



Published in final edited form as:

Arterioscler Thromb Vasc Biol. 2008 September ; 28(9): 1606–1613. doi:10.1161/ATVBAHA.108.169235.

Disruption of Ang-1/Tie-2 Signaling Contributes to the Impaired Myocardial Vascular Maturation and Angiogenesis in Type II Diabetic Mice

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Abstract

Objective—Microvascular insufficiency represents a major cause of end-organ failure among diabetics. The current studies were undertaken to determine whether dysregulation of the angiopoietins/Tie-2 system would result in an impairment of smooth muscle cell (SMC) recruitment and vascular maturation, which contributes to impaired angiogenesis in diabetes.

Methods and Results—Tie-2 expression was significantly attenuated, whereas angiopoietin-2 (Ang-2) was increased in db/db mice subjected to myocardial ischemia. Our morphological analysis showed that the number of SMC coverage area per neovessel was significantly reduced in db/db mice. This was accompanied by a significant reduction of myocardial capillary density and arteriole formation. Interestingly, Angiopoietin-1(Ang-1)–induced SMC recruitment and vessel outgrowth were severely impaired in db/db mice. Our *in vitro* studies further demonstrated that exposure of mouse heart endothelial cells to high glucose resulted in a significant upregulation of Ang-2 and a downregulation of Tie-2 expression. These alterations led to a significant impairment of Ang-1–induced Akt and eNOS phosphorylation, along with a remarkable impairment of Ang-1–induced endothelial cell migration and endothelial cell spheroid sprouting. Ang-1 gene transfer restored Tie-2 expression and rescued these abnormalities in diabetes.

Conclusions—Our findings underscore the important role of Ang-1–Tie-2 signaling in the diabetes-induced impairment of vascular maturation and angiogenesis.

Keywords

hyperglycemia; angiopoietins/Tie-2; myocardial ischemia; angiogenesis; type II diabetes

This study was to determine whether dysregulation of the angiopoietins/Tie-2 system would result in an impairment of smooth muscle cell recruitment, vascular maturation, and angiogenesis in diabetes. Our studies demonstrated that disruption of Angiopoietin-1/Tie-2

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Disclosures

None.

signaling and vascular maturation is an important contributor to the impaired angiogenesis in diabetes.

Angiogenesis requires coordinated signaling events among a variety of angiogenic factors and their receptors. Tie-2 is endothelial-specific receptor tyrosine kinase. Angiopoietin-1 (Ang-1) and Angiopoietin-2 (Ang-2) are the two key ligands for the Tie-2 receptor. Ang-1 binds to the Tie-2 receptor and induces Tie-2 phosphorylation.¹⁻³ Ang-2 has been identified as a natural antagonist of Ang-1, inhibiting Ang-1-mediated Tie-2 phosphorylation and angiogenesis.⁴ Angiogenesis is mainly regulated by the interplay between vascular endothelial growth factor (VEGF) and angiopoietins.^{5,6} VEGF is required to initiate neovessel formation, whereas Ang-1 is required for further maturation of the neovessel by recruitment of smooth muscle cell (SMC).^{5,6} Disruption of Ang-1-Tie-2 signaling has been shown to impede vascular maturation and disrupt vessel formation in the developing embryo.⁴ Ang-2 has been identified as a vessel-destabilizing agent that plays a predominant role in controlling vessel regression. In the absence of VEGF, increased Ang-2 leads to endothelial cell apoptosis, immature neovessel destabilization and neovessel regression.⁷

In patients with diabetes mellitus, coronary collateral vessel formation is significantly impaired during myocardial ischemia.⁸ The molecular mechanisms underlying the impairment of angiogenesis in diabetes have generated much interest but, so far, have remained largely unidentified. Recent studies have revealed that diabetic abnormal angiogenesis is closely associated with an abnormality of the angiopoietins/Tie-2 system.⁹ Our previous studies have demonstrated that disturbed angiopoietins/Tie-2 balance contributes to the hyperglycemic exacerbation of myocardial infarction and impairment of myocardial ischemia-induced neovessel formation in type 1 diabetes.¹⁰ However, what might be wrong with myocardial ischemia-induced neovessel formation and what is the molecular basis that leads to these abnormalities remain unknown. Ang-1 is required for SMC recruitment and neovessel maturation during myocardial ischemia.¹¹ After an early phase of plasticity associated with robust angiogenic response, SMCs were recruited into the infarct neovessels to form muscular arteries and veins, which improve myocardial blood supply and facilitate myocardial repair.¹¹⁻¹⁵ To date, little is known about the role of the angiopoietins/Tie-2 system on diabetic impaired myocardial angiogenesis and vascular maturation. Whether disruption of Ang-1-Tie-2 signaling occurs in the diabetic heart, which may contribute to the formation of immature vasculature, and thus leading to neovessel regression and reduction of angiogenesis in response to myocardial ischemia, has not yet been determined.

In this study, we hypothesized that diabetes impairs myocardial angiogenesis by a mechanism involving disruption of Ang-1-Tie-2 signaling and vascular maturation. To test our hypotheses, we characterized the expression of the angiopoietins/Tie-2 in vivo using a db/db mouse model subjected to myocardial ischemia, and, in vitro, using mouse heart myocardial endothelial cells (MHMECs) under hyperglycemic conditions. We examined SMC recruitment, neovessel maturation, and the angiogenic response to ischemia or Ang-1 stimulation. Our data indicated that SMC recruitment and myocardial ischemia-induced neovessel maturation were severely impaired in db/db mice. Furthermore, hyperglycemia

disrupted Ang-1–Tie-2 signaling and attenuated Ang-1–induced SMC recruitment and angiogenesis.

