Glycation of vitronectin inhibits VEGF-induced angiogenesis by uncoupling VEGF receptor-2–αvβ3 integrin cross-talk

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Glycation of vessel wall proteins is thought to have an important role in the pathogenesis of vascular complications in diabetes mellitus. However, no previous study has implicated glycated vitronectin (VN) in the control of vascular endothelial growth factor (VEGF) signaling. To explore whether the glycation of VN affects angiogenic signaling and to understand the molecular mechanisms involved, we synthesized glycated VN by incubating VN with methylglyoxal (MGO) in vitro and identified the formation of glycated VN by an LC–ESI–MS/MS-based method. We tested the hypothesis that glycation of VN downregulates VEGF receptor-2 (VEGFR-2) activation by uncoupling the interaction between VEGFR-2 and αvβ3. Unmodified and MGO-glycated VN were used as substrates for human umbilical vein endothelial cells (HUVECs). The effects of glycated VN on VEGF signaling in HUVECs were investigated. The glycation of VN inhibited VEGF-induced phosphorylation of VEGFR-2 and the intracellular signaling pathway downstream of VEGFR-2. Glycated VN inhibited the binding of VEGFR-2 to β3 integrin and inhibited the phosphorylation of β3 integrin. Furthermore, glycation of VN significantly decreased VEGF-induced migration of HUVECs in vitro and vessel outgrowth in an ex vivo angiogenesis model. Collectively, these data indicate that the glycation of VN inhibits VEGF-induced VEGFR-2 activation by uncoupling VEGFR-2–αvβ3 integrin cross-talk. The glycation of VN causes a reduction in the migration of endothelial cells and vessel outgrowth. This may provide a mechanism for the failure of collateral sprouting in diabetic microangiopathy.

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Type 2 diabetes is a chronic hyperglycemic condition that causes both microvascular and macrovascular complications. Impaired angiogenesis contributes to the development of various vascular complications in diabetes mellitus, and a number of abnormalities associated with angiogenesis have been observed in people with type 2 diabetes.¹,² The accumulation of advanced glycation end products (AGEs) may have an important role in the neovascularature of vascular complications in diabetes.³–⁶ Angiogenesis is triggered by angiogenic growth factors and vascular endothelial growth factor (VEGF) is a major angiogenic mediator under physiological and pathophysiological conditions.⁷ However, people with diabetes mellitus often show a poor response to therapeutic angiogenesis⁸ and develop VEGF resistance, an impairment in VEGF-induced signal transduction, which has been demonstrated as a molecular basis for the impaired angiogenesis in diabetes mellitus.⁹ The molecular mechanisms underlying VEGF resistance in diabetes mellitus are not fully understood.

The accumulation of AGEs in the vessel wall may impair vascular cell structure and function. Furthermore, AGEs may modify the extracellular matrix (ECM) through direct modification of RGD (arg-gly-asp) motifs, causing loss of charge and structural distortion. The ECM has been shown to potentiate VEGF signaling by interacting with cell surface integrins. Vitronectin (VN), one of the components of the ECM, is a multifunctional glycoprotein that is present in the plasma and is localized into the ECM of various tissues. Most plasma VN is an inactive monomer.¹⁰ ECM VN is present as an active multimeric form that binds to various ligands, such as integrins, plasminogen activator inhibitor-1 and urokinase receptors. VN also has an important role in regulating VEGF-induced angiogenesis. A cooperative binding interaction between VEGF receptor-2 (VEGFR-2) and αvβ3 integrin has a key role in regulating VEGF signaling in endothelial cells.¹¹ This receptor cross-talk depends on the binding of αvβ3 to VN.¹²,¹³ It has been demonstrated that glycation alters some functional properties of collagen,¹⁴–¹⁶ laminin,¹⁶–¹⁸ fibronectin¹⁷,¹⁹,²⁰ and VN.²⁰ However, no previous studies have implicated the glycation of VN in VEGF signaling.

In this study, we glycated VN in vitro using methylglyoxal (MGO) and characterized the structure of glycated and unmodified VN. We further tested the hypothesis that the glycation of VN contributes VEGF-mediated endothelial cell activation by disrupting VEGFR-2–αvβ3 cross-talk.

