Kallistatin Inhibits Vascular Inflammation by Antagonizing Tumor Necrosis Factor-α–Induced Nuclear Factor κB Activation

Hang Yin, Lin Gao, Bo Shen, Lee Chao, Julie Chao

Abstract—Kallistatin is a plasma protein with anti-inflammatory properties. In this study, we investigated the role and mechanisms of kallistatin in inhibiting endothelial inflammation through its heparin-binding domain. We showed that recombinant wild-type kallistatin dose-dependently competed with tumor necrosis factor (TNF)-α binding to TNF-α receptor in endothelial cells, whereas kallistatin mutant at the heparin-binding domain had no effect. Kallistatin, but not kallistatin mutant at the heparin-binding domain, abrogated TNF-α–induced endothelial cell activation, as evidenced by inhibition of TNF receptor 1–associated death domain protein activation, inhibitor of nuclear factor κB (NF-κB) degradation, nuclear factor κB translocation, and p38 mitogen-activated protein kinase phosphorylation, as well as cell adhesion molecule and cytokine expression. Moreover, kallistatin, but not kallistatin mutant at the heparin-binding domain, inhibited TNF-α–induced human monocyteic THP-1 cell adhesion to endothelial cells and prevented vascular endothelial growth factor–induced endothelial permeability. In mice, kallistatin gene delivery prevented vascular leakage provoked by complement factor C5a, whereas delivery of kallistatin heparin mutant gene had no effect. Similarly, gene transfer of kallistatin, but not the kallistatin heparin mutant, inhibited collagen/adjuvant-induced arthritis in rats. These results indicate that kallistatin’s heparin-binding site plays an essential role in preventing TNF-α–mediated endothelial activation and reducing vascular endothelial growth factor–induced vascular permeability, resulting in attenuation of vascular inflammation in cultured endothelial cells and animal models. This study identifies a protective role of kallistatin in vascular injury, thereby implicating the therapeutic potential of kallistatin for vascular and inflammatory diseases. (Hypertension. 2010;56:260-267.)

Key Words: kallistatin ■ tumor necrosis factor-α ■ vascular endothelial growth factor ■ nuclear factor κB ■ inflammation ■ vascular permeability

Kallistatin (KS) was first discovered as a tissue kallikrein-binding protein and a unique serine proteinase inhibitor (serpin).1–3 KS was subsequently identified to be a potent vasodilator independent of its interaction with tissue kallikrein.4 Moreover, our previous study indicated that KS inhibited angiogenesis and tumor growth in cultured cells and tumor xenografts in nude mice.5 In addition, transgenic mice overexpressing KS are resistant to lipopolysaccharide-induced mortality.6 Local delivery of the human KS gene significantly reduced joint swelling and inflammatory cytokine levels in a collagen-induced rat arthritis model.7 KS gene transfer into rat hearts improved cardiac function and reduced ventricular remodeling, oxidative stress, cardiomyocyte apoptosis, and inflammatory cell accumulation after acute myocardial ischemia/reperfusion and chronic heart failure.8,9 Furthermore, KS attenuated salt-induced renal injury, oxidative stress, inflammation, and fibrosis.10 Collectively, these findings indicate that KS may play an important role in the protection against oxidative stress-induced inflammatory responses and organ damage. However, the molecular mechanism by which KS modulates inflammation has not been determined.

The cytokine tumor necrosis factor (TNF)-α, through its heparin-binding domain, binds to TNF-α receptor 1 or receptor 2 in a variety of cells to initiate inflammatory signaling cascades.11 KS also possesses a heparin-binding site, which is localized in the region between the C2 sheet and H helix.12 Double mutation of 2 lysine residues to alanine (K312A/K313A) in KS resulted in a marked reduction of heparin-binding activity.12 Moreover, the heparin-binding site of KS prevented vascular endothelial growth factor (VEGF) binding to endothelial cells and inhibited VEGF-induced endothelial cell proliferation, adhesion, and migration.13 Therefore, we speculate that KS may act as an anti-inflammatory agent by competing with TNF-α binding to heparan-sulfate proteoglycans, a low affinity-binding site,
and, thus, prevent TNF-α-mediated inflammatory signaling. In the present study, we investigated the role of the heparin-binding domain of KS in TNF-α-mediated inflammatory signaling pathways and VEGF-induced permeability in cultured endothelial cells, as well as edema and vessel leakage in a mouse model and inflammatory arthritis in a rat model. Our present studies indicate that KS’s heparin-binding domain is essential for its anti-inflammatory actions by preventing endothelial cell activation, adhesion, and permeability in vitro and in animal models.