Materials and Methods

Experimental Diabetic Mouse Myocardial Ischemia Model^{10,16}

C57BLKS/J and db/db mice (12 to 14 weeks of age) were purchased from Jackson Laboratories (Bar Harbor, Me). Myocardial ischemia was achieved by ligation of the left anterior descending coronary artery (LAD). The sham control underwent the surgery without the LAD ligation. All procedures were in conformance with the Institute for Laboratory Animal Research Guide for the Care and Use of Laboratory Animals and were approved by the Vanderbilt University Institutional Animal Care and Use Committee.

Western Blot Analysis

For angiopoietins/Tie-2 and VEGF expression, the membranes were immunoblotted with Ang-1, Ang-2, Tie-2, and VEGF antibodies (1:1000, Santa Cruz). For eNOS and Akt phosphorylation, the membranes were immunoblotted with rabbit antiphospho-Akt and antiphospho-eNOS (1:1000, Cell Signaling). Total eNOS, Akt, and loading control β -Actin were detected using anti-Akt, Anti-eNOS, and anti- β -Actin (1:1000, Cell Signaling Technology) on the same nitrocellulose blots after stripping.

Analysis of Myocardial Capillary and Arteriole Densities¹⁰

Sections were incubated with fluorescerin-labeled Isolectin B4 (1:200, Molecular Probe, Invitrogen) and Cy3-conjugated anti- α smooth muscle actin (SMC, 1:100; Sigma). Myocardial capillary and arteriole (SMA-positive coated neovessels) densities were measured using image acquisition and analysis software (Image J, NIH). To assess the acquisition of a muscular coat by infarct neovessels, the density of coated vessels in the infarcted border zone area was stained for SMA. SMC coverage per neovessel in the infarcted border zone area was measured using image analysis software (Image J, NIH).

Mouse Aortic Ring Sprouting Ex Vivo Model^{16,17}

Mouse aortas were isolated from C57BLKS/J and db/db mice. Vessel outgrowth at day 5 was examined using a Nikon TE-300 microscope. The area of vessel outgrowth was quantified using image acquisition and analysis software (Image J, NIH).

Endothelial Cell and Smooth Muscle Cell Immunostaining¹⁸

To characterize Ang-1–induced SMC recruitment, specific EC and SMC markers were directly applied to ex vivo aortic culture explants. Briefly, the cultured explants were incubated with specific cell markers: fluorescein isothiocyanate (FITC)-labeled mouse CD31 antibody (1:100, BD Biosciences) for ECs; and Cy3-conjugated anti- α smooth muscle actin (1:100) for SMCs. SMC recruitment was quantified by measuring the relative area of SMC/EC coverage using image acquisition and analysis software (Image J, NIH).

Endothelial Cell Migration and Spheroid Angiogenesis Assays

Endothelial cell migration and endothelial cell spheroid sprouting assays were performed as previously described.^{16,17}

Statistical Analysis

The results are expressed as the mean±SD. Statistical analysis was performed using ANOVA followed by a *t* test corrected for multiple comparisons (Student–Newman–Keuls). Significance was set at $P<0.05$.

Results

Dysregulation of the Myocardial Angiopoietins/Tie-2 System in db/db Mice Subjected to Myocardial Ischemia

Wild-type (WT) mouse hearts exposed to ischemia for 24 hours showed a significant increase in Tie-2 expression, whereas myocardial ischemia (IS)-induced Tie-2 expression was significantly blunted in db/db mouse hearts (Figure 1A). Relative to WT mice, db/db mice had enhanced Ang-2 expression which was further increased by myocardial ischemia (Figure 1B). Ang-1 expression did not change significantly in either WT or db/db mouse hearts (data not shown). To examine the correlation between angiopoietins/Tie-2 and VEGF in diabetes-induced impairment of angiogenesis, we also examined myocardial VEGF level. Myocardial ischemia–induced VEGF expression was diminished in db/db mouse hearts subjected to ischemia for 24 hours (Figure 1C).

Impaired Akt and eNOS Phosphorylation in db/db Mice

Next, we examined Akt and eNOS phosphorylation in db/db mice. Our Western blot analysis data showed that Akt and eNOS phosphorylation was significantly reduced in the db/db mouse hearts (Figure 2A and 2B). Additionally, total Akt and eNOS expression was also significantly reduced in db/db mice both at basal level and subjected to myocardial ischemia (Figure 2C and 2D).

Myocardial Ischemia–Induced Capillary and Arteriole Formation Are Impaired in db/db Mice

WT mouse hearts subjected to ischemia showed a significant increase in myocardial capillary density in the border zone of the infarcted myocardium (Figure 3A). Furthermore, the number of arterioles in the border zone of the infarcted myocardium was also significantly increased (Figure 3B). Myocardial ischemia–induced capillary and arteriole formation in the border and remote area of the infarcted myocardium were significantly less in the hearts of db/db mice compared to WT mice (Figure 3A and 3B; supplemental Figure I and II, available online at <http://atvb.ahajournals.org>). To further determine whether rescuing impaired Ang-1–Tie-2 signaling can reverse impaired angiogenesis in the diabetic heart, diabetic db/db mice were given an intravenous injection of 1×10^9 PFU Ad-Ang-1. Ang-1 protein expression was significantly increased in the hearts of Ad-Ang-1–treated db/db mice. Ang-1 gene transfer resulted in the restoration of Tie-2 expression in db/db mice (Figure

3C). Furthermore, Ang-1 gene transfer led to a significant improvement in myocardial capillary formation in the db/db mice (Figure 3D).