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Abbreviations: AGE, advanced glycation end product; ECM, extracellular matrix; ERK, extracellular signal-regulated kinase; HUVEC, human umbilical vein endothelial cell; MGO, methylglyoxal; VEGF, vascular endothelial growth factor; VEGFR-2, VEGF receptor-2; VN, vitronectin; PECAM-1, platelet endothelial cell adhesion molecule-1; STZ, streptozotocin

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Results

Identification of specific glycation sites in human plasma VN. To explore whether glycation could be involved in causing VN conformational change, we examined VN expression by SDS-polyacrylamide gel electrophoresis (PAGE) under reducing and non-reducing conditions using 5–20% gradient gels. Multimeric VN or monomeric VN were incubated with MGO for 72 h at 37 °C. Western blotting analysis of incubates was performed using anti-human VN antibody. As shown in Figure 1a, normal VN-positive bands (65/75 kDa) vanished in multimeric VN and monomeric VN in the presence of MGO and multimeric-VN treated by MGO clearly shifted to a higher molecular mass, which indicated the changes in glycosylation and the existence of covalently cross-linked products. The production of glycated VN (VN-AGEs) was identified with fluorescence spectrophotometer measuring AGE-specific fluorescence at an emission of 440 nm and an excitation of 370 nm. We observed that the fluorescence of VN-AGEs was about three times as much as unmodified VN (Supplementary Figure 1a), suggesting VN-AGEs had been successfully produced in vitro.

To unequivocally identify glycated sites and peptides, glycated VN were digested using trypsin and PNGaseF followed by LC–ESI–MS/MS system analysis. The amino acid sequence of VN is displayed, showing glycosylation sites identified in this study, and an O-glycan attachment site was found (T44) (Supplementary Figure 1b). In total, 18 peptides were identified by their fragmentation patterns in the glycated VN. These peptides were annotated according to the amino acid sequence and their details are listed in Table 1. The results of peptide mass spectra from the glycated VN revealed O-glycosylation on T55 in tryptic peptide TAECK (Figure 1b). The mass of the modification was 203, which suggested that the O-glycan is N-acetylgalcosamine, indicating that VN-AGEs were successfully produced in vitro.

Furthermore, we observed that the O-glycan site overlapped with the somatomedin B domain and it was located near the RGD-containing peptide. In addition, the three N-glycosylation sites were identified from the sequence of VN. The results from these analyses are summarized in Figure 1c.

Glycation of VN impairs VEGFR-2 signaling. VN is a major ligand for αvβ3 and it significantly enhances VEGF-mediated activation of endothelial cells via VEGF-2. We hypothesized that the glycation of VN may impair VEGF-induced activation of VEGF-2. To test this hypothesis, human umbilical vein endothelial cells (HUVECs) were cultured in wells coated with unmodified or glycated VN and were exposed to VEGF or vehicle control for 10 min. Cell lysates were then prepared, separated by SDS-PAGE and analyzed by immunoblotting. VEGF significantly increased VEGF-2 phosphorylation in HUVEC cells grown on VN. However, the stimulatory effect of VEGF on VEGF-2 phosphorylation was inhibited in cells grown on glycated VN (Figure 2a). Intracellular signaling pathways activated by binding of VEGF to VEGFR-2 were also examined. Glycated VN also significantly inhibited VEGF-induced phosphorylation of Akt and extracellular signal-regulated kinase1/2 (ERK1/2) (Figure 2a). As a whole, these results indicated that the glycation of VN impairs VEGF-2 phosphorylation and downstream signaling in HUVEC cells.

αvβ3 Integrin-augmented VEGFR-2 phosphorylation is dependent on the formation of the VEGFR-2–αvβ3 complex. Therefore, we examined the effects of glycated VN on the VN-dependent binding interaction between VEGF-2 and αvβ3. VEGF-2–αvβ3 integrin complexes were captured by an immobilized anti-VEGFR-2 antibody and detected by immunoblotting with an anti-β3 integrin antibody. The results showed that VEGF significantly enhanced the coimmunoprecipitation of VEGFR-2 and β3 integrin in HUVEC cells grown on VN, but no enhancement was seen in cells grown on glycated VN.
grown on MGO-glycated VN (Figure 2b). These results suggested that VEGF induces the formation of VEGFR-2–αvβ3 integrin complexes, and that the glycation of VN inhibits this process.

