consequence of AT2R activation, previous studies have reported that AT2R exerts antigrowth and proapoptotic effects in vascular smooth muscle cells in vitro and in vivo.[41,42] Furthermore, other studies have demonstrated that AT2R stimulation increased the formation of the SHP-1/ATIP complex, leading to its translocation to the nucleus, which caused inhibition of smooth muscle cell proliferation, inflammation, and oxidative stress in femoral artery after cuff placement.[43,44] Thus, the deletion of AT2R in smooth muscle cells may have participated to vessel maturity and stability because of the proliferation of smooth muscle cells. In addition, we cannot ignore the potential impact of AT2R activation on bone marrow–derived progenitor cell function. Previous study demonstrated that Ang II can induce epicardial progenitor cell differentiation into smooth muscle cells.[45] However, further experiments will be required to investigate the effects of AT2R activation

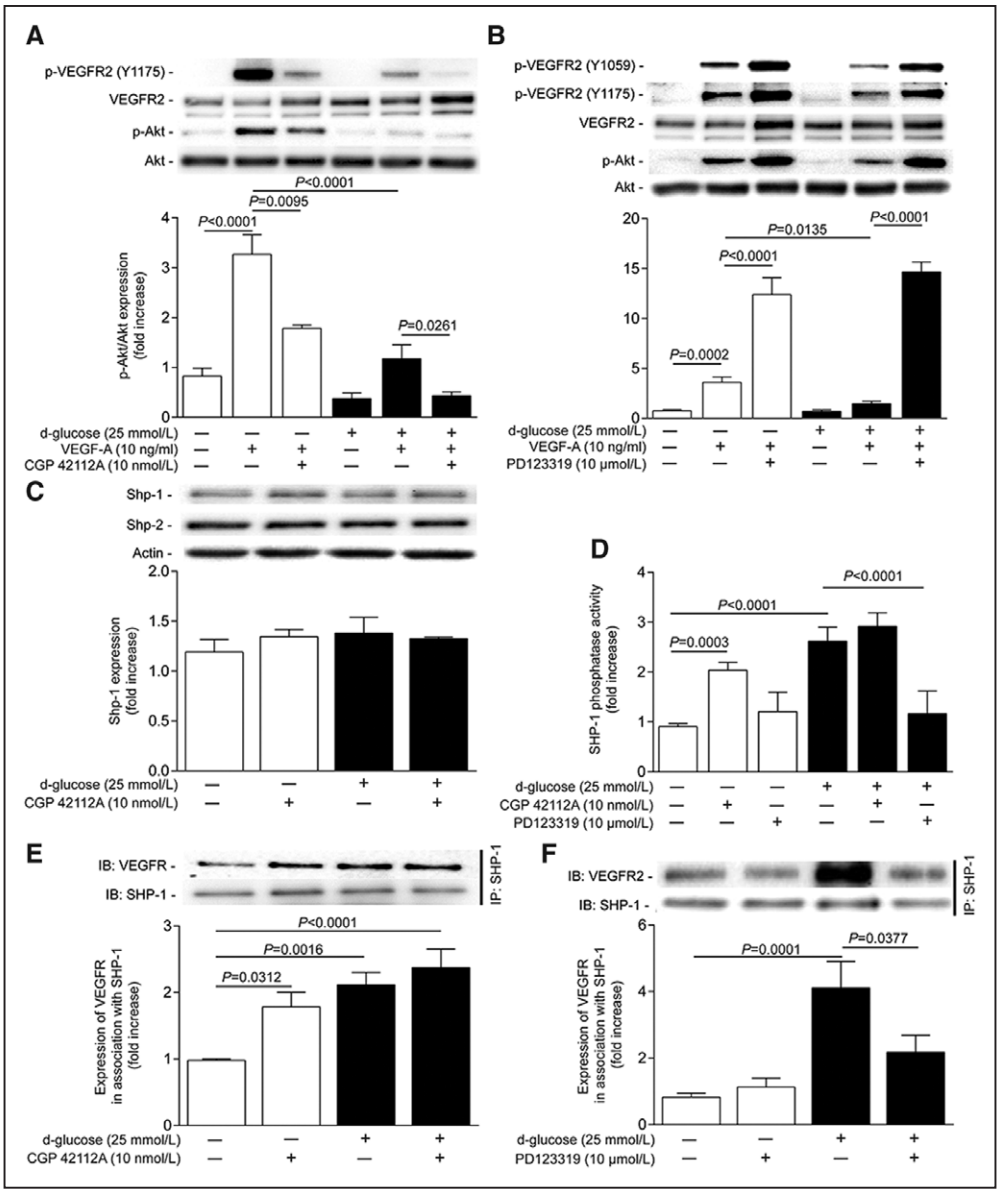

Figure 6. Increased activity of SHP-1 (SH2 domain–containing phosphatase 1) caused inhibition of VEGF (vascular endothelial growth factor) signaling pathways in high glucose condition and with the AT2 (angiotensin II type 2) receptor agonist. Endothelial cells were incubated with normal glucose (NG; 5.6+19.4 mmol/L of mannitol; white bars) and high glucose (HG; 25 mmol/L; black bars) for 24 h in presence of CGP42112A or PD123319 and then stimulated with VEGF for 10 minutes. **A** and **B**, Phosphorylation of VEGF and Akt (protein kinase B), as well as total expression VEGF and Akt. **C**, Protein expression of SHP-1 and SHP-2 were detected by immunoblot