**Methods**

**Cell Culture**

Human umbilical vein endothelial cells (HUVECs; Clonetics) were cultured in endothelial basal medium 2 supplied with EGM-2 SingleQuots (Lonza). Experiments were performed between cell passages 3 and 5. Human THP-1 monocytes (American Type Culture Collection) were cultured in RPMI 1640 with 10% FBS.

**Generation of Adenovirus Carrying Human KS and Purification of Recombinant KSs**

Adenoviral vectors carrying the wild-type human KS and KS heparin mutant (K312A/K313A) cDNA under the control of the cytomegalovirus enhancer/promoter (Ad.KS and Ad.KS-HM) or the adenoviral vector alone (Ad.Null) were constructed and prepared as described previously.14 Expression and purification of recombinant KS and KS heparin mutant (KS-HM) proteins were performed as described.12

**Competitive Binding of 125I-Labeled TNF-α With KS to Endothelial Cells**

Competitive binding of 125I-labeled TNF-α with KS to the endothelial cell surface was performed as described previously.13 Quiescent HUVECs were incubated with 1 ng/mL of 125I-labeled TNF-α in binding buffer (DMEM supplemented with 20 mmol/L of HEPES [pH 7.4] and 0.1% BSA) at 4°C for 2 hours with or without increasing concentrations of KS or KS-HM (0.01 to 1.00 μmol/L). Unbound 125I-labeled TNF-α was removed by washing, and bound protein was solubilized in 250 μL of 0.3 mol/L of NaOH. Radioactivity was determined using a Multigamma counter. Nonspecific binding was assessed by measuring binding in the presence of excess unlabeled TNF-α. Specific binding was calculated by subtracting the nonspecific binding from the total binding.

**Assessment of TNF-α–Induced Signaling by Western Blot**

To study the effects of TNF-α on inhibitor of nuclear factor κB (NF-κB; IκB)-α and NF-κB signaling, endothelial cells were treated with KS or KS-HM (0.1 to 0.5 μmol/L) in the presence or absence of TNF-α (10 ng/mL). Western blot analysis of cytosolic extracts was performed using the following primary antibodies: anti-TNF receptor 1–associated death domain protein (TRADD), antiphospho–IκB-α (Ser 32), anti–IκB-α, antiphospho-p38 mitogen-activated protein kinase (MAPK), and antiphospho-p38 MAPK. Nuclear extracts were used for Western blot analysis using the primary antibodies antiphospho–NF-κB (Ser 536) and anti–NF-κB. Protein concentration in cytosolic or nuclear extracts was determined by Bio-Rad DC Protein Assay kit (Bio-Rad Laboratories).

**Proinflammatory Gene Expression by Quantitative Real-Time PCR**

Total RNA was extracted from cultured cells using TRIzol reagent (Invitrogen). cDNA was transcribed from 2 μg of RNA using a high cDNA archive kit (Applied Biosystems) following the manufacturer’s instructions. The quantitative real-time PCR was carried out using the Gene Expression Assay on a 7300 real-time PCR system (Applied Biosystems). Quantification was determined by Relative Quantification Software (Applied Biosystems). The effect of p38MAPK inhibitor (SB 202190, 5 μmol/L, Calbiochem) on the expression of vascular cell adhesion molecule (VCAM) 1, intercellular adhesion molecule (ICAM) 1, and monocyte chemoattractant protein (MCP) 1 was determined.

**NF-κB Luciferase Assay**

NF-κB driven luciferase plasmid (1 μg) was transfected into HUVECs with a HUVEC Nucleofector kit (Lonza) according to the manufacturer’s instructions. Transfections were performed in triplicates. Luciferase activity was measured using the Luciferase Assay System (Promega) according to the manufacturer’s instructions. The light produced was measured using a luminometer. Luciferase activity was normalized to total cell protein.9

**Adhesion of Endothelial Cells to Monocytes**

HUVECs in 24-well plates were pretreated with KS or KS-HM (0.05 to 0.2 μmol/L) for 1 hour, followed by incubation with TNF-α (10 ng/mL) for 24 hours. The cells were rinsed, and THP-1 monocytic cells were then added to each well at a density of 2.5×10⁶ cells per well and incubated for 90 minutes. The nonadherent THP-1 cells were rinsed off, and monocytic cells adherent to endothelial cells were counted as the number of translucent cells per visual field using a phase-contrast microscope.15