**Glycation of VN inhibits VEGF signaling.**

β3 Integrin phosphorylation is complementary to VEGF-induced tyrosine phosphorylation of VEGFR-2. VEGFR-2 activation can induce β3 integrin phosphorylation, which in turn is required for VEGFR2–αvβ3 integrin association and maximum phosphorylation of VEGFR-2. Therefore, the effects of glycated VN on β3 phosphorylation were observed. Stimulation with VEGF for 10 min induced a significant increase in β3 phosphorylation in HUVEC cells grown on unmodified VN, but not in those grown on glycated VN (Figure 3a). These results suggested that VN glycation-induced VEGF resistance is associated with an inhibition of β3 phosphorylation stimulated by VEGF.

We further examined the effects of blockade of αvβ3 on VEGF-2 signaling. HUVEC cells grown on VN were pretreated with LM609 or vehicle control for 30 min, followed by stimulation with VEGF for 10 min. Cell lysates were separated by SDS-PAGE and analyzed by immunoblotting. Anti-αvβ3 blocking antibody significantly inhibited VEGF-induced phosphorylation of VEGFR-2, Akt and ERK1/2.

**Table 1** VN peptides detected by LC–MS/MS

| Scan Peptide | Start | Stop | Calculated mass | Measured mass |
|--------------|-------|------|-----------------|---------------|
| 4613 CTEGFNVDKK | 28    | 37   | 1196.5          | 1196.9        |
| 4168 TAECK T+203 | 55    | 59   | 810.3           | 810.8         |
| 7630 GNPEQTPVLKPEEAPAEVG | 108   | 128  | 2187.1          | 2186.3        |
| 5860 PFDAFTDLK | 160   | 168  | 1052.5          | 1052.0        |
| 7296 AFRGQVCYELDEK | 174   | 186  | 1677.7          | 1677.4        |
| 3378 AVRPGYPK | 187   | 194  | 886.5           | 886.3         |
| 11410 LIRDWVGIEGPIADAFFTR | 195   | 212  | 2028.1          | 2029.4        |
| 11294 DWGIEGPIADAFFTR | 198   | 212  | 1645.8          | 1645.1        |
| 4870 TYLFK | 219   | 223  | 670.4           | 670.0         |
| 3738 GSQYWR | 224   | 229  | 795.4           | 795.1         |
| 7840 FEDGVLDPDYPR | 230   | 241  | 1421.6          | 1421.2        |
| 2370 TSAGTR | 325   | 330  | 591.3           | 590.3         |
| 6629 HGVPGQVDAMAGR | 340   | 353  | 1364.7          | 1364.3        |
| 3916 PSLAK | 363   | 367  | 514.3           | 514.4         |
| 3206 FFSGDK | 438   | 443  | 699.3           | 699.8         |
| 5068 YYRNLIR | 444   | 450  | 982.5           | 981.9         |
| 5702 VDPYPR | 457   | 463  | 842.4           | 842.5         |
| 10263 SIAQYWLGCPAPGHHL | 464   | 478  | 1668.8          | 1668.2        |

Abbreviations: LC, liquid chromatography; MS/MS, tandem mass spectrometry; VN, vitronectin.
These results further suggested that β3 is required for VEGF-stimulated activation of VEGFR-2.

Glycation of VN impairs VEGF-induced endothelial cell migration and angiogenesis ex vivo. We examined the effects of glycated VN on the physiological responses of endothelial cells to VEGF stimulation. HUVEC cells were added to the upper chambers containing unmodified or glycated VN-coated porous filters and were exposed to VEGF. After 24 h, cells that migrated to the lower chamber of the membrane were counted. VEGF significantly increased the migration of HUVEC cells grown on unmodified VN. However, the glycation of VN inhibited the migration induced by VEGF (Figure 4a). To further examine the consequences of αvβ3-integrin-blocking antibodies on cell migration, cells were seeded onto VN-coated upper chambers and were pretreated with αvβ3 integrin antibody for 30 min. Cells were then stimulated by VEGF for 24 h. LM609 also significantly inhibited VEGF-induced migration of HUVEC cells (Figure 4b).