analysis. **D**, Phosphatase activity of SHP-1 and coimmunoprecipitation assay of SHP-1 and VEGFR2 in presence of (**E**) CGP42112A or (**F**) PD123319. The densitometry quantification was measured. Results are shown as mean±SEM of 6 to 8 independent experiments.

in smooth muscle cells, as well as in endothelial progenitor cell function in the context of diabetes mellitus.

In conclusion, our article provided evidence that the AT2 receptor in diabetes mellitus promoted the activity of SHP-1, which was associated with the VEGF receptor, inhibition of VEGF actions on endothelial angiogenesis, and poor collateral vessel formation.

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Disclosures

None.

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Highlights

- Deletion of the AT2 (angiotensin II type 2) receptor improved vascular density and blood flow reperfusion of the hindlimb after ischemia in diabetic mice.
- Activation of AT2 receptor and high glucose level exposure enhanced SHP-1 (SH2 domain–containing phosphatase 1) activity in endothelial cells.
- SHP-1 interacted with VEGFR2 (vascular endothelial growth factor receptor 2), reducing its tyrosine phosphorylation on AT2 receptor stimulation and high glucose level exposure in endothelial cells.
- Inhibition of AT2 receptor prevented high glucose level–induced SHP-1 activity and restored VEGF angiogenic actions on endothelial cell proliferation and migration.

Arteriosclerosis, Thrombosis, and Vascular Biology

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Deletion of AT2 Receptor Prevents SHP-1–Induced VEGF Inhibition and Improves Blood Flow Reperfusion in Diabetic Ischemic Hindlimb

