Effects of Angiotensin II on NO Bioavailability Evaluated Using a Catheter-Type NO Sensor

Toshio Imanishi, Katsunobu Kobayashi, Akio Kuroi, Seiichi Mochizuki, Masami Goto, Kiyoshi Yoshida, Takashi Akasaka

Abstract—We investigated the acute or chronic effects of angiotensin (Ang) II on the bioavailability of NO in Ang II–infused rabbits using the catheter-type NO sensor. Male New Zealand White rabbits were infused with vehicle (sham), Ang II at a rate of 200 ng/kg per minute, either alone or in combination with hydralazine, Ang II type I receptor antagonist (valsartan), or an antioxidant (tempol) for 24 hours or 14 days. Plasma NO concentration was measured using the catheter-type NO sensor located in the aorta. We then infused saline (vehicle) and acetylcholine (ACh) into the aortic arch with or without pretreatment with N\textsuperscript{6}-methyl-L-arginine. An increase in plasma NO levels in response to ACh was significantly attenuated in the Ang II group compared with the control group. The decrease in the basal plasma NO concentration was significantly lower in the Ang II group than in the control group. Plasma peroxynitrite concentrations in Ang II group were significantly higher than in the control group. The negative effects of Ang II, that is, the decrease in basal and ACh-induced NO production and the increase in oxidative stress, were significantly suppressed by the cotreatment with either valsartan or tempol. Short-term treatment with Ang II significantly increased the ACh-induced increase in plasma NO concentration, as well as basal NO release. Although Ang II stimulates release of NO in the short term, chronic treatment with Ang II elicits the decreased NO bioavailability in the aorta of the Ang II–infusion rabbit model. (Hypertension. 2006;48:1058-1065.)

Key Words: nitric oxide ■ angiotensin II ■ oxidative stress ■ NO sensor ■ rabbit

In patients with coronary artery disease (CAD), endothelial dysfunction contributes to the abnormal vasomotor response to exercise, exposure to cold, or mental stress and may trigger myocardial ischemia.\textsuperscript{1} The endothelium is a dynamic autocrine and paracrine organ that regulates anti-inflammatory, mitogenic, and contractile activities of the vessel wall, as well as the hemostatic process within the vessel lumen. NO produced by endothelial cells is a highly reactive molecule with many biological effects.\textsuperscript{2} A dysfunctional endothelium, characterized by decreased NO synthesis and/or NO bioavailability, facilitates inflammation, smooth muscle cell proliferation and extracellular matrix deposition, and vasoconstriction, as well as a prothrombotic state within the vessel lumen.\textsuperscript{3,4} In addition, the long-term follow-up of patients with endothelial dysfunction suggests that reduced bioavailability of NO may even have prognostic implications and contributes to the progression of coronary atherosclerosis, because impaired endothelium-mediated vasomotion is associated with future cardiovascular events.\textsuperscript{5-7} Therefore, the appropriate treatment of vascular inflammation–mediated change in NO production should be further explored for the potential to prevent human atherosclerosis and unstable atheroma.

Previously, it has been considered that NO, once released from vascular endothelial cells into the bloodstream, is immediately oxidized or inactivated by dissolved oxygen, oxyhemoglobin, and/or oxygen radical species.\textsuperscript{8,9} However, growing experimental and clinical evidence suggests that NO remains active in the bloodstream, causing remote vasodilatory responses.\textsuperscript{10,11} As a high-temporal resolution method, electrochemical measurement methods for NO, that is, NO sensors, have been developed by several groups.\textsuperscript{12,13} These sensors enable us to evaluate dynamic changes in NO concentration in solutions and tissues in response to agonists, NO-generating reagents, and physical stimuli.\textsuperscript{14,15} However, electrical interference vibration, poor durability of the sensor-tip coatings, and other factors have made in vivo NO measurement very difficult. To overcome these drawbacks, a new NO sensor, which encloses both the working and reference electrodes within a highly gas-permeable and robust enclosure, has been developed.\textsuperscript{16-18} In addition, we have recently developed a catheter-type NO sensor.\textsuperscript{17,18}