Impairment of Neovessel Maturation in db/db Mice Subjected to Myocardial Ischemia

As shown in Figure 4A, the border zone of the infarcted myocardial area contained a significant number of mature, coated neovessels in WT mice, whereas few coated neovessels were found in db/db mouse hearts after 14 days of ischemia (Figure 4A). Quantitative analysis of SMC coverage further revealed that 62.6% of the neovessels in the border zone of the infarcted myocardial area were stained positive for SMA after 14 days of myocardial ischemia in WT mice. The relative ratio of SMC/neovessel coverage was considerably reduced; only 27.9% of neovessels were covered with SMA in db/db mouse hearts (Figure 4A).

Ang-1–Induced SMC Recruitment Is Impaired in db/db Mice

Stimulation of aortic rings isolated from WT mice with Ang-1 (250 ng/mL) led to a significant increase in SMC recruitment (Figure 4B). Ang-1–induced SMC/EC coverage was significantly less in aortic rings isolated from db/db mice than in those from WT mice (Figure 4B). The area of vessel outgrowth in response to Ang-1 stimulation was significantly reduced in the aortic rings isolated from db/db diabetic mice than in those from WT mice (supplemental Figure III).

High Glucose Dysregulates Angiopoietins/Tie-2 Expression and Impairs Ang-1–Induced Angiogenesis

Exposure of MHMECs to high glucose (HG; 25 mmol/L) resulted in a significant increase in Ang-2 expression; this was accompanied by a dramatic decrease in Tie-2 expression as compared to low glucose conditions (LG; 5 mmol/L; Figure 5A). Ang-1 expression was undetectable in MHMECs both under LG and HG conditions (data not shown). Stimulation of MHMECs with Ang-1 (250 ng/mL) led to a gradual increase in Akt and eNOS phosphorylation under LG conditions. Ang-1–induced Akt and eNOS phosphorylation were significantly blunted under HG conditions (Figure 5B and 5C). This was accompanied by a significant reduction of MHMEC migration in response to Ang-1 (supplemental Figure IV). Furthermore, Ang-1–induced endothelial cell spheroid sprouts were dramatically suppressed under HG conditions (supplemental Figure IV).

To examine whether overexpression of Ang-1 to rescue Ang-1–Tie-2 signaling can restore angiogenesis under HG conditions, MHMECs were first infected with Ad–Ang-1 for 24 hours. The infected cells were then exposed to HG conditions for 72 hours. Overexpression of Ang-1 significantly improved the HG-induced impairment of MHMEC spheroid sprouting (supplemental Figure V).

Discussion

In this study, we have demonstrated that Tie-2 expression was significantly attenuated, whereas Ang-2 expression was increased under hyperglycemia and in db/db mice subjected to myocardial ischemia. Ang-1 plays a critical role in regulating endothelial sprouting and

SMC recruitment, whereas Ang-1–induced SMC recruitment and ischemia-induced neovessel maturation were impaired in db/db mice. These were accompanied by a significant reduction of angiogenesis. Restoration of Tie-2 expression by Ang-1 gene transfer reversed the impaired angiogenesis in db/db mouse hearts. Our present study has provided additional mechanistic evidence that hyperglycemia may impair angiogenesis by a mechanism involving disruption of Ang-1–Tie-2 signaling in diabetes.

Diabetic reduced angiogenesis is associated with defective angiogenic growth factor expression as well as the impairment of angiogenic signaling transduction, such as the loss Akt/eNOS signaling.^{19–21} Tie-2 expression is specifically localized to the vascular endothelium and is upregulated by hypoxia and ischemia.^{22–26} Reduction of Tie-2 expression has been found in the experimental pulmonary hypertension model. Furthermore, restoration of Tie-2 expression by Ang-1 gene transfer protects the pulmonary microvasculature and attenuates pulmonary hypertension progression.²⁷ Our data for the first time have demonstrated that myocardial ischemia–induced Tie-2 expression was significantly blunted in db/db mice. Exposure of MHMECs to high glucose resulted in a significant reduction of Tie-2 expression. This was accompanied by a significant impairment of Ang-1–induced Akt/eNOS phosphorylation and angiogenesis. These results led us to speculate that reduction of Tie-2 expression and disruption of Ang-1–Tie-2 signaling under hyperglycemic conditions may be the underlying cause of impaired angiogenesis in diabetics. This notion was further substantiated by our data which revealed that overexpression of Ang-1 restored Tie-2 expression and significantly improved angiogenesis both in vitro and in vivo myocardial angiogenesis models.

Sustained elevation of Ang-2 expression has been shown to disrupt the formation of capillary-like structures and impaired angiogenesis.^{28,21} Hindlimb ischemia–induced collateral artery growth and angiogenesis were significantly impaired in the transgenic overexpression of Ang-2 mice.²⁹ Furthermore, chronic systemic delivery of Ang-2 resulted in a dramatic reduction of myocardial vasculature.³⁰ Recent clinical studies revealed that the plasma Ang-2 level was increased in patients with diabetes.^{31–34} Intriguingly, administration of Ang-2 into the eyes of normal rats led to a dose-dependent pericyte loss whereas heterozygous Ang-2 deficiency completely prevented diabetes-induced pericyte loss.³⁵ Exposure of mesangial cells to high glucose dramatically increased Ang-2 expression and significantly inhibited capillary tubule formation.⁹ Consistent with these findings, our data showed that Ang-2 expression was upregulated in MHMECs under hyperglycemic conditions and in diabetic db/db mice. Furthermore, Ang-1–induced endothelial cell spheroid sprouting was significantly decreased in MHMECs in the presence of exogenous Ang-2 (unpublished data). As such, we reasoned that elevation of Ang-2 might be competition with Ang-1 and negatively interfere with Ang-1–Tie-2 signaling and impair angiogenesis in diabetes. Our data showed that Ang-1 gene transfer rescued the hyperglycemia-induced impairment of endothelial sprouting, further suggesting the possibility that the overexpression of Ang-1 may overcome endogenous blocking by Ang-2, thereby restoring angiogenesis in diabetes.