**Endothelial Cell Monolayer Permeability**

HUVECs were seeded onto the upper chambers of collagen IV–coated (50 μL, 50 μg/mL) transwell filters (0.4-μm pore size) at a density of 2×10⁵ cells per well in 12-well plates and incubated at 37°C in growth medium for 4 days with changes of fresh medium daily. Fluorescein isothiocyanate–labeled albumin (Sigma) was added to growth medium in the upper chamber, and 1 mL of growth medium containing 1% BSA was added into the lower chamber. Immediately afterward, VEGF (100 ng/mL) was added in the presence or absence of KS or KS-HM (0.5 μmol/L) for 24 hours. From the lower chamber, aliquots (100 μL) were aspirated and diluted with PBS before the concentration of fluorescein isothiocyanate–labeled albumin was measured, as an index of monolayer permeability. The amount of fluorescein isothiocyanate–labeled albumin was determined using a fluorescent spectrophotometer.16

**Vascular Permeability in a Mouse Model**

All of the procedures complied with the standards for care and use of animal subjects as stated in the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Resources, National Academy of Sciences). The protocol for the animal studies was approved by the Medical University of South Carolina Institutional Animal Care and Use Committee. Three days before inflammation induction, adenoviral solution of Ad.KS or Ad.KS-HM (9×10⁸ plaque-forming units in PBS) was delivered into male wild-type C57BL/6 mice (Jackson Laboratory) through tail vein injection. Ad.Null was used as the control. Three days after adenovirus delivery, Evans blue dye (6.25 mg/mL in 200 μL of PBS) was injected intravenously. Ten minutes later, mice were given a bolus dose of recombinant human C5a (2 μg/kg in 200 μL) intravenously. At 1 hour after injection, mice were euthanized, and paws, intestine, and lung were removed for leakage density evaluation. Vascular permeability after C5a injection was assessed as described previously.13 Expression of recombinant human KS was measured in mouse serum by ELISA, as described previously.18

**Rat Arthritis Model**

All of the procedures were approved and complied with the standards as indicated above. Male Sprague-Dawley rats (Harlan) were anesthetized by inhaled isoflurane and immunized by intradermal injection of a collagen II/Feud complete adjuvant mixture into the tail.
and 4 sites of both knees, as described previously. One week after initial injection, booster was administered on both knees intra-articularly using 200 μL of collagen II/Freund complete adjuvant mixture. At the same time, both ankles were injected with Ad.KS, Ad.KS-HM, or Ad.Null (8×10^7 plaque-forming units in PBS). Five weeks after initial collagen/adjuvant injection, ankle arthritis parameters, including measurements of articular index scores, ankle circumference, and paw thickness, were assessed as described previously.

**Statistical Analysis**

Data are expressed as mean±SEM and were compared between experimental groups with the use of 1-way ANOVA followed by Fisher protected least significant difference. Probability values of P<0.05 were considered statistically significant.

**Results**

**KS Antagonizes TNF-α Binding to TNF-α Receptor on Endothelial Cells**

To test the hypothesis that KS may act to prevent endothelial inflammation by competing with TNF-α binding to heparan-sulfate proteoglycans and, thus, to inhibit the TNF-α-mediated inflammatory cascade, we first determined whether KS is capable of competing with TNF-α in binding to endothelial cells. Figure 1A shows that recombinant KS dose-dependently inhibited TNF-α binding to endothelial cells. The specific binding of ^125^I-labeled TNF-α was reduced by 55.7% in the presence of 1 μmol/L of KS. In contrast, KS mutant K312A/K313A lacking heparin-binding activity (KS-HM) at all of the doses did not interfere with TNF-α binding to endothelial cells (Figure 1A). The effect of KS on TRADD, an adaptor protein of the TNF-α receptor 1, was determined by Western blot analysis. TNF-α treatment before the addition of KS, or TNF-α alone, significantly increased TRADD levels (Figure 1B). However, KS treatment before the addition of TNF-α, or simultaneous treatment of TNF-α and KS, not only effectively reduced TRADD levels but also caused a marked rise in IκB-α compared with TNF-α alone, indicating that KS inhibited TNF-α–induced NF-κB activation by preventing IκB-α degradation (Figure 1B). These findings demonstrate that KS through its heparin-binding domain prevents TNF-α–mediated effects by antagonizing TNF-α binding to the TNF-α receptor on the endothelial cell surface.