In atherosclerosis\textsuperscript{19} and some forms of hypertension, levels of angiotensin (Ang) II may be increased. Ang II reportedly impairs vascular relaxation, in part by increasing the production of superoxide anion via a membrane-bound reduced nicotinamide-adenine dinucleotide phosphate oxidase.\textsuperscript{20} In Ang II–induced hypertension in rats, the impairment of vasodilator
responses to acetylcholine and calcium ionophore is overcome by treatment with liposome-encapsulated superoxide dismutase.\(^{21}\) On the basis of these findings, endothelial dysfunction in response to long-term Ang II treatment may be secondary to increased superoxide production within the endothelium.\(^{21,22}\) However, the limitation of these studies is that the release of NO from endothelium could only be inferred on the basis of a comparison of vessel relaxation. Meanwhile, the direct effect of Ang II on vascular smooth muscle cells might mask the endothelium-dependent action of NO. Therefore, direct in vivo measurements of intra-arterial NO concentration in blood would contribute to the detailed evaluation of endothelial function. The present studies were performed in rabbits to determine whether acute or chronic elevations in Ang II affect dynamic changes in plasma NO concentrations in Ang II–infusion rabbit models using the catheter-type NO sensor.

**Methods**

**Catheter-Type NO Sensor**

Integrated architecture and performance of the catheter-type NO sensor have been described previously.\(^{17,18}\) In brief, an NO sensor (amino-700 XL, Innovative Instruments, 700 \(\mu\)m in diameter at the detection tip) was mounted in a 4-Fr catheter (1200 mm long; Hirakawa Hewtech) and fixed with silicon adhesive. A soft protection tip of polyurethane was attached at the edge of the detection tip to prevent the vessel wall from physical damage, and 2 metal wires were also attached along the detection tip to provide the electrodes a mechanical support. The oxidative current of NO was monitored using the catheter-type NO sensor.17,18 Briefly, NO-saturated pure water was prepared by bubbling pure NO gas in oxygen-free pure water. Using a gas-tight syringe, 5 \(\mu\)L were injected into a well-stirred saline solution (50 mL) in which the NO sensor was immersed (final NO concentration: 190 nM) as described previously.\(^{16-18}\) The baseline (0 level) is set arbitrarily in the amperometric method, and, thus, a change in the current form from the baseline is used and is expressed as “change in NO concentration (nM),” as described previously.\(^{16-18}\)

**Animal Preparation**

We confirmed with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health. In addition, the study protocol was approved by the Institutional Animal Care and Use Committee of the Wakayama Medical University. Male New Zealand White rabbits (2.0 to 2.5 kg) were maintained on tap water and standard diet. The animals were randomized into 1 of 6 groups. Group 1 (\(n = 6\)) received vehicle (0.154 mol/L of NaCl), whereas groups 2 through 6 (\(n = 6\) per group) were prepared by the infusion of human Ang II (Peninsula Laboratories) at 60 ng/kg per minute (Ang II 60; group 2) and 200 ng/kg per minute (Ang II 200; groups 3 to 6) via an osmotic minipump (Alzet; DURET Corp) implanted SC for 14 days. In addition to Ang II, groups 4 through 6 also received, respectively, 10 mg/kg per day of hydralazine, 5 mg/kg per day of valsartan (Novartis Pharma AG), or 10 mmol/L of tempol (a superoxide dismutase mimetic; Sigma Chemical Co)\(^{23,24}\) in their drinking water. Rabbits were anesthetized with xylazine (10 mg/kg IM), ketamine (50 mg/kg IM), and pentobarbital sodium (10 mg/kg IV), followed by heparin (1000 U IV) for anticoagulation. A catheter for acetylcholine (ACh) infusion was located in the aortic arch from the external carotid artery, and the NO sensor was inserted through the left femoral artery and located in the abdominal aorta. Aortic blood pressure was simultaneously monitored using a stilt cannula with a strain gauge pressure transducer (Nihon Kohden). In addition, to examine the acute response to Ang II, the animals received either vehicle (0.154 mol/L of NaCl) or human Ang II at 200 ng/kg per minute via an osmotic minipump implanted SC for 24 hours. They then investigated the short-term effect of Ang II on the ACh-induced NO and the basal NO release as described above.

