Mechanism of Endothelial Nitric Oxide Synthase Phosphorylation and Activation by Thrombin

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Abstract—Thrombin has been shown to activate endothelial NO synthase (eNOS) leading to endothelium-dependent vasorelaxation. In addition to its activation by Ca\(^{2+}\)/calmodulin, eNOS has several regulatory sites. Ser\(^{179}\) phosphorylation of eNOS by the phosphatidylinositol 3-kinase–dependent Akt stimulates its catalytic activity. In this study, we have elucidated the signaling mechanism of thrombin-induced phosphorylation of eNOS in the regulation of NO production. Immunoblot analysis showed that thrombin rapidly phosphorylates eNOS at Ser\(^{179}\) in cultured bovine aortic endothelial cells. Also, thrombin was unable to stimulate eNOS if the Ser\(^{179}\) was mutated to Ala. Akt is phosphorylated in response to thrombin at Ser\(^{273}\) at a later time point than eNOS. In this regard, a phosphatidylinositol 3-kinase inhibitor, LY294002, blocked Akt phosphorylation without affecting eNOS phosphorylation and cGMP production by thrombin. The Ca\(^{2+}\) ionophore A23187 stimulated eNOS phosphorylation, as well as cGMP production, and pretreatment with intracellular or extracellular Ca\(^{2+}\) chelators inhibited thrombin-induced eNOS phosphorylation and cGMP production. Moreover, infection of bovine aortic endothelial cell with adenovirus encoding dominant-negative mutants of protein kinase C (PKC) α and PKCβ pretreatment of bovine aortic endothelial cells with PKC inhibitors revealed that PKCβ is indispensable for thrombin-induced eNOS phosphorylation and activation. From these data, we concluded that thrombin induces the Ser\(^{179}\) phosphorylation-dependent eNOS activation through a Ca\(^{2+}\)-dependent, PKCβ-sensitive, but phosphatidylinositol 3-kinase/Akt-independent pathway. (Hypertension. 2007;49:577-583.)

Key Words: thrombin ■ NO ■ endothelial NO synthase ■ protein kinase C

Thrombin has been shown to induce endothelium-dependent relaxation in many types of vascular tissue. In most cases, production of an endothelium-derived relaxing factor, NO, is, at least in part, responsible for the thrombin-induced vasorelaxation. NO is the most potent endogenous vasodilator known. NO also inhibits platelet adherence and aggregation, reduces adherence of leukocytes to the endothelium, and suppresses proliferation of vascular smooth muscle cells. In this regard, endothelial dysfunction in various cardiovascular disorders is characterized by decreased NO availability, justifying the number of studies dealing with signal transduction involved in the regulation of endothelial NO synthase (eNOS), the enzyme responsible for endothelial NO production. In addition to well-known Ca\(^{2+}\)/calmodulin-dependent activation of eNOS, recent studies revealed additional modes of posttranslational eNOS regulation involving its phosphorylation, dimerization, protein/protein interactions, and subcellular localization. For example, eNOS phosphorylation at Ser\(^{177}\)/human (Ser\(^{1179}\)/bovine) by phosphatidylinositol 3-kinase (PI3-K)–dependent Akt plays a critical role in its activation by various agonists, such as vascular endothelial growth factor.

Much effort has been made to delineate the molecular mechanisms by which thrombin affects cellular function. Thrombin exerts its cellular effects by activation of G protein–coupled protease-activated receptors. Through specific protease-activated receptors coupled to various G proteins (G\(_{i}\), G\(_{s}\), G\(_{i}\), and G\(_{12/13}\)), thrombin induces an array of intracellular signal transduction pathways in endothelial cells, such as phospholipase C, phospholipase A2 protein kinase C (PKC), small G proteins (Ras, Rho, and Rac), PI3-K, and several protein kinases including Akt/protein kinase B, Ca\(^{2+}\)/calmodulin-dependent kinase II, and mitogen-activated protein kinases. In the present study, we have investigated the roles of PI3-K, Akt, Ca\(^{2+}\), and specific PKC isoforms in regulating eNOS Ser\(^{179}\) phosphorylation by thrombin. Here, we demonstrated that thrombin induces phosphorylation and activation of eNOS through a Ca\(^{2+}\)-dependent and PKCβ-sensitive pathway that is independent of the PI3-K/Akt pathway.