To determine the role of glycated VN in VEGF-induced angiogenesis, we cultured segments of the aorta from wild-type (WT) and VN-deficient (VN−/−) mice ex vivo in Matrigel in the presence or the absence of glycated VN. As shown in Figures 4c and d, VEGF-induced angiogenesis was significantly impaired in VN−/− mice compared with WT mice. The capacity of VEGF to stimulate microvessel sprouting was significantly greater in unmodified VN than in glycated VN in WT mice. These results suggested that the glycation of VN has a key role in contributing VEGF-induced angiogenesis in vivo.

Discussion

Hyperglycemia and diabetes mellitus have direct effects on the vessel wall by promoting glycation and cross-linking of long-lived ECM, leading to the production of one form of the AGEs, which has been implicated in diabetic vascular complications. Studies suggest that the formation and accumulation of VN have been proposed to be involved in the evolution of diabetic microangiopathy.3,4 However, it is not fully understood what leads to VN accumulation and in which form VN exerts its antiangiogenic effects. In this study, we

Figure 3 Glycation of VN inhibits VEGF-induced phosphorylation of β3 integrin. (a) HUVEC cells were cultured on VN or glycated VN and stimulated by VEGF (50 ng/ml) or vehicle control for 10 min. Cell lysates were prepared and incubated with a resin-bound anti-β3 integrin antibody. Captured proteins were analyzed by western blotting with anti-phosphotyrosine antibodies. Representative images of three independent experiments and densitometric analysis are shown. Data are shown as mean ± S.D. and are presented as fold changes. **P < 0.01. (b) αvβ3 integrin antibody (LM609) inhibits VEGF-induced VEGFR-2 signaling. HUVEC cells were cultured on VN and were pretreated with LM609 (20 μg/ml) or vehicle control for 30 min, followed by stimulation with VEGF (50 ng/ml) for 10 min. Cell lysates were prepared and subjected to western blotting, to detect the phosphorylation of VEGFR-2, Akt and ERK1/2 and total VEGFR-2, Akt and ERK1/2. Representative images of three independent experiments and densitometric analysis of phosphorylated VEGFR-2, Akt and ERK1/2 normalized to total VEGFR-2, Akt and ERK1/2 are shown. Data are shown as mean ± S.D. for triplicate experiments and presented as fold changes. **P < 0.01.
have investigated the effects of MGO modification of VN on conformational and structural properties. We synthesized glycated VN in vitro and evaluated the modification. Our findings were similar to a previous study, which observed that a conformational change in glycated VN yielded high-molecular-weight SDS-resistant products. By mass spectrometry analysis, we observed that the glycated peptide is overlapped by the plasminogen activator inhibitor-1-binding domain (Somatomedin B domain), which is located near the RGD-containing peptide. AGE formation leads to a reduction in the binding of collagen and heparan sulfate to VN. The characterized altered structure of glycation on VN results in the loss of binding between RGD-binding integrins and their ligands. Thus, it is likely to be that the alteration blocks the adhesion and migration of endothelial cells, thereby inhibiting angiogenesis.

The interaction between multimeric VN and αvβ3 integrin is known to potentiate VEGF-induced angiogenesis. The results of the present study showed significantly decreased VEGF-R2 phosphorylation and downstream signaling activation in HUVEC cells grown on MGO-modified VN compared with cells grown on unmodified VN, suggesting a serious impairment of glycated VN in VEGF signaling. Previous studies showed that αvβ3 integrin and VN potently potentiate VEGF-R2 activation by VEGF, demonstrating a critical role of αvβ3–VEGF-R2 cross-talk in VEGF signaling and the VN-dependent interaction. Our experiments suggest that the modification of the αvβ3–VN binding interaction by the presence of glycosylation directly uncouples αvβ3–VEGF-R2 cross-talk and downregulates VEGF-R2 activation.

Specifically, MGO modification of arginine residues within the RGD and GFOGER motifs can lead to integrin inactivation and disengagement from the ECM. Numerous reports suggest that VEGF is a critical growth factor for angiogenesis under pathological conditions and EC migration is a key event for angiogenesis in vivo. Using a solid-phase assay, we previously showed that multimeric VN binds VEGF, and such interactions could localize VEGF and further bind to VEGF-R2. Our data suggest that the pro-angiogenic (endothelial cell migration and tube sprouting) effect of VN was lost by conformational changes in αvβ3 integrin and/or VEGF-VN binding under glycosylation.