**Experimental Protocol**

To measure the endothelium-dependent NO production, either 5 or 20 \(\mu\)g/kg of ACh was administered at 1 mL/min for 5 minutes. To inhibit NO synthesis in the endothelium, 5 mg/kg of N\(^{-}\)-methyl-L-arginine ((L-NMMMA) NO synthase inhibitor) was infused at 1 mL/min for 10 minutes. We then repeated ACh infusions at 2 doses for each animal. Plasma NO concentration in the abdominal aorta was monitored over the entire time course.

**Measurement of Plasma Nitrotyrosine**

Active NO metabolites can react with superoxide to form peroxynitrite, a strong oxidant. Subsequent reaction of peroxynitrite with proteins results in nitrotyrosine formation. As a stable end product of peroxynitrite-mediated oxidation/nitration, nitrotyrosine can be used as a surrogate index of NO-dependent damage in vivo. Therefore, we investigated the effect of Ang II on nitrotyrosine formation. Plasma nitrotyrosine concentrations were measured using the NWLSS nitrotyrosine ELISA kit (Northwest Life Science Specialties, LLC) according to the manufacturer’s protocol. The NWLSS nitrotyrosine ELISA assay measures nitrosylated protein and not “free” nitrotyrosine. Samples do not need to be hydrolyzed, and data should be interpreted as being inclusive of nitrotyrosine still bound to soluble proteins.\(^{25}\) The kit is a simple “sandwich” ELISA using a plate-bound capture nitrotyrosine antibody and a biotinylated secondary tracer antibody. The addition of streptavidin-peroxidase followed by tetramethylbenzidine facilitates color development. The reaction is stopped using a citric acid solution, and the amount of bound protein in each well was measured using a microplate reader at 450 nm.

**Statistical Analysis**

Differences in values between groups and treatments were tested using 2-factor repeated-measures ANOVA. When appropriate, posthoc comparisons between groups were made with Student t test. A \(P\) value of \(<0.05\) was considered significant. All of the data were expressed as the mean±SEM based on \(\geq 6\) independent experiments.

**Results**

**Calibration of Sensors**

The basic performance of the integrated catheter-type NO sensors had been reported.\(^{17,18}\) The NO sensors showed no noticeable response to changes in concentrations of oxygen, ACh, valsartan, hydralazine, tempol, or solution mixing, indicating a high specificity for NO (data not shown). The mean peak response of NO value of the 7 sensors used in the present study was 321±12 pA/nM. This value is comparable to the values obtained with the original in vivo sensor.\(^{16}\)

**Evaluation of Aortic NO Production**

Although ACh infusion (4 \(\mu\)g/kg per minute for 5 minutes) caused a significant fall in mean arterial pressure (MAP), as well as heart rate (HR), pretreatment with L-NMMA caused an attenuation of ACh-induced hypotensive actions and bradycardia (Table 1).

Plasma NO concentration was successfully measured in the aorta by the catheter-type NO sensor in all of the rabbits studied. Figure 1A shows representative tracings of the plasma NO concentration in the aorta after infusion of saline, ACh (1 and 4 \(\mu\)g/kg per minute), and ACh (4 \(\mu\)g/kg per minute) after L-NMMA. Intra-aortic infusion of saline (vehicle) caused only a small fluctuation in the plasma NO concentration (Figure 1A). Plasma NO concentration increased sharply after intra-aortic injection of ACh (Figure 1A). ACh (1 or 4 \(\mu\)g/kg per minute)
increased the plasma NO concentration, which was demonstrated as the peak response, as well as the integrated response over the entire period, in a concentration-dependent manner (Figure 1B and 1C). The ACh-induced increase in NO concentration was significantly attenuated after the pretreatment with L-NMMA (Figure 1).

**TABLE 1. Hemodynamic Data in This Study**

<table>
<thead>
<tr>
<th>Injection</th>
<th>MAP, mm Hg</th>
<th>HR, bpm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre-ACh</td>
<td>Post-ACh</td>
</tr>
<tr>
<td>Vehicle</td>
<td>68.8±1.5</td>
<td>69.5±1.1</td>
</tr>
<tr>
<td>ACh, 1 μg/kg per minute</td>
<td>70.1±1.8</td>
<td>66.5±2.4</td>
</tr>
<tr>
<td>ACh, 4 μg/kg per minute</td>
<td>69.9±2.1</td>
<td>52.4±2.2</td>
</tr>
<tr>
<td>L-NMMA+ACh, 4 μg/kg per minute</td>
<td>72.5±2.1</td>
<td>62.1±1.9</td>
</tr>
</tbody>
</table>

*Data are the mean±SEM. *P<0.05 vs pre-ACh.