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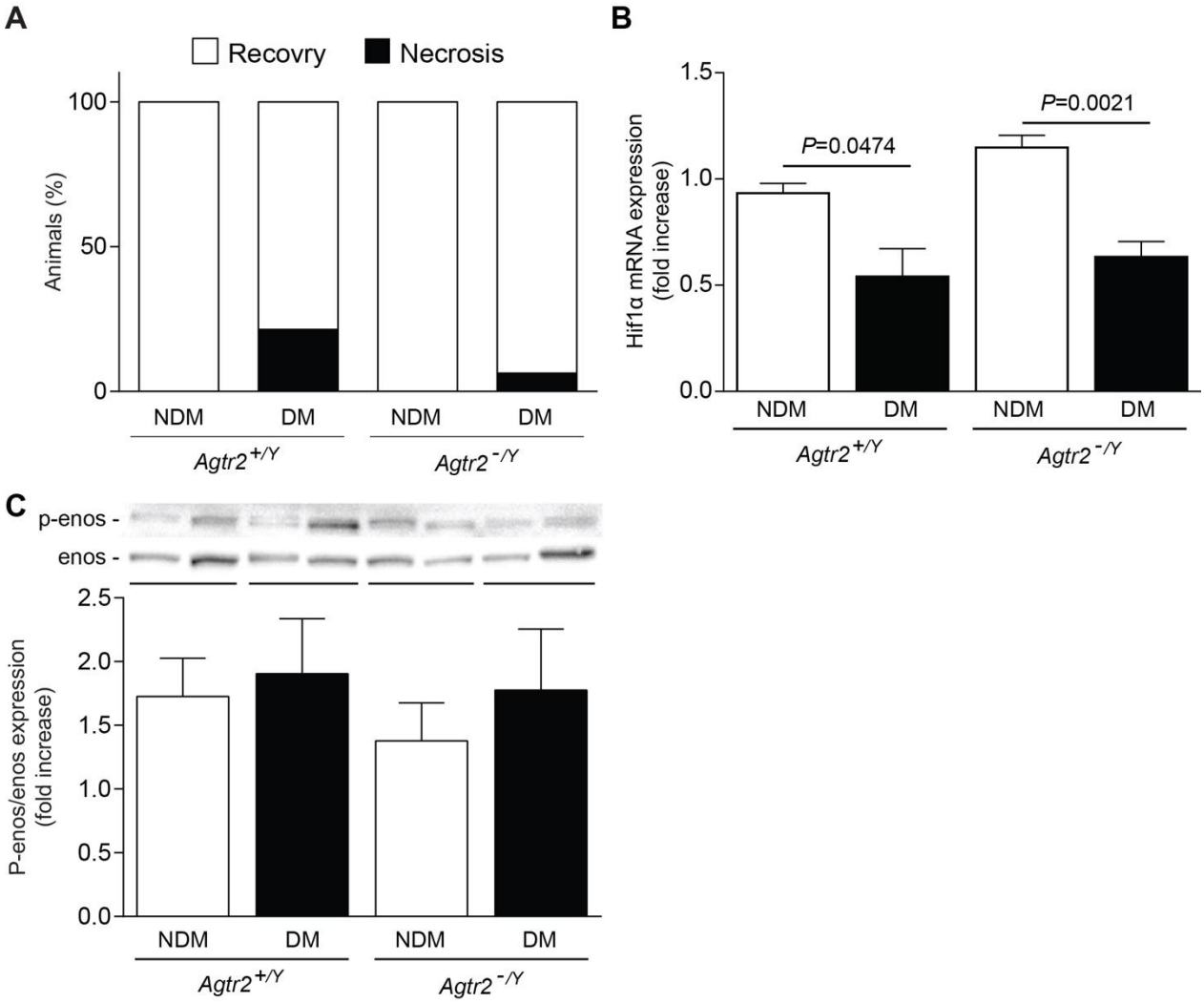