Methods

Reagents

Thrombin from bovine plasma and EGTA were purchased from Sigma Chemical Co. N\(^{6}\)-nitro-L-arginine methyl ester, phorbol 12-
myristrate 13-acetate (PMA), LY294002, 1,2-bis(2-aminoxyethyl)ethane-N,N,N',N'-tetraacetic acid tetraacetoxy-methyl ester, GF109203X, and rottlerin were purchased from Calbiochem. YM-254890 was a gift from Astellas Pharma Inc. Antibodies that selectively recognize Ser1179-phosphorylated eNOS, Thr497-phosphorylated eNOS, Ser473-phosphorylated Akt, and total Akt were purchased from Cell Signaling Technology, and antibody to detect Tyr311-phosphorylated PKC\(\alpha\) was purchased from Bio-source International. Total eNOS antibody was purchased from BD Transduction Laboratories. Total PKC\(\alpha\), PKC\(\delta\), and extracellular signal-regulated kinase 2 antibodies were purchased from Santa Cruz Biotechnology, Inc.

**Cell Culture**

Bovine aortic endothelial cells (BAECs) were purchased from BioWhittaker and cultured in DMEM containing 10% FBS, penicillin, and streptomycin. Cells from passage 4 to 12 were grown to \(\approx 90\%\) confluence and serum depleted for 48 hours before the experiments. Cultured human umbilical vein endothelial cells were a gift from Dr Yi Wu (Temple University School of Medicine). Primary vascular smooth muscle cells (VSMCs) from rat aorta were obtained by the explant methods and subcultured for the experiment as described previously.12

**Immunoblotting**

Cell lysates were subjected to SDS-PAGE gel electrophoresis and electrophoretically transferred to a nitrocellulose membrane as described previously.13 The membranes were then exposed to primary antibodies overnight at 4°C. After incubation with the peroxidase-linked secondary antibody for 1 hour at room temperature, immunoreactive proteins were visualized by enhanced chemiluminescence reagent.13 Results were expressed as percentage increase in which the maximum response to thrombin (10 U/mL) is defined as 100%, because the basal signals are more varied depending on film exposure than the stimulated signals.

**Adenovirus Infection**

The generation and characterization of adenovirus encoding wild-type and dominant-negative mutants of PKC\(\alpha\) and PKC\(\delta\) were described in detail elsewhere.14 Adenovirus encoding wild-type and the Ser1179 mutant, S1179A, were kindly provided by Dr William Sessa.15 BAECs or VSMCs were infected with the adenovirus for 2 days as described previously before treatment with thrombin.16

**Intracellular cGMP Accumulation**

BAECs were incubated with agonists at 37°C for 20 minutes in the presence of 0.5 mmol/L of methylisobutylxanthine,17 and intracellular cGMP was determined using an enzyme immunoassay kit (Cayman Chemical) under the manufacturer’s instruction. Results were expressed as percentage increase in which the maximum response to thrombin (10 U/mL) is defined as 100%.

**Statistical Analysis of Data**

Results shown in the blots are representative of \(\geq 3\) independent experiments. Densitometry data and cGMP data were analyzed using 1-way ANOVA with a significance level of \(P<0.05\) (results shown are the mean±SEM of data from \(\geq 3\) separate experiments).

**Results**

**Thrombin Induces Ser1179 Phosphorylation and Activation of eNOS**

First, we examined whether or not thrombin phosphorylates eNOS at Ser1179, a catalytically positive regulatory site, in BAECs. As shown in Figure 1A, thrombin stimulated eNOS phosphorylation in a concentration-dependent manner. Figure 1B demonstrates that thrombin stimulates a significant increase in cGMP production that was completely inhibited by an eNOS inhibitor N\(^2\)-nitro-L-arginine methyl ester (100 \(\mu\)mol/L) for 30 minutes and stimulated with thrombin (10 U/mL) for 20 minutes. C, VSMCs infected with adenovirus encoding eNOS (WT), eNOS-S1179A (SA), or control vector (Vec) were stimulated with thrombin (10 U/mL) for 20 minutes, and cGMP concentrations were measured. Also, expression of eNOS in the infected VSMCs was evaluated. The data shown are mean±SEM (\(P<0.05\)).