**KS Through Its Heparin-Binding Site Inhibits TNF-α–Induced IκB-α Degradation and NF-κB-65 Phosphorylation**

We next investigated the effect of KS on TRADD-mediated TNF-α signaling pathways in endothelial cells. KS, but not KS-HM, significantly decreased TRADD levels in a dose-dependent manner, indicating that KS blocks TNF-α receptor activation (Figure 2A). Furthermore, after TNF-α treatment, KS increased IκB-α levels but decreased phospho–IκB-α levels in cytosolic extracts, whereas KS-HM had no effect. The inhibition of NF-κB activation by KS was confirmed by reduced phospho–NF-κB-65 levels in nuclear extracts. These results indicate that the heparin-binding domain is essential for KS to regulate TNF-α–mediated NF-κB nuclear translocation and activation.

**KS Through Its Heparin-Binding Site Inhibits TNF-α–Induced p38MAPK Phosphorylation and VCAM-1, ICAM-1, and MCP-1 Expression in Endothelial Cells**

KS treatment reduced TNF-α–induced p38MAPK phosphorylation, although KS-HM had no effect (Figure 2B). KS, but not KS-HM, suppressed TNF-α–induced NF-κB activation. Inhibition of p38MAPK by SB 202190, a p38MAPK inhibitor, also resulted in reduced TNF-α–induced NF-κB activity (Figure 3A). Likewise, KS, but not KS-HM, suppressed TNF-α–induced VCAM-1, ICAM-1, and MCP-1 expression through p38MAPK activation (Figure 3B through 3D). These results indicate that KS attenuates TNF-α–induced proin
Inflammatory gene expression by suppressing p38MAPK-dependent NF-κB activation.

**KS Inhibits Adhesion of Activated Endothelial Cells to THP-1 Monocytes**

It is well known that monocyte adhesion to endothelial cells is an important event in the initiation of inflammation development. Therefore, we examined THP-1 cell adhesion to TNF-α-stimulated endothelial cells to assess the mechanism responsible for the anti-inflammatory effect of KS. As shown in Figure 4, THP-1 cell adhesion to HUVECs was substantially increased when HUVECs were stimulated with TNF-α but significantly suppressed by KS (456.0±37.5 versus 238.0±15.0 cells per high-power field; n=4; P < 0.05). Conversely, KS-HM treatment had no effect on TNF-α-induced THP-1 adhesion to endothelial cells.

**KS Inhibits Vascular Permeability in Cultured Cells and in Mice**

We further studied the effect of KS on vascular permeability using cultured endothelial cells and passive cutaneous anaphylaxis in mice. Recombinant KS, but not the heparin mutant, inhibited VEGF-induced endothelial permeability in cultured endothelial cells (Figure 5A). Our results showed that recombinant human KS levels were 17.2±3.7 µg/mL (n=10) in mouse serum 3 days after systemic injection of adenovirus carrying human KS gene but were not detected in mice injected with control adenovirus. In mice, vascular leakage was determined by Evans blue dye extravasation from the bloodstream into tissues at 1 hour after C5a-induced passive cutaneous anaphylaxis. Representative images showed that, 3 days after gene transfer, KS, but not KS heparin mutant, markedly inhibited vascular leakage in paws.
triggered by C5a injection (Figure 5B). Evans blue densities in bowel and lung were also significantly reduced by KS gene administration (Figure 5C and 5D). However, gene delivery of KS heparin mutant had no effect on in vivo vascular leakage. These findings demonstrate that KS, through its heparin-binding site, blocks vascular permeability.

**KS’s Heparin-Binding Domain Is Essential to Prevent Inflammation in Rat Arthritis**

We analyzed the role of KS’s heparin-binding domain on joint swelling and inflammation using an established ankle joint arthritis model in rats. Expression of human KS in rat joint tissue after local gene delivery was identified by immunohistochemical staining and Western blot, as reported previously7 (data not shown). Representative ankle joint images showed that KS gene transfer significantly reduced joint swelling, whereas the KS heparin mutant had no effect (Figure 6A). Parameter analysis demonstrated that KS, but not KS heparin mutant, significantly reduced ankle circumference, articular index, and paw thickness from 3 to 5 weeks after induction of ankle joint arthritis (Figure 6B through 6D). These findings further confirm that KS’s heparin-binding domain is necessary to prevent joint arthritis in vivo.