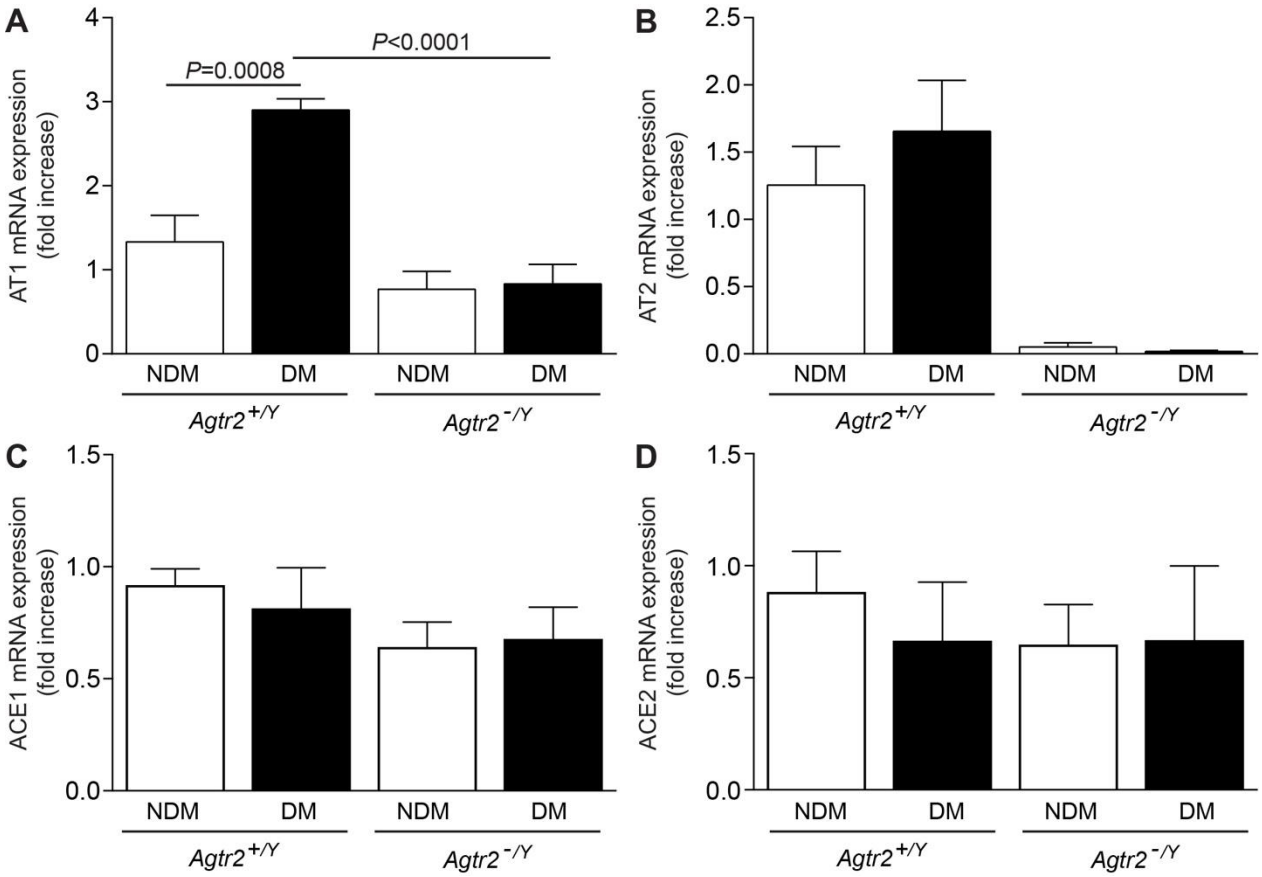
SUPPLEMENTAL MATERIAL