![Figure 1. The effect of thrombin on eNOS phosphorylation and cGMP formation. BAECs were stimulated with the indicated concentrations of thrombin for 1 minute, and (A) phosphorylation of eNOS at Ser1179 was determined using immunoblot analysis with a phosphospecific antibody, or (B) cGMP was measured as described in the Methods section. BAECs were also pretreated with the eNOS inhibitor N\(^2\)-nitro-L-arginine methyl ester (100 \(\mu\)mol/L) for 30 minutes and stimulated with thrombin (10 U/mL) for 20 minutes. C, VSMCs infected with adenovirus encoding eNOS (WT), eNOS-S1179A (SA), or control vector (Vec) were stimulated with thrombin (10 U/mL) for 20 minutes, and cGMP concentrations were measured. Also, expression of eNOS in the infected VSMCs was evaluated. The data shown are mean±SEM (\(P<0.05\)).](http://hyper.ahajournals.org/doi/fig/10.1161/HYPERTENSIONAHA.106.137857)
inhibitor, N\textsuperscript{G}-nitro-L-arginine methyl ester. To examine the functional relevance of Ser1179 phosphorylation of eNOS by thrombin, cultured rat VSMCs with no detectable eNOS expression were infected with adenovirus encoding wild-type or S1179A eNOS, and thrombin-induced cGMP production was evaluated. Despite comparable expression and induction of equivalent basal cGMP production, thrombin was able to further stimulate cGMP production in VSMCs expressing wild-type eNOS but not in VSMCs expressing S1179A, suggesting a positive contribution of Ser1179 phosphorylation in eNOS activation by thrombin (Figure 1C).

**Thrombin-Induced Phosphorylation and Activation of eNOS Is Independent From the PI3-K/Akt Pathway**

Because eNOS phosphorylation at Ser\textsuperscript{1179} and subsequent eNOS activation are mediated by PI3-K/Akt activation in response to several distinct eNOS activators, such as vascular endothelial growth factor, in cultured endothelial cells,\textsuperscript{6} we investigated whether thrombin activates Akt and whether the activation precedes eNOS phosphorylation. Activation of Akt was assessed by its phosphorylation at Ser\textsuperscript{473}. As shown in Figure 2A and 2B, thrombin-induced eNOS phosphorylation could be observed as early as 0.5 minutes with maximum phosphorylation occurring at 1 minute. In contrast, we observed that thrombin induces the phosphorylation of Akt as early as 5 minutes (Figure 2A and 2B), which was much later than the thrombin-induced phosphorylation of eNOS (Figure 2C). These data indicate that Akt may not participate in the phosphorylation of eNOS by thrombin in BAECs.

To further explore our interpretation that the PI3-K/Akt signaling pathway is not required for thrombin-induced eNOS phosphorylation, we pretreated the BAECs with LY294002, an inhibitor of PI3-K before exposure to thrombin. Figure 3A and 3B show that LY294002 did not inhibit thrombin-induced eNOS phosphorylation at Ser\textsuperscript{1179} but that thrombin-induced Akt phosphorylation was completely inhibited by the agent. In addition, LY294002 had no inhibitory effect on cGMP production stimulated by thrombin in BAECs (Figure 3B). These studies demonstrate that thrombin activation of the PI3-K/Akt pathway is not essential for thrombin-induced phosphorylation and activation of eNOS in BAECs.

**Involvement of Intracellular Calcium in eNOS Phosphorylation and Activation**

Thrombin is known to cause the intracellular release of Ca\textsuperscript{2+} stores and the influx of extracellular Ca\textsuperscript{2+} in endothelial cells.\textsuperscript{18,19} This may stimulate eNOS activity through a Ca\textsuperscript{2+}/calmodulin-dependent mechanism, whereas the possible contribution of intracellular Ca\textsuperscript{2+} to the phosphorylation of eNOS remains uncertain. We observed that A23187, a Ca\textsuperscript{2+} ionophore, stimulated eNOS phosphorylation at Ser\textsuperscript{1179} in BAECs (Figure 4A). In Figure 4B, we observed a comparable increase in the production of cGMP by thrombin and A23187. Moreover, we
found a critical role for Ca\(^{2+}\) in the thrombin-induced phosphorylation of eNOS. Pretreatment with an intracellular Ca\(^{2+}\) chelator, 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid tetraacetoxy-methyl ester, an intracellular Ca\(^{2+}\) chelator, for 30 minutes (C and E) or 5 mmol/L EGTA, an extra Ca\(^{2+}\) chelator, for 3 minutes (D and F) and stimulated with or without 10 U/mL thrombin for 1 minute (C and D) or for 20 minutes (E and F), eNOS phosphorylation at Ser\(^{1179}\) was observed by immunoblot analysis with a phosphospecific antibody (C and D), as well as cGMP production using an assay kit (E and F). The data shown are mean ± SEM (\(P < 0.05\) vs basal; †\(P < 0.05\) vs stimulated control).