Neovessel maturation is critical for the stabilization and repairing of the myocardial infarcted area. After an early phase of plasticity associated with robust angiogenic response,

SMCs were recruited into the neovessels to form muscular arteries and veins, which improve myocardial blood supply and facilitate myocardial repair.^{12-15,36} Recruitment of SMCs in the myocardial infarcted area is also important for the prevention of neovessel from regression.^{12-15,36} VEGF is required to initiate neovessel formation, whereas Ang-1-Tie-2 signaling is required for SMC recruitment and neovessel maturation.^{5,6} Importantly, Ang-1-Tie-2 signaling is crucial in their development as stable and leak-resistant neovessels during myocardial ischemia. Data from our laboratory and other investigators revealed that Ang-1 gene transfer improved neovessel density in acute myocardial infarction rat¹¹ and STZ hyperglycemic mice.¹⁰ Furthermore, Ang-1 gene transfer stabilized the angiogenic response for longer duration, and led to the formation of larger number of matured vascular structures.¹¹ In light of these findings, we have examined SMC recruitment and neovessel maturation in diabetic d/db mouse using ex vivo and in vivo models. Our data clearly demonstrated that Ang-1-induced SMC recruitment and myocardial ischemia-induced neovessels maturation in the border zone of infarcted area were severely impaired in db/db mice, suggesting that reduction of SMC coverage and formation of immature neovessels in the border zone of infarcted area might be another possible mechanistic explanation for the impairment of myocardial angiogenesis in diabetes.

There is a notable dearth of knowledge about the impairment of VEGF expression, which has been linked to insufficient angiogenesis in diabetic wound healing.³⁷⁻³⁹ Our present studies have extended these findings, demonstrating that myocardial ischemia-induced VEGF expression is also impaired in db/db mice. In contrast to these results, recent studies also revealed that the expression of VEGF remained unchanged in the myocardium of diabetic patients. However, VEGFR phosphorylation and its downstream Akt and eNOS phosphorylation were severely impaired.⁴⁰ Furthermore, in diet-induced diabetic mice, plasma VEGF level was increased whereas VEGFR, Akt, and eNOS phosphorylation were decreased.⁴¹ Taken together, these findings suggest that impaired angiogenesis in diabetes is not only attributable to the reduction of the VEGF level, but is also involved in the impairment of VEGF/VEGFR signaling transduction. Our present data demonstrated that VEGF expression and Ang-1-Tie-2 signaling were significantly blunted after coronary occlusion in db/db mouse hearts, further supporting the notion that deficits in postischemic angiogenesis in diabetes were associated with the dysregulation of a complex angiogenesis-regulatory factors and its signaling.^{41,42}

In summary, our present data demonstrate that, in the absence of VEGF, hyperglycemia disrupts the angiopoietins/Tie-2 balance in favor of Ang-2, and blunts Ang-1-Tie-2 angiogenic signaling, thus leading to the impairment of SMC recruitment, immature neovessel formation, and reduced angiogenesis (Figure 6). Given that disruption of Ang-1-Tie-2 signaling and vascular maturation is an important contributor to the impairment of angiogenesis in diabetes, therefore, rescue of immature vasculature should be considered as a novel therapeutic strategy in treatment of diabetic impaired myocardial collateral and angiogenesis complications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Sources of Funding This work was supported by grants from the American Heart Association grant 0565196B and NIH grant DK074995 to J.X. Chen.