Supplementary figure I: (A) Morphological and observational analysis of necrosis and amputation of post-surgery. Quantitative real-time PCR of (B) HIF-1α mRNA expression and (C) protein expression of phospho-eNOS and eNOS in ischemic adductors muscles of non-diabetic (white bars) and diabetic (black bars) *Atgr2*$^{+/Y}$ and *Atgr2*$^{-/Y}$ mice. The densitometry quantitation was measured. Results are shown as mean ± SEM of 6 mice.



Supplementary figure II: Expression of the RAS pathway. Quantitative real-time PCR of (A) AT1 receptor, (B) AT2 receptor, (C) ACE1 and (D) ACE2 mRNA expression in ischemic adductors muscles of non-diabetic (white bars) and diabetic (black bars) *Atgr2*$^{+/Y}$ and *Atgr2*$^{-/Y}$ mice. Results are shown as mean ± SEM of 6 mice.

Supplementary table I. Sequences of primers

Gene	Forward	Reverse
mVEGF	GGAGTACCCCGACGAGATAGAGTA	AGCCTGCACAGCGCATC
mKDR/Flk-1	AGAACACCAAAAGAGAGAGGAACG	GCACACAGGCAGAAACCAGTAG
meNOS	GTTTGTCTGCGGCGATGTC	GAATTCTCTGCACGGTTTGCA
mSHP-1	ATCAATGCCAACTACGTGAAGAAC	GGCTGGCGATGTAGGTCTTAGA
mSHP-2	CCTCAACACAACTCGTATCAATGC	TGTTGCTGGAGCGTCTCAAA
mAT1	AACCTGCGGAGTAGCAACGT	AGCAGCGAGGCACTTCCA
mAT2	CCACCAGCAGAAACATTACC	GGACTCATTGGTGCCAGTT
mACE1	TCCAGAGGGCATTGACCTAGA	TTGGGCTGTCCGGTCATA CT
mACE2	GAAGCGGGAGATCGTTGGT	GGGTCACAGTATGTTTCATCATGA G
mHIF-1α	CACCGATTCGCCATGGA	TCGACGTTCAGAACTCATCTTTTT
mGAPDH	GCATGGCCTTCCGTGTTC	GATGTCATCATACTTGGCAGGTTT
bosSHP-1	AGCGCTCGGGCATGGT	CGATGGCCACGTAGATGAACT
bosSHP-2	GGAGAGAAATTTGCCACTTTGG	TAGTTGCCCGTGATGTTCCA
bosVeptp	GGTAGGACTGGAACCTTTATTGCA	TCCATAAATGTCCACAGAATCTTTG
BosGAPDH	TGGAAAGGCCATCACCATCT	GCATCACCCCACTTGATGTTG

Supplementary Table II. Body weight and fasting glucose levels of non-diabetic (*Ins2*$^{+/+}$) and diabetic (*Ins2*$^{+/C96Y}$) *of Agtr2*$^{+/Y}$ and *Agtr2*$^{-/Y}$ mice.

	Agtr2$^{+/Y}$		*Agtr2*$^{-/Y}$	
	Ins2$^{+/+}$	*Ins2*$^{+/C96Y}$	*Ins2*$^{+/+}$	*Ins2*$^{+/C96Y}$
Body Weight (g)	29.6 ± 2.7	20.1 ± 1.4	27.0 ± 3.9	20.8 ± 1.6
Blood glucose levels (mg/dl)	139 ± 37	435 ± 61	134 ± 31	404 ± 60

Research Design and Methods

Reagents and antibodies – Primary antibodies for immunoblotting were obtained from commercial sources, including actin (horseradish peroxidase [HRP]; I-19), GAPDH HRP (V18), SHP-1 (C19), VEGF (147), and nitric oxide synthase (NOS) 3 (C-20) from Santa Cruz Biotechnology Inc (Dallas, TX); protein kinase B (Akt), phospho-Akt (D9E),p-VEGF receptor 2 (VEGFR2; Y1175 and Y1059), VEGFR2, p-ENOS (s1177) from Cell Signaling (Danver, MA); anti-α smooth muscle actin from Abcam (Toronto, ON); anti-CD31 from BD Bioscience(Mississauga, ON); and anti-SHP-2 from Millipore. Secondary antibodies of anti-rabbit and -mouse peroxidase-conjugated were purchased from Cell Signaling. Fetal bovine serum (FBS), phosphate-buffered saline (PBS), penicillin-streptomycin, and DMEM low glucose (31600-034) were obtained from Invitrogen (Burlington, ON). VEGF-A_{165} was purchased from R&D (Minneapolis, MN). CGP-42112A (CGP) was purchased from Bachem (Torrance, CA). (H-9395). Collagenase type 1 was purchased from Wortington Biochemical Corporation (Lakewood, NJ). All other reagents used, including EDTA, d-glucose, d-mannitol, leupeptin, phenylmethylsulfonyl fluoride, aprotinin, and Na3VO4 were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise stated.