**Effect of Chronic Treatment With Ang II on Aortic NO Concentration**

MAP in Ang II (60) rabbits did not differ significantly from that in vehicle (control) rabbits. In contrast, Ang II (200) rabbits showed a significant increase in MAP; this effect was significantly reduced by the cotreatment of valsartan or hydralazine but not tempol (Table 2). Similarly, Ang II (200) rabbits showed a significant increase in HR; this effect was significantly abolished by the cotreatment of valsartan or hydralazine but not tempol (Table 2). Long-term Ang II treatment resulted in a significant reduction in the increase in plasma NO concentration, which was demonstrated as the peak response, as well as the integrated response over the entire period (Figure 2). In contrast, Ang II type 1 receptor antagonist (valsartan), but not hydralazine, significantly reversed the Ang II–induced decrease of ACh-induced NO concentration.

**Figure 1.** ACh-induced change in NO concentration and the effect of NO synthase inhibition. (A) Typical tracings of the plasma NO concentration in the abdominal aorta. (a) Saline (vehicle) was injected at the arrow for 5 minutes. ACh at (b) 1 or (c) 4 μg/kg per minute was injected at the arrow for 5 minutes. ACh at 4 μg/kg per minute was injected at the arrow for 5 minutes after inhibition of NO synthesis by infusion of L-NMMA for (d) 10 minutes. The injection of saline caused only a treatment fluctuation but no increase in NO concentration. In contrast, ACh increased NO concentration markedly in a dose-dependent manner. L-NMMA attenuated the increase of NO concentration by ACh. Plasma NO concentration measured by the (B) peak response, as well as the (C) integrated response over the entire period, was increased by ACh in a dose-dependent manner (1 and 4 μg/kg per minute). L-NMMA suppressed the increase of NO concentration by ACh significantly. Data are the mean±SEM (n=6). *P<0.05 vs vehicle (control). #P<0.05 vs ACh (4 μg/kg per minute).
NO production (Figure 2). In addition, an antioxidant, tempol, also significantly reversed Ang II–induced decrease of ACh-induced NO production (Figure 2).

Effect of Chronic Treatment With Ang II on Basal Plasma NO Concentration

The effect of Ang II on basal plasma NO concentration was evaluated using an NO synthesis inhibitor, L-NMMA. Ang II affected basal plasma NO concentration (Figure 3). That is, the decrease in the basal plasma NO concentration by L-NMMA infusion was significantly lower in the Ang II 200 group than in the untreated (control) group, indicating a lower basal NO bioavailability (Figure 3). Furthermore, the inhibitory effect of Ang II 200 on basal plasma NO concentration was significantly reversed by cotreatment with either valsartan (Figure 3).

Effect of Chronic Treatment With Ang II on Plasma Nitrotyrosine

The Ang II 200 group had significantly higher plasma nitrotyrosine levels than the control group. Both valsartan and tempol significantly reduced the plasma nitrotyrosine level (Figure 4).

Acute Response to Ang II

Because a number of studies have demonstrated that Ang II increases NO release and that blockade of NO synthase (NOS) leads to a potentiation of Ang II-induced hypertension,26–28 we examined the short-term effect of Ang II on the ACh-induced NO response and the basal NO release. In

![Figure 2](http://hyper.ahajournals.org/)

**Figure 2.** Effect of chronic treatment with Ang II on ACh-induced plasma NO concentration. (A) Typical tracings of the plasma NO concentration induced by ACh in the aorta treated with vehicle (control; a), Ang II 60 (b), Ang II 200 (c), Ang II 200+valsartan (d), and Ang II 200+tempol (e; n=6 for each group). ACh-induced increases in the NO concentration measured by the peak response (B), as well as in the integrated response over the entire period (C) in aorta treated with vehicle, Ang II 60, Ang II 200, Ang II 200+hydralazine, Ang II 200+valsartan, and Ang II 200+tempol. The decrease of plasma NO concentration in Ang II 200 rabbits was significantly reversed by cotreatment of either valsartan or tempol but not hydralazine. Data are expressed as an absolute value. Bars represent mean±SEM.