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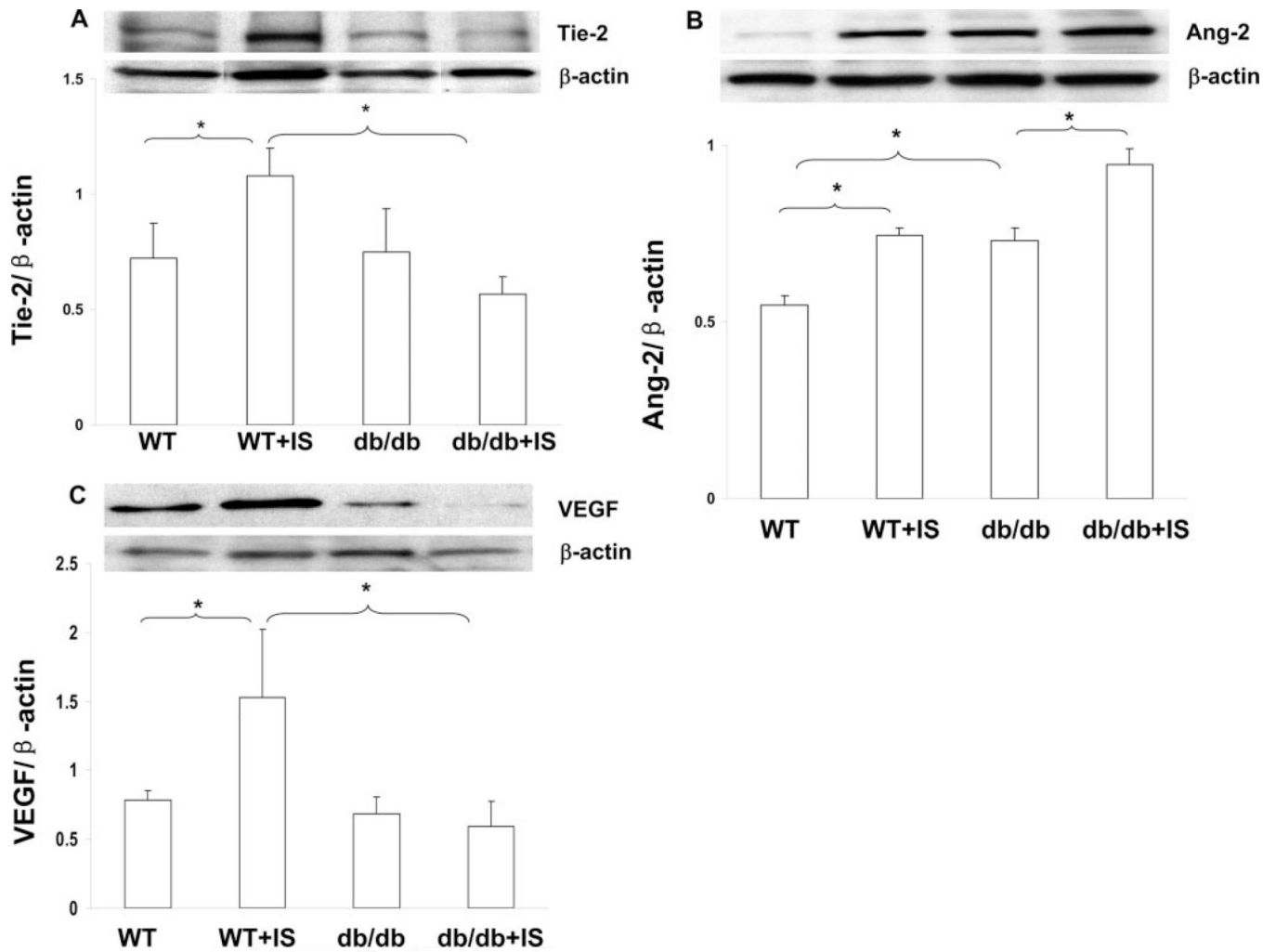


Figure 1.

Representative Western blot and densitometry analysis of myocardial angiopoietins/Tie-2 and VEGF expression after 24 hours myocardial ischemia: Tie-2 was downregulated (A), whereas Ang-2 was upregulated in db/db mice (B). VEGF expression was diminished in db/db mice (C). n = 5 to 10, * $P < 0.05$.