Alternatively, VN is believed to interact with collagen and VN–collagen interactions regulate VN-mediated cell adhesion and migration. We and others observed that collagen binding of VN can be modulated by its glycosylation status. Thus, the presence of glycosylation on VN decreases its binding activity, which results in an inhibitory effect on cell adhesion and migration, suggesting that the proangiogenic effect of VEGF requires physical binding to VN.

The attenuated VEGF signal transduction, or VEGF resistance, has been established as one of the mechanisms underlying the dysfunction of angiogenesis in people with type 2 diabetes. Our experiments involving cell migration in vitro and microvessel sprouting from aortic rings in vivo demonstrated that glycated VN significantly decreased VEGF-induced cell migration and vessel outgrowth. Consistent with these findings, we showed evidences for AGEs cross-linking of VN in the ischemic muscle of diabetic mice, and associated with an impairment in capillary and arteriole density after
induction of hindlimb ischemia. Previous studies have shown that collateral arteriole development after femoral artery occlusion are dependent on VEGFR-2 activation by VEGF. Therefore, our results support the in vivo relevance of the inhibition of VEGFR-2 activation by the formation of VN-AGEs. A limitation of our hindlimb ischemia model experiment is that we cannot definitively conclude the negative effect of VN-AGE formation on VEGFR-2 activation, as formation of VN-AGEs could potentially modulate the angiogenic response to ischemia by VEGFR-2-independent pathways. Nevertheless, our hindlimb ischemia model data support the significance of our proposed molecular mechanisms in a clinically relevant in vivo context, thereby complementing our cell culture, and ex vivo aortic ring data, which demonstrated that glycated VN inhibits VEGFR-2 activation. Additional in vivo studies will be necessary to further dissect and better characterize the significance of our newly reported regulatory pathway on VEGF-dependent angiogenic signaling in other disease models and diabetic patient samples.

In summary, we have found that the formation of glycated VN by MGO inhibits the pro-angiogenic effect of VEGF with mechanisms involving the inactivation of the VEGFR-2 pathway and disruption of the pro-angiogenic binding interaction between VEGFR-2 and αvβ3. These data reveal that the underlying mechanism of diabetic microangiopathy may be through increased formation of glycated VN.

Materials and Methods

Reagents and chemicals. MGO, STZ and human VN were from Sigma (St Louis, MO, USA). Recombinant VEGF-A was from R&D Systems (Minneapolis, MN, USA). Growth-factor-reduced BD Matrigel Matrix (BD Biosciences, San Jose, CA, USA) was from Chemicon International (Temecula, CA, USA). Antibodies to phosphorylated VEGFR-2, total VEGFR-2, phosphorylated Akt, total Akt, phosphorylated ERK1/2, total ERK1/2 and β3 integrin were from Cell Signaling Technology (Beverly, MA, USA). Antibodies to platelet endothelial cell adhesion molecule-1 (PECAM-1) and smooth muscle α-actin were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibody to AGEs was from Abcam (Cambridge, MA, USA). Antibody to VN was from R&D Systems. LM609 (anti-αvβ3 antibody) and anti-phosphotyrosine antibodies were from Merck Millipore (Watford, UK).

Cell culture. HUVEC cells (Cascade Biologics, Portland, OR, USA) were grown in Medium 200 (Cascade Biologics) containing low-serum growth supplement. Cells used were passaged 3–7 times.

Animals. C57BL/6J mice were from Jackson Labs (Bar Harbor, ME, USA). C57BL/6J-congenic Vn−/− mice were a gift from Dr David Ginsburg, University of Michigan, Ann Arbor, MI, USA. All animal care and experimental procedures were approved by the University of Missouri Animal Care and Use Committee.

Glycation of VN. Glycation of VN was performed as described previously. Briefly, VN was modified by incubating the protein (10 μg/ml) with MGO (500 μM) in 100 mM sodium phosphate buffer, pH 7.4, at 37 °C for 72 h. Control VN was
subjected to the same conditions, except that MGO was omitted. The production of VN-AGEs was identified with western blotting and fluorescence spectrophotometer (Molecular Devices, Sunnyvale, CA, USA) measuringAGE-specific fluorescence at emission of 440 nm and excitation of 370 nm.