*P<0.05 vs control. #P<0.05 vs Ang II 200.
contrast with chronic administration of Ang II, the short-term administration of Ang II significantly increased the ACh-induced increase in plasma NO concentration, as well as basal NO release (Figure 5A and 5B, respectively). Interestingly, in contrast with chronic administration of Ang II, plasma peroxynitrite concentration in the short-term Ang II–treated group was only a slight, but not significant, increase compared with the control group (Figure 5C). Furthermore, neither MAP nor HR was significantly different between the Ang II–treated group and the control group (MAP: 73.5 ± 1.5 versus 69.0 ± 2.5, P value not significant; HR: 172 ± 2 versus 170 ± 3, P value not significant, respectively). These results suggest that Ang II stimulates the release of NO in the short term.

**Discussion**

This study, using the newly developed NO sensor, reports for the first time that long-term Ang II treatment leads to the decrease in NO bioavailability in a concentration-dependent manner because of the increase in oxidative stress. From an analytical point of view, detection of NO in biological fluids is a challenging topic. NO produced by endothelial cells diffuses into the flowing blood, where it reacts with blood components, such as erythrocytes and proteins. It has been assumed that NO is rapidly oxidized by its reaction with the ion-containing heme groups of oxyhemoglobin. The short biological half-life of NO means that it is most likely that the bioactivity of NO is confined to the vicinity of its production site. However, Stamler et al11 have suggested that S-nitrosohemoglobin is a source of bioactive NO and a crucial component of the cardiorespiratory cycle. They propose that S-nitrosohemoglobin forms in tissues at high oxygen tensions, whereas at low oxygen saturations it decomposes and releases NO, which dilates blood vessels. Whatever mechanisms turn out to lie behind the role of NO in the cardiorespiratory cycle, it is now most possible that NO may be transported throughout the body in the manner of a hormone. Actually, growing experimental and clinical evidence suggests that NO remains active in the bloodstream, causing remote vasodilatory responses.10,11

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**Figure 3.** Effect of chronic treatment with Ang II on basal plasma NO concentration by L-NMMA. (A) Typical tracings of the basal plasma NO concentration induced by L-NMMA in the aorta treated with vehicle (control; a), Ang II 200 (b), Ang II + valsartan (c), and Ang II + tempol (d). Basal plasma NO concentration measured by the peak response (B), as well as the integrated response over the entire period (C) in aorta treated with vehicle, Ang II 60, Ang II 200, Ang II 200 ± hydralazine, Ang II 200 ± valsartan, and Ang II ± tempol in the presence of 100 μmol/L L-NMMA. After pretreatment for 10 minutes with L-NMMA, NO release was measured by NO sensor. Data are the mean ± SEM (n = 6). *P < 0.05 vs vehicle; #P < 0.05 vs Ang II 200.
Detection of NO has been based mostly on the measurement of biologically inactive products of NO (nitrite and nitrate) or bioassays that rely on secondary effects of NO (e.g., vasorelaxation after denudation of endothelial cells or NO synthesis inhibition). On the other hand, the catheter-type NO sensors, which we used in the present study, showed high sensitivity (320 pA/nM) with satisfactory stability and reliability in measurements of plasma NO concentration in the rabbit aorta. As reported previously, direct exposure of the new NO sensor to ACh solution did not cause any significant change in the baseline current (data not shown). The NO sensor also showed no noticeable change in the baseline current because of solution mixing, suggesting that it was not affected by fluid (blood) motion (data not shown). Thus, this system has high specificity for NO, and the monitored current reflected the change in plasma concentration of NO released from the endothelium after ACh infusion. The present study demonstrated that the catheter-type NO sensor is applicable to the direct in vivo measurement of endothelium-derived NO in the rabbit model. We found that NO concentration increased in blood during ACh infusion. The base-to-peak increase in plasma NO concentration during ACh infusion was found to be ≈8 nM. Although the dose of 1-NMMA used in this study (5 mg/kg for 10 minutes) was reported to inhibit NO synthesis greatly, administration of this dose could not achieve complete inhibition of NO production by ACh. We believe that under in vivo circumstances with stimulatory mechanical stresses, it may be extremely difficult to completely abolish the NO production by using the competitive inhibitors of NOS. These data are in good agreement with those of the previous experiments using dogs. 1-NMMA infusion significantly attenuated the decrease in systemic pressure by ACh, as well as the increase in plasma NO, demonstrating the systemic hypotensive effect of endothelium-derived NO. We have demonstrated in the present study that the decrease in MAP, as well as HR, by ACh infusion was attenuated by pretreatment with 1-NMMA. These results are in line with previous reports. That is, Rees et al have shown that blockade of the NO system with 1-NMMA inhibits the hypotensive action of ACh, suggesting a role for NO in the regulation of blood pressure in anesthetized rabbits. In addition, they have demonstrated that pretreatment with 1-NMMA attenuated ACh-induced bradycardia, although the mechanisms remain to be ascertained. Related to these observations, Togashi et al have shown that NO decreases central sympathetic outflow. However, a decreased HR may not simply be an indicator of decreased sympathetic activity; further studies will be needed to clarify this observation.