**The Role of PKC\(\delta\) in the Thrombin-Induced Phosphorylation and Activation of eNOS**

Thrombin activates several PKC isoforms, such as PKC\(\alpha\) and PKC\(\delta\), in endothelial cells, and each isoform appears to mediate distinct downstream functions.\(^{11}\) We observed that rottlerin, a PKC\(\delta\) inhibitor, attenuates the phosphorylation of eNOS. Rottlerin, but not LY294002, also blocked the phosphorylation of eNOS by thrombin in human umbilical vein endothelial cells (Figure 1A, available online at http://hyper.ahajournals.org). In contrast, nonsubtype selective PKC inhibitor GF10932X had only a small inhibitory effect on the phosphorylation of Ser\(^{1179}\) eNOS (Figure 5A). Thrombin-induced cGMP production was almost completely inhibited by rottlerin (Figure 5B), consistent with the importance of PKC\(\delta\) in eNOS activation by thrombin. In contrast to A23187, a nonsubtype selective PKC activator, PMA, dephosphorylated eNOS at Ser\(^{1179}\) (Figure 5C). Thrombin-induced cGMP production was almost completely inhibited by rottlerin (Figure 5B), consistent with the importance of PKC\(\delta\) in eNOS activation by thrombin. In contrast to A23187, a nonsubtype selective PKC activator, PMA, dephosphorylated eNOS at Ser\(^{1179}\) (Figure 5C). When both agonists are combined, A23187 seems to have the dominate effect on the Ser\(^{1179}\) phosphorylation of eNOS. In addition, PMA did not stimulate cGMP production in BAECs (Figure 5D). We also observed that thrombin rapidly induced phosphorylation of PKC\(\delta\) Tyr\(^{311}\), the site linked to its activity\(^{20}\) (Figure 1B). To study the relationship between Ca\(^{2+}\) and PKC\(\delta\) in eNOS phosphorylation, the effect of rottlerin on A23187-induced eNOS phosphorylation was examined. Rottlerin markedly inhibited A23187-induced eNOS phosphorylation in BAECs (Figure 1C).

To complement these studies with rottlerin, which may have nonspecific effects, the role of PKC\(\delta\) was further studied by infection of BAECs with adenovirus encoding dominant-negative (dn) mutants of PKC\(\delta\) or PKC\(\alpha\). As shown in Figure 6A, infection of BAECs with dnPKC\(\delta\) adenovirus enhanced both basal- and thrombin-induced Ser\(^{1179}\) phosphorylation of eNOS, whereas infection of BAECs with dnPKC\(\delta\) adenovirus inhibited thrombin-induced Ser\(^{1179}\) phosphorylation of eNOS. We also observe that dnPKC\(\delta\), but not dnPKC\(\alpha\), causes a
significant reduction in cGMP production induced by thrombin in BAECs (Figure 6B). These findings suggest that PKC isoforms have distinct regulatory roles in eNOS phosphorylation and activation, and the positive regulatory role of PKC\(\delta\) on Ser\(^{1179}\) is overridden if other negative regulatory PKCs, such as PKC\(\alpha\), are simultaneously activated by PMA in BAECs. Because reciprocal negative eNOS regulation through eNOS Thr\(^{497}\) phosphorylation has been reported,\(^{21}\) we have further examined phosphorylation of this site by thrombin in BAECs pretreated with or without PMA. Consistent with a recent report,\(^{22}\) thrombin also stimulated eNOS Thr\(^{497}\) phosphorylation. Pretreatment with PMA further enhanced Thr\(^{497}\) phosphorylation but partially inhibited Ser\(^{1179}\) phosphorylation induced by thrombin (Figure 6D).