**Discussion**

This is the first study to demonstrate a novel role of KS as an inhibitor of inflammation and vascular permeability and that KS’s heparin-binding domain plays a crucial role in its anti-inflammatory actions. Endothelial cells lining the blood vessels are actively involved in many physiological processes, such as regulation of selective permeability, recruitment, and homing of inflammatory cells to the injured tissues. The endothelial cell is the principal physiological target of the proinflammatory cytokine TNF-α. TNF-α elicits a broad spectrum of biological effects, including the proliferation, differentiation, and apoptosis of endothelial cells and other cell types.20 Previous studies suggest that its receptor, TNFR1, primarily mediates TNF-α-induced inflammation and cell death, whereas TNFR2 serves to enhance TNFR1-induced cell death or promote cell activation, migration, growth, or proliferation in a cell type–specific manner.21–23 It is believed that the 2 receptors initiate distinct signal transduction pathways by interacting with different protein complexes. Although TNFR2 can inhibit apoptosis,24,25 the role of the proteins recruited to TNFR2 and the downstream signals involved have not been defined. In contrast, many proteins have been shown to be recruited by TNFR1, including TRADD. TRADD functions as a platform adaptor that recruits other proteins to form a TNFR1-signaling complex, which subsequently activates several distinct signaling cascades, including MAPKs, NF-κB, and caspase-dependent apoptotic pathways.21 Our present study indicated that KS, through its heparin-binding site, prevents TNF-α receptor activation in endothelial cells, resulting in blockade of TNF-α–induced signaling cascades and inhibition of monocyte adhesion. Previous studies showed that binding of VEGF to heparan-sulfate proteoglycans on the endothelial cell surface enhances its association with VEGF receptor 2 (Flk-1/KDR).26,27 Mutations that affect the heparin-binding activity lead to a
Kallistatin Inhibits Endothelial Inflammation

Figure 4. Effects of KS and KS heparin mutant on TNF-α-induced THP-1 monocyte adhesion on HUVECs. A, Representative images show adherent THP-1 cells to HUVECs. Endothelial cells were pretreated with KS (0.2 μmol/L) or KS heparin mutant (0.2 μmol/L) for 1 hour, followed by incubation with TNF-α (10 ng/mL) for 90 minutes. B, Quantitative analysis of adherent cells was determined by counting the number of translucent cells per visual field using the ×100 objective of a phase-contrast microscope. Values represent mean±SEM (n=4).

Reduction in the affinity of VEGF164 binding specificity to VEGF receptor 1 (Flt-1). Moreover, VEGF binding to the KDR receptor is necessary and sufficient for potentiation of VEGF-induced cytokine expression in cultured endothelial cells. Therefore, KDR receptor signaling governs the synergistic up-regulation of tissue factor induced by VEGF and TNF-α. Therefore, it is likely that competition of KS’s heparin-binding domain with VEGF binding to endothelial cells may prevent the synergistic VEGF/TNF-α upregulation of cytokine expression and, thus, inhibit local inflammation. We demonstrated previously that KS is an angiogenesis inhibitor and that the heparin-binding domain of KS is essential for blocking the effects of VEGF by competing with 125I-labeled VEGF and VEGF receptor binding in a concentration-dependent manner. Wild-type KS and the KS active-site mutant A377T, but not KS mutant lacking heparin-binding activity (K312A/K313A), competes with VEGF binding, inhibits VEGF-induced endothelial cell proliferation and migration, and attenuates VEGF-induced capillary density in mice. In the current study, we demonstrated an essential role of the heparin-binding domain in mediating KS’s effects on TNF-α- and VEGF-induced endothelial activation, adhesion, and permeability.