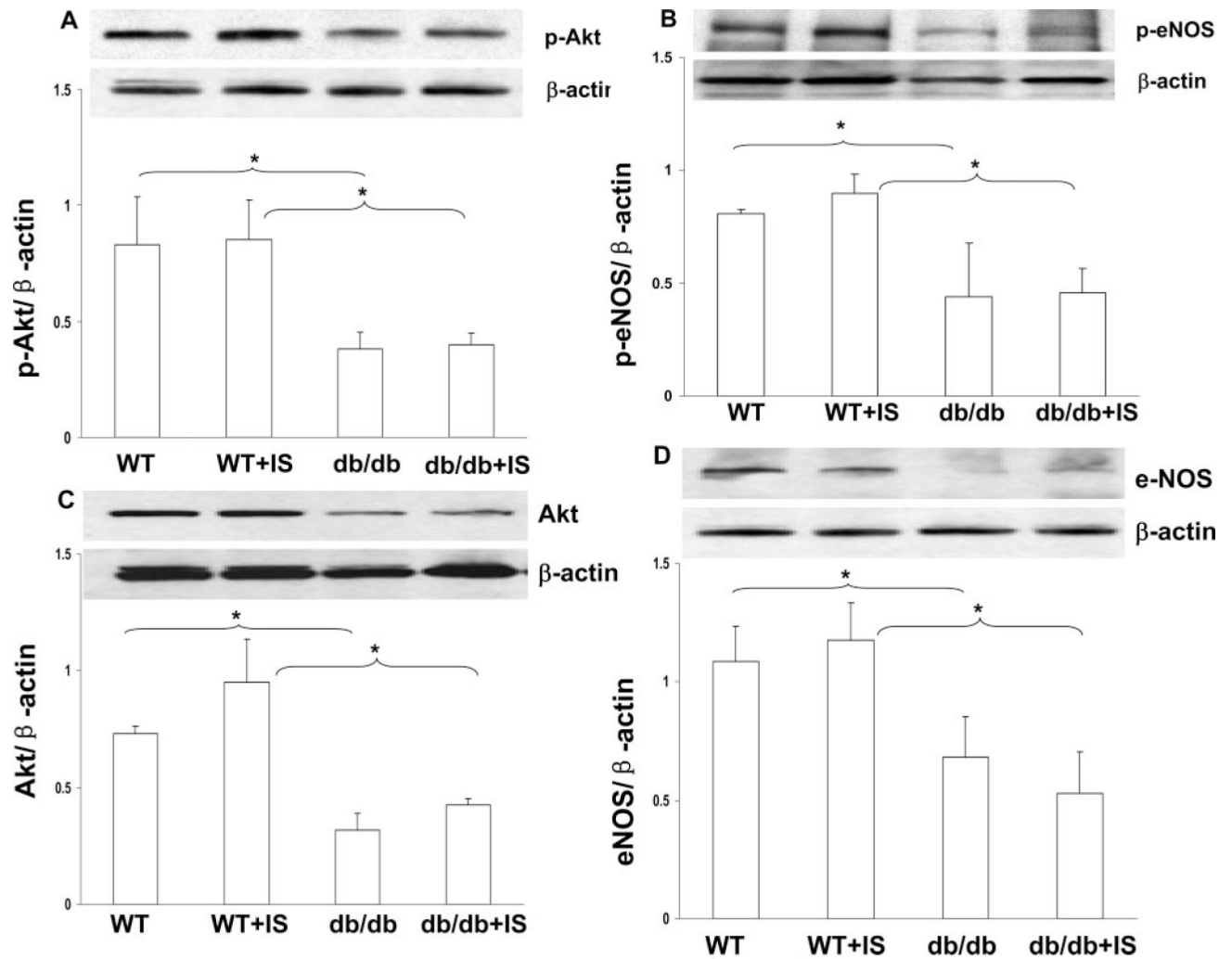


Figure 2. Diabetic db/db mice demonstrated decreased Akt (A) and eNOS (B) phosphorylation compared with WT mice. Total Akt (C) and eNOS (D) protein expression was reduced in db/db mouse hearts compared with WT mice at the baseline level and after myocardial ischemia. n = 3 to 4, * P <0.05.

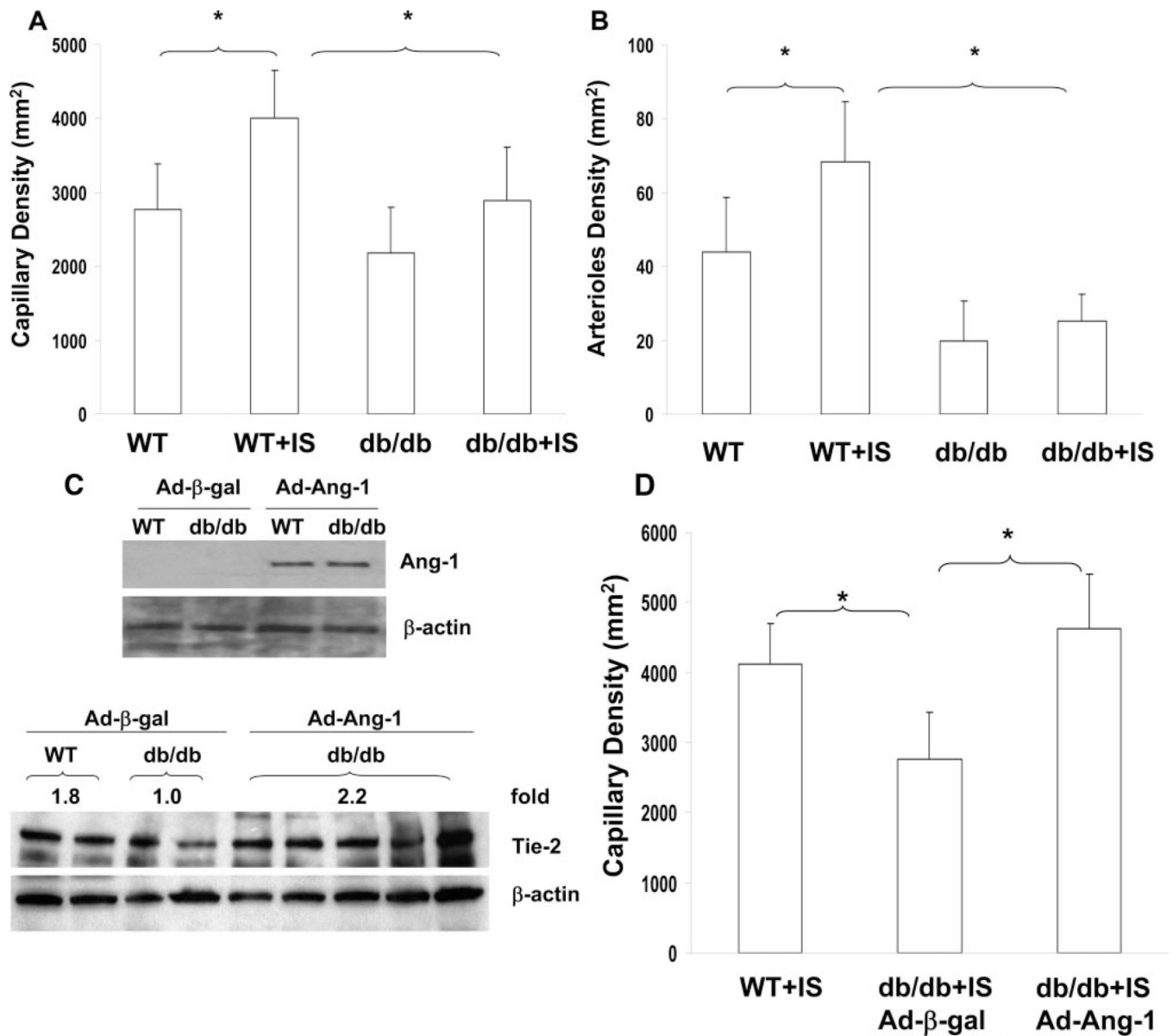


Figure 3.