The requirement of Ca\(^{2+}\) and a PKC isoform for thrombin-induced Ser\(^{1179}\) eNOS phosphorylation suggests that \(G_q\) may be required for the phosphorylation of eNOS at Ser\(^{1179}\). In fact, a selective \(G_q\) inhibitor, YM-254890,\(^{23}\) inhibited thrombin-induced Ser\(^{1179}\) phosphorylation of eNOS (Figure 6C). YM-254890 also blocked thrombin-induced cGMP production in BAECs (Figure 6D). Taken together, these data indicate that, in addition to a previously known Ca\(^{2+}\)/calmodulin-dependent mechanism, phosphorylation of Ser\(^{1179}\) eNOS is mediated through the activation of \(G_q\) and subsequent elevation of intracellular Ca\(^{2+}\) combined with the activation of PKC\(\delta\), which are indispensable for activation of eNOS by thrombin in endothelial cells (Figure 7).

**Figure 6.** The effects of dominant negative (dn) PKC and a \(G_q\) inhibitor on thrombin-induced phosphorylation and activation of eNOS. BAECs were infected with adenovirus encoding dn adenoviruses for PKC\(\alpha\) or PKC\(\delta\) (100 multiplicities of infection) for 48 hours and then stimulated with 10 U/mL of thrombin for 1 minute (A) or for 20 minutes (B). BAECs were pretreated with or without 10 \(\mu\)mol/L YM-254890 for 10 minutes and stimulated with or without 10 U/mL thrombin for 1 minute (C) or 20 minutes (D). eNOS phosphorylation at Ser\(^{1179}\) was observed by immunoblot analysis with a phosphospecific antibody (A and C). These data are representative of 3 separate experiments giving similar results. cGMP production was measured using an assay kit (B and D). The data shown are mean±SEM (*\(P<0.05\) vs basal; †\(P<0.05\) vs stimulated control).

**Figure 7.** Proposed signal transduction cascade by which thrombin mediates eNOS activation. CaM indicates calmodulin.
this study, we have demonstrated that the PI3-K/Akt pathway is not necessary for eNOS regulation by thrombin in endothelial cells. Several lines of evidence support this notion: (1) Akt phosphorylation occurs later than eNOS phosphorylation, rather than preceding it; (2) a PI3-K inhibitor, LY294002, did not inhibit thrombin-induced Ser1179 phosphorylation of eNOS, although it inhibited the phosphorylation of Akt induced by thrombin under the same condition; and (3) LY294002 does not inhibit thrombin-induced cGMP production, which is known to result from eNOS activation. We verified that thrombin induced cGMP production via eNOS activation by using N^\* -nitro-L-arginine methyl ester, an eNOS inhibitor, which attenuated thrombin-induced cGMP production.

Thrombin is known to cause the intracellular release of Ca^{2+} stores and the influx of extracellular Ca^{2+} in endothelial cells.18,19 This may be sufficient to stimulate eNOS activity through a Ca^{2+}/calmodulin-dependent mechanism. Although phosphorylation of eNOS by thrombin at Ser1179 has been demonstrated recently,24 the functional relevance of this phosphorylation is uncertain. Our experiment using the S1179A mutant strongly suggests the relevance of Ser1179 phosphorylation in eNOS activation by thrombin. We also confirmed recently the requirement of Ser1179 phosphorylation for eNOS activation via a G_i-coupled angiotensin II receptor.25 Moreover, the relationship between Ca^{2+} and the regulation of eNOS phosphorylation by G protein–coupled receptors (GPCRs) has not been established previously. Therefore, our findings are novel in that Ca^{2+} is also required for eNOS activation by a GPCR agonist through its input on the phosphorylation of Ser1179.