Animal and experimental design – C57BL/6J (*Ins2*$^{+/+}$) and diabetic heterozygous male *Ins2*$^{+/C96Y}$ (DM; Akita) mice were purchased from the Jackson Laboratory and bred with the *Agtr2*$^{-/Y}$ mice (generous gift from Prof. N. Gallo-Payet, originated from Prof. T. Inagami, Department of Biochemistry, Nashville University, Nashville, TN, USA)[1] in our animal facility. Throughout the period of study, animals were provided with free access to water and standard rodent chow (Harlan Teklad). All experiments were conducted in accordance with the Canadian Council of Animal Care and Institutional Guidelines and were approved by the Animal Care and Use Committees of the University of Sherbrooke, according to National Institutes of Health (NIH) guidelines.

Hindlimb ischemia model – We have assessed blood flow in non-diabetic (NDM; *Ins2*$^{+/+}$) and four months-old diabetic (DM; *Ins2*$^{+/C96Y}$) *Agtr2*$^{+/Y}$ and *Agtr2*$^{-/Y}$ mice. Animals were anesthetized and the entire lower extremity of each mouse was shaved. A small incision was made along the thigh all the way to inguinal ligament and extending superiorly towards the mouse abdomen. Femoral artery was isolated from the femoral nerve and vein, and ligated distally to the origin of the arteria profunda femoris. The incision was closed by interrupted 5-0 sutures (Syneture).

Laser Doppler Perfusion Imaging and physical examination – Hindlimb blood flow was measured using a laser Doppler perfusion imaging (PIMIII) system (Perimed Inc) as previously described[2]. Consecutive perfusion measurements were obtained by scanning the region of interest (hindlimb and foot) of anesthetized animals. Measurements were performed pre- and post-artery ligation, and additionally on postoperative days 7, 14, 21 and 28. To account for variables that affect blood flow temporally, the results at any given time were expressed as a ratio against simultaneously obtained perfusion measurements of the right (ligated) and left (non-ligated) limb. Tissue necrosis was scored to assess mice that had to be euthanized during the course of the experiment due to necrosis/loss of toes.

Histopathology –Right and left abductor muscles from NDM and DM *Agtr2*$^{+/Y}$ and *Agtr2*$^{-/Y}$ mice were harvested for pathological examination and sections were fixed in 4% paraformaldehyde (Sigma-Aldrich) for 18 h and then transferred to 70% ethanol for light microscopy. Paraformaldehyde-fixed tissue was embedded in paraffin and 4 μm sections were stained with hematoxylin & eosin (Sigma-Aldrich).

Immunofluorescence – Cross-sections of adductor muscles of each group were blocked with 10% goat serum for 1 h and were exposed in sequence to primary antibodies (CD31 (1:50) and α-smooth muscle actin, 1:200) overnight, followed by incubation with secondary antibodies Alexa-488 conjugated anti-rabbit IgG and Alexa-594 conjugated anti-mouse (1:400), purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). Apoptotic cells were detected using the TACS 2 Tdt-Fluor in situ apoptosis detection kit (Trevigen, Gaithersburg, MD) according to the manufacturer's instructions. Briefly, sections were incubated with CD31 (1:50) overnigh followed by incubation with secondary antibody Alexa-488 conjugated anti-body. Proteinase K treatment was perform 30min at 37 ˚C. Anti-streptavidin 647 was used to observe apoptotic cells. Images were captured on a Nikon eclipse Ti microscope. Three cross-sections, 50 μm apart, were taken per animal and all images were taken at the same time under identical settings and handled in Adobe Photoshop similarly across all images.

Immunoblot analysis – Adductors muscles were lysed in RIPA buffer (50 mmol/L Tris [pH 6.8], 150 mmol/L NaCL, 0.1% SDS, 1% triton, 0.5% sodium deoxycholate and 2 mmol/L EDTA) containing protease inhibitors (1 mmol/l phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mmol/l Na_3VO_4; Sigma). Protein amount was measured with DC kit (Bio-Rad). The lysates (20-50 μg protein) were separated by SDS-PAGE, transferred to PVDF membrane, and blocked with 5% skim milk. Primary antibodies were incubated overnight at 1:1000 in 5% skim milk or 1:500 in 5% BSA for p-VEGFR and p-eNOS. Antigens were detected using anti-rabbit HRP-conjugated antibody 1:10 000 (or 1:2000 for for p-VEGFR and p-eNOS) and detected with the either ECL system (Pierce Thermo Fisher, Piscataway, NJ) or Luminata forte western HRP substrate (millipore, Etobicoke, ON). Protein content quantification was performed using computer-assisted densitometry ImageLab imaging software (Chemidoc, BioRad).