A and B, Myocardial ischemia-induced capillary and arteriole formation was significantly reduced in db/db mice. C, Myocardial Ang-1 and Tie-2 expression was increased in Ad-Ang-1-treated db/db mice. D, Systemic administration of Ad-Ang-1 significantly increased capillary density in db/db mice. n = 6 to 8, * $P < 0.05$.

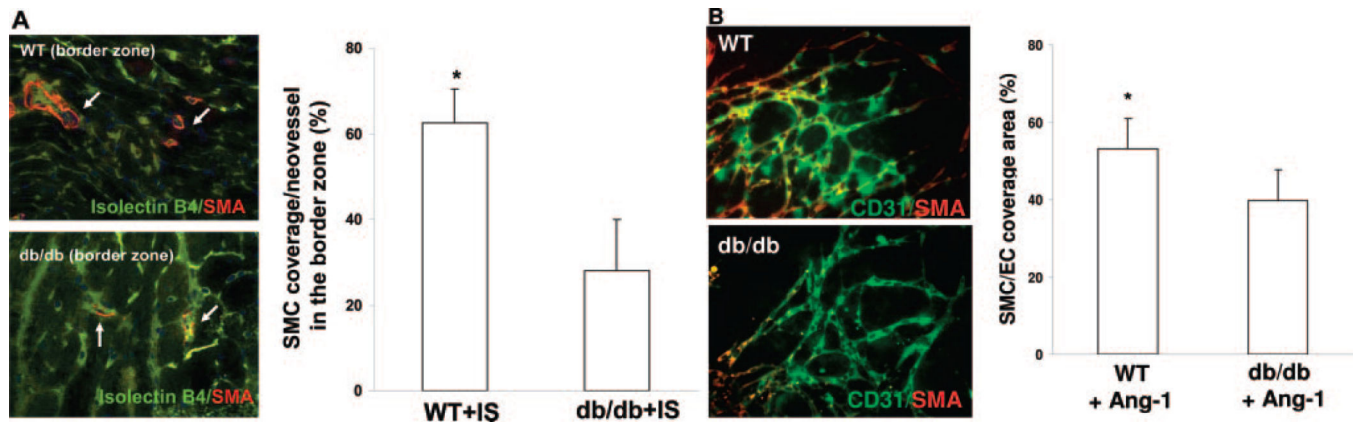


Figure 4.

A, Immunostaining and quantitative analysis of SMC coverage of neovessels in the infarcted myocardial border zone (n = 6 mice). B, CD31/SMC coverage of Ang-1–induced SMC recruitment in aortic ring explants (n = 10 mice), * $P < 0.05$.