In addition to Akt, several protein kinases have been shown recently to phosphorylate eNOS at Ser1179. These include protein kinase A, 5'-AMP-activated protein kinase, and calmodulin-dependent kinase II.6,7 Because the amino acid sequences surrounding the Ser1179 site do not exactly match the consensus substrate sequences of PKC\(\delta\), it is possible that one or more of these Ser1179 kinases, upon activation/modulation by PKC\(\delta\), phosphorylate eNOS in response to thrombin. Although 5'- AMP–activated protein kinase was proposed to phosphorylate eNOS at Ser1179 by thrombin,26 a recent study has clearly eliminated this possibility.20

Thrombin exhibits its effect through the activation of G protein–coupled protease-activated receptors.8,9 Protease-activated receptor activation in endothelial cells results in intracellular signal transduction, involving G\(_i\)-stimulated phosphatidylinositol metabolism via phospholipase C-\(\beta\), resulting in an increase in intracellular Ca^{2+} mobilization and PKC activation.10,11 This is in line with the findings presented here demonstrating that G\(_i\), Ca^{2+}, and PKC\(\delta\) are all necessary components in the thrombin-induced phosphorylation of Ser1179 and activation of eNOS. Although we have not studied the molecular link between Ca^{2+} and PKC\(\delta\) Tyr311 phosphorylation in the present study, we have shown recently that the elevation of Ca^{2+} could lead to PKC\(\delta\) Tyr311 phosphorylation, a site critical for its enzymatic activity in VSMCs stimulated by the GPCR agonist angiotensin II.27 Therefore, it is likely that, in parallel with a phospholipase C/diacylglycerol-dependent PKC\(\delta\) activation, intracellular Ca^{2+} elevation stimulates PKC\(\delta\) activity through its Tyr311 phosphorylation in BAECs stimulated by thrombin.

Thors et al24 used human umbilical vein endothelial cells and ruled out the PKC pathway by using the data that PMA does not cause Ser1179 phosphorylation of eNOS. Although we have obtained similar results here, the findings could be interpreted as the complex regulation of eNOS by distinct PKC isozymes rather than the lack of positive regulation by the PKC pathway. Partovian et al28 demonstrated recently that PKC\(\alpha\) is required for Ser1179 phosphorylation of eNOS and subsequent NO release in BAECs and human umbilical vein endothelial cells stimulated by a growth factor, FGF2. However, in the present study, dnPKC\(\alpha\) has no inhibitory effect on Ser1179 eNOS phosphorylation by thrombin. FGF receptors activate phospholipase C\(\gamma\) causing distinct time courses/patterns/amounts of intracellular Ca^{2+} formation and diacylglycerol production, which will likely lead to activation of distinct sets of PKC isozymes and eNOS regulation.

It should be noted that our findings are in contrast to previous publications dealing with Ser1179 phosphorylation of eNOS by stimulation of other GPCRs, including sphingosine 1-phosphate/the endothelial differentiation gene receptor, bradykinin/B2 receptor, and endothelin-1/endothelin B receptor. Sphingosine 1-phosphate/endothelial differentiation gene–stimulated Ser1179 eNOS phosphorylation is reported to require G\(_i\), Ca^{2+}, vascular endothelial growth factor receptor (Flk-1/kinase insert domain receptor) transactivation, PI3-K, and Akt.29–31 BK/B2-dependent phosphorylation partially requires Flk-1/kinase insert domain receptor transactivation and PI3-K as well.32,33 Endothelin-1/endothelin B receptor–dependent phosphorylation again requires G\(_i\), G\(_{\text{q,y}}\), PI3-K, and Akt.34 We have confirmed recently that thrombin-induced Ser1179 eNOS phosphorylation is not blocked by pertussis toxin (a G\(_i\) inhibitor) or overexpression of C terminus of G protein receptor kinase-2 (a G\(_{\text{q,y}}\) inhibitor; H Suzuki, S Eguchi, unpublished observation, 2006). Taken together, there should be at least 2 independent mechanisms of eNOS phosphorylation by GPCRs; 1 is used by a G\(_i\)-coupled receptor involving Flk-1/kinase insert domain receptor, PI3-K, and Akt, and the other is used by a G\(_{\text{q,y}}\)-coupled receptor involving Ca^{2+} and PKC\(\delta\).

Perspectives

Thrombin induces Ser1179 eNOS phosphorylation and activation through a Ca^{2+}-sensitive, but PI3-K/Akt-independent pathway. Because dysfunction of endothelial NO production is one of the major predictors of cardiovascular events, these findings will contribute to a better understanding of the signaling mechanisms of thrombin in regulating the endothelial function. Therefore, further in vivo studies focusing on human pathophysiology in this area will contribute to the development of specific-acting drugs that can be used to treat and/or prevent endothelial dysfunctions.

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Disclosures

None.
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