Endopeptidase trypsin (modified, sequencing grade) was purchased from Promega (Madison, WI, USA). Pepsin was obtained from Roche (Indianapolis, IN, USA). PNAase F was purchased from New England Biolabs (Ipswich, MA, USA). All other chemicals and enzymes used in proteolytic digestion and high-performance liquid chromatography (HPLC) were obtained from Sigma. The quadrupole ion trap mass spectrometer (LTQ) used in the proteomic analysis was manufactured by Thermo (Palo Alto, CA, USA). The VN sample was denatured in 8 M Urea, treated with DTT to reduce disulfide bonds and then reacted with iodoacetamide to alkylate cysteine residues. The sample was cleaned up by dialysis to remove all chemicals. The cleaned sample was digested with trypsin and then treated with PNAse F.

The digested peptide mixture was analyzed by an LC–ESI–MS/MS system, in HPLC for which a 75-μm inner diameter reverse-phase C18 column was on-line coupled with an ion trap mass spectrometer. The solvents used for HPLC were solvent A (98% H2O, 2% acetonitrile) and solvent B (10% H2O, 90% acetonitrile), both containing 0.025% TFA. The analysis time was 200 min for solution-digested samples. The data analysis was carried out with ProMody software suites (ProTech Inc., Phoenixville, PA, USA). All MS/MS data were searched by SV Finder software (Ver1.2, ProTech Inc.), to look for potential sequence modification. All hits from the search were manually validated and confirmed by manual MS/MS spectral assignment.

Cell migration assays. HUVEC cell migration assays were performed using transwell migration chambers with a 8-μm-size porous membrane (Corning Costar, Corning, NY, USA). The membranes were precoated with VN or MGO-glycated VN overnight at 4 °C, after which HUVEC cells (2 × 104) were added to the upper chambers and treated with VEGF (50 ng/ml) or vehicle control. In some experiments, cells were seeded onto VN-coated wells and pretreated with αVEGFR-2 experiments, cells were seeded onto VN-coated wells and pretreated with

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Ex vivo tissue culture. Tissue culture was performed as described previously, with minor modifications.31 Briefly, thoracic aortic rings isolated from WT and VN−/− mice were embedded between two layers of growth-factor-reduced Matrigel (250 μl/layer) in a 24-well plate, in the presence of VN or glycated VN, followed by exposing to Medium 200 (Cascade Biologics; 500 μl) with or without VEGF (50 ng/ml). Medium was changed every 3 days. Aortic rings were photographed 14 days later. The number of sprouting microvessels was quantified by computer-assisted images analysis using Image-Pro Plus software.25

Mouse diabetes model. To induce diabetes, mice were injected i.p. with 55 mg/kg STZ in 0.05 M sodium citrate buffer, pH 4.5, daily for 5 days. Seven days after the last injection, blood glucose levels were measured. Only mice with blood glucose levels ≥ 11.1 mM were used in the present study. Age-matched non-diabetic mice were injected with sodium citrate buffer and served as controls. After 3 weeks of treatment, mouse hindlimb ischemia model was performed in untreated control and STZ-induced diabetic mice.

Mouse hindlimb ischemia model. Unilateral hindlimb ischemia was induced in mice by ligation and excision of a segment of the left femoral artery, as previously described.31 Mice were euthanized 7 days after surgery. Ischemic gastrocnemius muscle was excised, embedded in paraffin and cross-sections were prepared for immunohistochemical analysis.

Immunohistochemistry. Tissue sections were incubated with rabbit antibody directed against AGE formation (10 μg/ml) and FITC-conjugated species-specific secondary antibody (1:200). Costaining was then performed with a rat antibody directed against VN (20 μg/ml) and Texas red-conjugated species-specific secondary antibody (1:200). Arterioles within gastrocnemius muscle were immunostained with anti-smooth muscle α-actin antibody (1:100) and capillaries within gastrocnemius muscle were immunostained with anti-PECAM-1 antibody (1:100).

Data analysis. Data are presented as mean ± S.D. of mean. Results were analyzed by one-way analysis of variance followed by post hoc comparison. The level of significance was set at P < 0.05.

Conflict of Interest

The authors declare no conflict of interest.

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Author contributions

JW designed, carried out experiments and participated in writing the manuscript. XZ, NP, LM, YL, and NC performed experiments and analyzed data. MR and XD prepared samples. JW designed the experiments and wrote the manuscript, and all authors reviewed the manuscript.

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