C5a has been shown to induce the production of TNF-α and the corresponding upregulation of vascular ICAM-1 in acute lung inflammatory injury in rats. Moreover, TNF-α exposure increased transient receptor potential channel expression in human pulmonary artery endothelial cells, which results in marked endothelial barrier dysfunction and increased vascular permeability. TNF-α promotes adhesion of immune cells to endothelial cells and causes an increase in vascular permeability. A previous report indicated that intravitreal injection of C5a in wild-type mice induced VEGF expression in the choroid, whereas VEGF levels were greatly reduced in C5a receptor gene knockout mice. Furthermore, C5a treatment increased VEGF secretion in human retinal pigment epithelial cells, whereas the C5a receptor antagonist, NDT9513727, inhibited C5a-induced VEGF expression. The current study, we found that KS through its heparin-binding site inhibited TNF-α-induced expression of cell adhesion molecules and cytokines, as well as monocyte adhesion in cultured endothelial cells. Our findings are supported by results obtained from both in vitro and in vivo studies, because KS, but not KS heparin mutant, reduced C5a/VEGF-induced endothelial permeability in vitro and in C5a-induced vascular leakage in mice by 50%. However, these measurements do not take into account the involvement of additional factors other than TNF-α and VEGF. Taken together, these results indicate that KS via the heparin-binding domain plays an important role in the protection against vascular injury by inhibiting TNF-α- and VEGF-induced vascular permeability and inflammatory gene expression.

In the present study, we showed that recombinant human KS at 0.01 to 1.00 μmol/L inhibited TNF-α binding to its receptor, and at 0.1 to 0.5 μmol/L significantly decreased TNF-α-induced NF-κB activation and proinflammatory gene expression in cultured human endothelial cells. We reported previously that endogenous KS levels are 20 to 25 μg/mL in rat plasma. These combined findings support the notion that endogenous levels of KS are high enough to oppose the actions of TNF-α and VEGF. Taken together, these results indicate that KS via the heparin-binding domain plays an important role in the protection against vascular injury by inhibiting TNF-α- and VEGF-induced vascular permeability and inflammatory gene expression.

Perspectives

Inflammation contributes broadly to a number of physiological and pathological processes, such as wound healing, infection, and cancer. Chronic inflammation of the blood vessel linings could cause altered or diminished synthesis or secretion of vasoactive substances by the endothelium. Prolonged inflammation may eventually lead to endothelial dysfunction and become a contributing factor in the development of hypertension and heart diseases. This study identified KS as a new endogenous plasma protein that is capable of inhibiting vascular inflammation. We showed that the heparin-binding domain of KS is essential for preventing TNF-α-mediated NF-κB activation and proinflammatory gene expression, thus suppressing endothelial cell activation,
leukocyte adhesion, and cellular permeability in cultured cells. Moreover, KS’s heparin-binding site plays a key role in prevention of vascular leakage in a mouse permeability model and joint inflammation in a rat arthritis model. We showed recently that KS inhibits endothelial inflammation by increasing endothelial NO synthase expression and NO formation in endothelial cells. KS thus inhibits inflammation by 2 unique and independent mechanisms: increased endothelial

![Graph A](image1)  
![Graph B](image2)  
![Graph C](image3)  
![Graph D](image4)

**Figure 5.** Effect of KS and KS heparin mutant on vascular permeability. A, Effect of KS and KS heparin mutant (0.5 μmol/L) on VEGF-induced albumin leakage in cultured endothelial cells as a measure of vascular permeability. Values represent mean±SEM (n=3). B, Representative images of Evans blue dye leakage in mouse paws. Evans blue dye leakage into (C) bowel and (D) lung. Values represent mean±SEM (n=10).

![Graph A](image5)  
![Graph B](image6)  
![Graph C](image7)  
![Graph D](image8)

**Figure 6.** Effects of KS and KS heparin mutant on collagen/adjuvant-induced arthritis in rats. A, Representative images show ankle and paw edema 5 weeks after collagen and Freund complete adjuvant injection. Quantitative analysis of (B) ankle circumference, (C) articular index scores, and (D) paw thickness. Values represent mean±SEM (n=10).
NO synthase expression and reduced TNF-α–induced signaling pathways. These results place KS at a unique position to foster the development of new strategies for treating a wide spectrum of conditions and diseases caused by vascular inflammation and endothelial dysfunction.

Sources of Funding
This work was supported by National Institutes of Health grants HL-44083 and HL-29397, and the Extramural Research Facilities Program of the National Center for Research Resources grant C06 RR105455.

Disclosures
None.

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Hypertension. 2010;56:260-267; originally published online June 21, 2010; doi: 10.1161/HYPERTENSIONAHA.110.152330
Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2010 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

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