Real time PCR analysis – Real-time PCR was performed to evaluate mRNA expressions of VEGF, KDR/Flk-1, eNOS, SHP-1, SHP-2, VEPTP, AT1, AT2, ACE1, ACE2 and HIF-1α of non-ischemic and ischemic limbs. Total RNA was extracted from adductor muscles with TRI-REAGENT (Burlington, ON), as described by the manufacturer. The RNA was treated with deoxyribonuclease I (DNase I; Invitrogen) to remove any genomic DNA contamination. Approximately 1 μg RNA was used to generate cDNA using SuperScript III reverse transcriptase and random hexamers (Invitrogen) at 50 ˚C for 60 min. PCR primers are listed in supplementary table 1. GAPDH expression was used for normalization. PCR products were gel purified, subcloned using QIA quick PCR Purification kit (Qiagen), and sequenced in both directions to confirm identity.

Phosphatase assay – We have assessed phosphatase activity of SHP-1 by using Tyrosine phosphatase assay system (V2471, Promega, Madison, WI) according to the manufacturer's instructions and as previously reported[3]. SHP-1 was immunoprecipitated from cell lysates with a polyclonal antibody (C-19) prebound to protein A Sepharose beads. The phosphatase activity was determined using the *Infinite M200 PRO* NanoQuant (Tecan Group Ltd).

Cells Culture – Bovine aortic endothelial cells (BAECs) were isolated from fresh harvested aortas that were obtained from a local abattoir. Cells were cultured in DMEM 2.5% FBS, 1% P/S. Cells were exposed in DMEM 0.1% FBS containing normal glucose (NG; 5.6 mmol/L + 19.4 mmol/L mannitol to adjust osmotic pressure) or high glucose (HG; 25 mmol/L) levels up to 48 h. Then, cells were treated with or without CGP (10 nmol/L), PD123319 (10 μmol/L) and stimulated with VEGF (10 ng/ml) for 5 min.

Proliferation assay – BAECs were seeded in a 96-wells plate at 5 000 cells per well for 4 h. Cells were then exposed to NG or HG concentrations for 48 h with or without VEGF 25 ng/ml and CGP 10 nmol/L treatment for the last 24 h. Cells were then fixed in 4% paraformaldehyde for 5 min, rinced twice for 2 min in PBS and stained with DAPI (Sigma-Aldrich) at 0.001 mg/ml for 10 min. Cells were counted using the NIS-Elements software of Nikon eclipse Ti microscope.

Migration assay – BAEC were seeded at 25 000 cells per chamber of Ibidi's culture-insert placed in a 12-wells plate for 4 h. Cells were then exposed to NG or HG for 24 h. Following this exposure, each insert was removed and the wells were filled with 1 ml of either NG or HG for another 24h, with or without VEGF-A (25 ng/ml) and CGP (10 nmol/L). Cell migration was evaluated using a Live Cell Imaging chamber adapted to Nikon eclipse Ti microscope in which images were taken immediately after removing the insert and at the end of the experiment. Analysis were performed with Image J software by measuring the difference of occupied surface prior VEGF treatment and after 24 h.

Statistical analyses – The data were shown as mean ± SEM for each group. Statistical analysis was performed by unpaired by one-way (Fig. 1, 2, 3 and 4) and two-way (Fig. 5 and 6) analysis of variance (ANOVA) followed by Tukey's test correction for multiple comparisons. Data in each group were checked for normal distribution using D'Agostino and Pearson normality test based on alpha = 0.05. All results were considered statistically significant at $P < 0.05$.

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