eNOS Activation Induced by a Polyphenol-Rich Grape Skin Extract in Porcine Coronary Arteries

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Abstract

Background/Aims: Drinking red wine is associated with a decreased mortality from coronary heart diseases. This study examined whether polyphenols contained in a grape skin extract (GSE) triggered the endothelial formation of nitric oxide (NO) and investigated the underlying mechanism.

Methods: Vascular reactivity was assessed in organ chambers using porcine coronary artery rings in the presence of indomethacin (a cyclooxygenase inhibitor) and charybdotoxin plus apamin (inhibitors of endothelium-derived hyperpolarizing factor-mediated responses). The phosphorylation level of Src, Akt and endothelial NO synthase (eNOS) were assessed by Western blot analysis, and the formation of reactive oxygen species (ROS) was investigated using dihydroethidine and dichlorodihydrofluorescein.

Results: GSE-induced endothelium-dependent relaxations were abolished by N G -nitro- L -arginine (an eNOS inhibitor) and ODQ (a soluble guanylyl cyclase inhibitor), and they were reduced by MnTMPyP, polyethylene glycol catalase, PP2 (an inhibitor of Src kinase) and wortmannin (an inhibitor of phosphoinositide 3-kinase). GSE caused phosphorylation of Src, which was prevented by MnTMPyP. It also caused phosphorylation of Akt and eNOS, which were prevented by MnTMPyP, polyethylene glycol catalase, PP2, wortmannin and LY294002. GSE elicited the formation of ROS in native and cultured endothelial cells, which was prevented by MnTMPyP. Conclusions: GSE causes endothelium-dependent NO-mediated relaxations of coronary arteries. This effect involves the intracellular formation of ROS in endothelial cells leading to the Src kinase/phosphoinositide 3-kinase/Akt-dependent phosphorylation of eNOS.

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Introduction

Epidemiological studies have indicated an inverse correlation between red wine consumption and the risk of cardiovascular diseases [1–3]. The protective effect of red...
wine on the cardiovascular system is due, at least in part, to the polyphenolic component [4]. Indeed, numerous studies indicate that both acute and chronic administration of various preparations from polyphenol-rich foods or beverages has a beneficial effect on hemodynamic parameters in patients and animals. For instance, chronic intake of 100 g of dark polyphenol-rich chocolate for 14 days decreased both diastolic and systolic blood pressure within 10 days in patients with mild isolated systolic hypertension [5]. In patients with at least 1 cardiovascular risk factor (coronary artery disease, hypertension, hyperlipidemia, diabetes or smoking), ingestion of 100 ml of a cocoa drink containing high amounts of flavonoids increased endothelium-dependent vasodilation, an effect associated with an increased plasma nitric oxide (NO) pool [6]. Furthermore, it has been observed that in healthy humans, regular intake of a flavonoid-rich cocoa for 4 days induced a prominent peripheral vasodilation via activation of the NO pathway [7]. Endothelium-dependent flow-mediated vasodilation is also improved after acute intake of 500 ml of red wine with or without alcohol in healthy men, as determined by ultrasonography of the brachial artery [8]. In addition, experimental evidence indicates that red wine polyphenols can induce pronounced endothelium-dependent relaxations of isolated arteries through an increased formation of both NO and endothelium-derived hyperpolarizing factor (EDHF), 2 endothelial factors that play major roles in the control of vascular homeostasis [9–15].

Recently, we have shown that chronic intake of the polyphenols contained in an alcohol-free lyophilized Brazilian red wine or in an alcohol-free grape skin extract (GSE) from Vitis labrusca reduced systolic, mean and diastolic arterial pressure in several experimental models of hypertension [16, 17]. Both types of extracts also caused endothelium-dependent vasodilation in the perfused rat mesenteric vascular bed by increasing the endothelial formation of both NO and EDHF [16–18]. The aim of the present study was to characterize the signaling pathway leading to the enhanced endothelial formation of NO in response to GSE using isolated coronary arteries and cultured coronary artery endothelial cells.

Methods

Preparation of the GSE

V. labrusca (Isabel variety) red grapes were obtained from selected vineyards located in the state of Rio Grande do Sul, Brazil. The grapes were washed in tap water and the skins were separated from the pulps. Approximately 100 g of skin were boiled in 400 ml of distilled water for 5 min and then minced. 400 ml of ethanol was added to the decoction, shaken for 4 h and kept in dark bottles inside a refrigerator (4°C) for 20 days. The hydroalcoholic extract of V. labrusca skins was filtered through Whatman No. 1 filter paper and the ethanol was evaporated under low pressure at 55°C. The extract was lyophilized and frozen at –20°C until the day of use. 100 g wet skins yield about 8.9 g of lyophilized extract. The concentration of polyphenols in lyophilized GSE was 55.5 mg/g, as measured by analysis of total phenol by the Folin-Ciocalteu procedure [19].