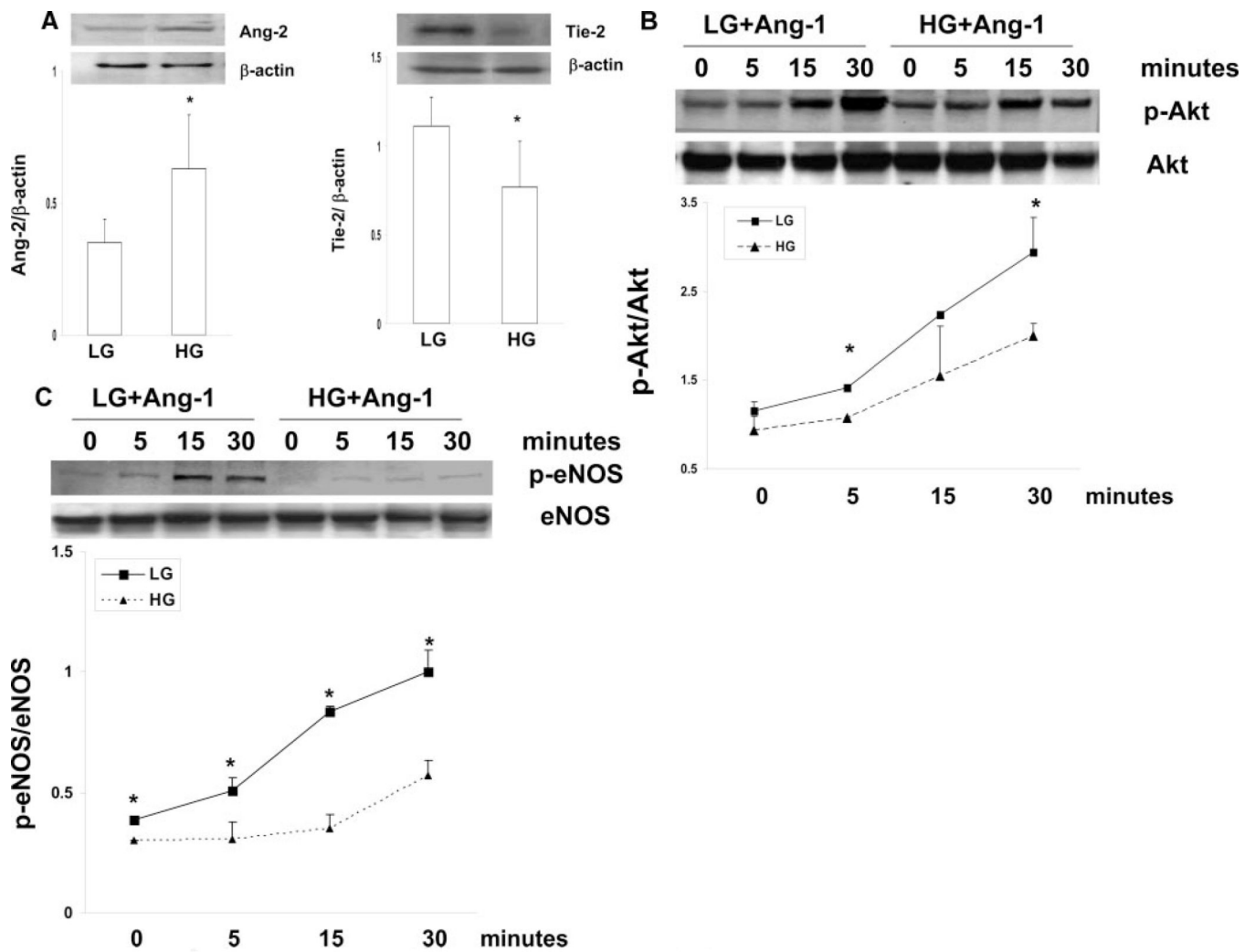


Figure 5.

A, Ang-2 and Tie-2 protein expression under LG or HG conditions in MHMECs (n = 5 to 6). B and C, Ang-1-induced Akt and eNOS phosphorylation under LG or HG conditions in MHMEC (n = 3 to 4), * $P < 0.05$.

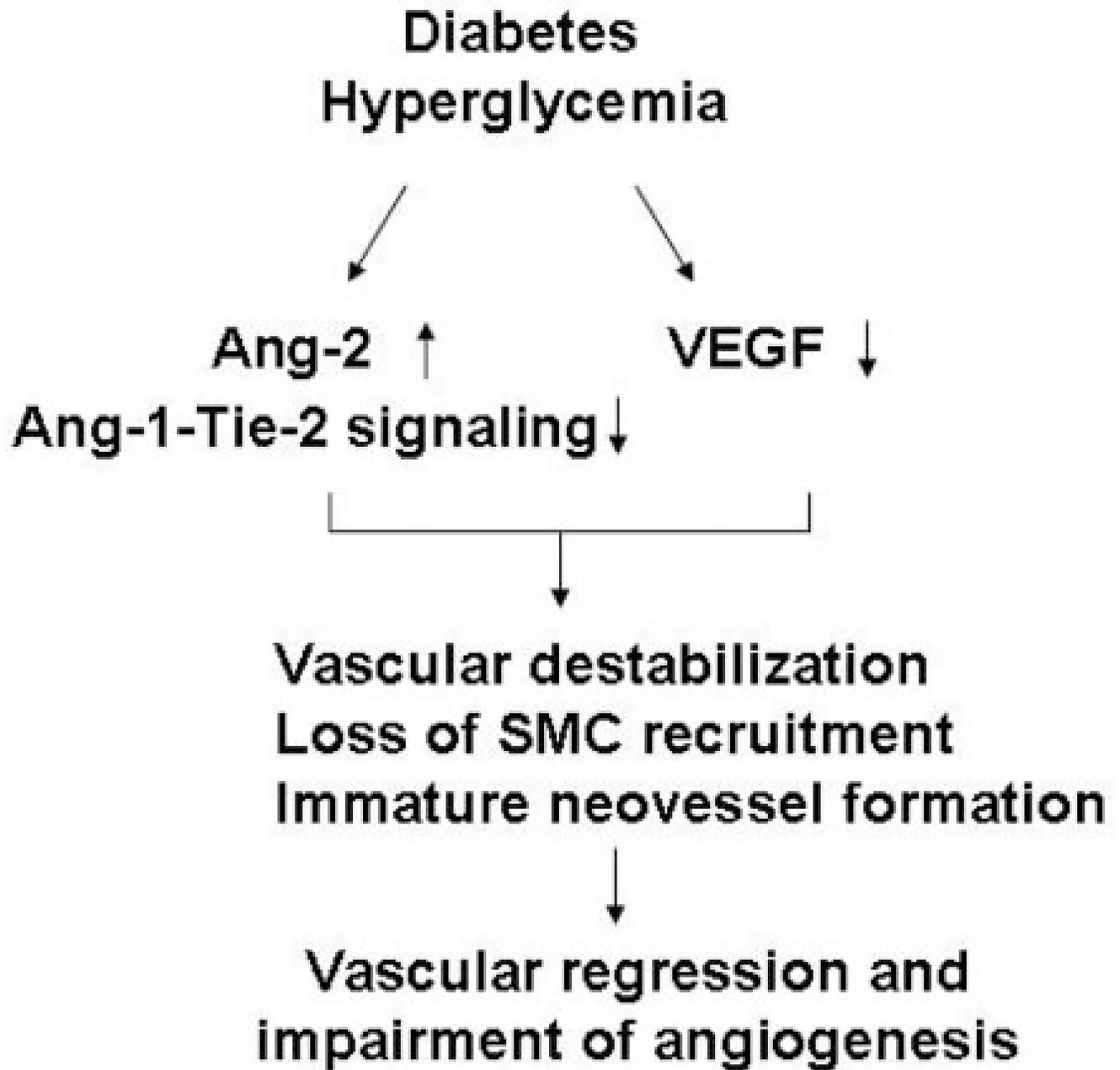


Figure 6.

Current working hypothesis of intracellular mechanisms by which hyperglycemia causes impairment of angiogenesis in diabetes. Hyperglycemia disrupts the angiopoietins/Tie-2 balance in favor of Ang-2 and attenuates Ang-1-Tie-2 signaling, thus leading to loss of SMC recruitment, immature neovessel formation, and neovessel regression in the deficiency of VEGF.