In the present study, we have demonstrated that long-term infusion of Ang II elicits a reduction in NO concentration measurable by the NO sensor. This may result from a decrease in NO synthesis or an increase in NO inactivation because of locally enhanced production of reactive oxygen species (ROS). This result is in line with previous findings. Mollnau et al have shown that NO in the vascular segments from the aorta, assessed with electron paramagnetic resonance, was markedly reduced in the Ang II–infusion rat model. They have proposed a mechanism where reduced nicotinamide-adenine dinucleotide phosphate oxidase–induced superoxide production may trigger NOS III uncoupling, leading to impaired NO/cGMP signaling and to endothelial dysfunction in this animal model. Moreover, an increase in the plasma NO level in response to ACh was significantly attenuated in the Ang II–treated group compared with the control group. Inactivation of NO occurs mainly through its interaction with ROS. In fact, in the present study, an antioxidant, tempol, significantly reversed the ACh-induced impairment of NO production induced by ACh.

In conclusion, we have elucidated for the first time how Ang II may alter plasma NO concentration in anesthetized animal using a catheter-type NO sensor. Short-term infusion of Ang II elicited the basal and ACh-induced increase of plasma NO concentration. In contrast, chronic Ang II infu-
Figure 5. (A) Effect of acute treatment with Ang II on ACh-induced plasma NO concentration. (a) Typical tracings of the plasma NO concentration induced by ACh in the aorta treated with vehicle (control; top) and Ang II at 200 ng/kg per minute (bottom). ACh-induced increases in the NO concentration measured by the peak response (b), as well as the integrated response over the entire period (c), in aorta treated with vehicle (control) and Ang II. Data are expressed as the mean±SEM (n=6). *P<0.05 vs control group. (B) Effect of acute treatment with Ang II on basal plasma NO concentration by L-NMMA. (a) Typical tracings of the basal plasma NO concentration induced by L-NMMA in the aorta treated with vehicle (control; top) and Ang II (bottom). Basal plasma NO concentration measured by the peak response (b), as well as the integrated response over the entire period (c), in aorta treated with vehicle (control) and Ang II. Data are expressed as the mean±SEM (n=6). *P<0.05 vs control group. (C) Effect of acute treatment with Ang II on plasma peroxynitrite. The levels of plasma peroxynitrite treated with vehicle (control) and Ang II were detected as described in the Methods section. Data are expressed as the mean±SEM (n=6). *P<0.05 vs control group.
tion elicited the impairment of the basal and ACh-induced decrease of plasma NO concentration possibly through a decrease in NO synthesis or an increase in NO inactivation because of locally enhanced production of ROS.

**Perspectives**

Impaired NO bioavailability represents the central feature of endothelial dysfunction and predisposes to a platelet-dependent arterial prothrombotic state, conditions that both contribute to the development of atherothrombosis. An exciting aspect of this emerging area of study is that NO research has led to the pharmacological modulation of NO, which may lead to restoration of endothelial function and reduced atherogenesis in several clinical correlations to laboratory animal experimental models. The catheter-type NO sensor has potential use in the study of the pharmacological modulation of NO, enabling the measurement of the basal and ACh-induced NO production in the aorta of experimental animal models. Furthermore, it may also be possible to apply the sensor to clinical diagnosis of endothelial dysfunction, that is, reduced endothelium-derived NO availability in the cardiovascular system.

**Disclosures**

None.

**References**

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