Vascular Reactivity Studies

Left anterior descending porcine coronary arteries (obtained from the local slaughterhouse) were cleaned of connective tissue and cut into rings. Rings were suspended in organ baths containing oxygenated (95% O2 and 5% CO2) Krebs bicarbonate solution (in mM: NaCl 119, KCl 4.7, KH2PO4 1.18, MgSO4 1.18, CaCl2 1.25, NaHCO3 25, and D-glucose 11; pH 7.4, 37°C) under a resting tension of 5 g for the determination of changes in isometric tension, as described previously [14]. Rings were constricted with U46619 before a concentration-relaxation curve to either GSE or bradykinin was constructed. All experiments (except as indicated) were performed in the presence of indomethacin, an inhibitor of cyclooxygenases, and the combination of charybotoxin and apamin (both inhibitors of calcium-activated potassium channels) to rule out the formation of vasoactive prostanooids and EDHF, respectively. In some experiments, rings were incubated with a modulator for 30 min before addition of U46619.

Culture of Coronary Artery Endothelial Cells

Porcine coronary artery endothelial cells were isolated by collagenase treatment (type I, Worthington; 1 mg/ml for 12 min at 37°C), and cultured as described previously [14]. All experiments were performed with confluent cultures of cells used at first or second passage. They were exposed to serum-free culture medium in the presence of 0.1% bovine serum albumin for 6 h prior to treatment.

Determination of the Phosphorylation Level of Src, Akt and Endothelial NO Synthase

Total protein (25 μg) was separated on SDS-polyacrylamide gels (12%) at 70 V for 3.5 h and then transferred electrophoretically (100 V for 2 h) to polyvinylidene difluoride membranes (Amersham-Pharmacia Biotech, Piscataway, N.J., USA). Immunodetection was carried out using primary antibodies directed against either phosphorylated Src, phosphorylated endothelial NO synthase (eNOS) or phosphorylated Akt (Cell Signaling Technology, Beverly, Mass., USA). The immunoreactive bands were detected by enhanced chemiluminescence (Amersham Biosciences). Ponceau staining was performed to verify the quality of the transfer and equal amounts of proteins in each lane.

Detection of Reactive Oxygen Species in Cultured and Native Endothelial Cells

The oxidative fluorescent dye dihydroethidine (DHE) was used to evaluate the production of reactive oxygen species (ROS) in cultured endothelial cells by use of a method described by Miller et al. [20]. DHE permeates freely into cells and in the presence of ROS is oxidized to ethidium bromide, which is trapped by intercalating with the DNA. Cultured coronary endothelial cells...
were rinsed in PBS and incubated in Hanks’ balanced salt solution containing DHE (10 μM) in a light-protected humidified chamber at 37°C. After 10 min, cells were exposed to either solvent (ethanol 0.5%) or GSE. In some experiments, cells were exposed to Mn(III)tetrakis(1-methyl-4-pyridyl)porphyrin (MnTMPyP; 100 μM) for 30 min before addition of GSE. Images were obtained with a Bio-Rad MRC-1024 laser scanning confocal microscope equipped with a krypton/argon laser. Laser settings were identical for acquisition of images from solvent and GSE-treated cells. Ethidium bromide was excited at 488 nm with an emission spectrum of 610 nm. Fluorescence was detected with a 585-nm long-pass filter.

The in situ formation of ROS was assessed in coronary artery sections using the redox-sensitive fluorescent probe 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (DCHF; Invitrogen™). Coronary artery rings were incubated in PBS for 30 min at 37°C in the absence or presence of a modulator before being embedded in Tissue-Tek OCT compound (Qaigen, Hilden, Germany), and frozen in liquid nitrogen. These unfixed frozen artery rings were cut into 25-μm thick sections and placed on polylysine-coated plus glass slides. Artery sections were exposed to DCHF for 15 min at 37°C before the treatment with GSE in the absence or presence of the respective modulator. Thereafter, DCHF fluorescence was determined by confocal microscopy (1024 MRC; Bio-Rad, Hercules, Calif., USA) with a ×10 epifluorescence objective (Nikon, Tokyo, Japan). After excitation at 488 nm with a krypton/argon laser, the emission signal was recorded with a Zeiss 565–610 nm filter. Images were analyzed by the Confocal Assistant™ (CAS 40 version 4.02, 1024 × 728 pixels, 32 bits per pixel).

Chemicals
N-acetylcysteine, N⁶-glyco-L-arginine (L-NA), 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), apocynin, sulfaphenazol, rotenone, allopurinol, apamin, charybdotoxin, indomethacin, bradykinin, superoxide dismutase, polyethylene-glycol-superoxide dismutase, catalase, polyethylene-glycol-catalase (PEG-catalase) and DHE were obtained from Sigma (St. Louis, Mo., USA). 9,11-dideoxy-11α,9β-epoxymetha-prostaglandin F2α (U46619) was purchased from Cayman Chemical (Ann Arbor, Mich., USA). Wortmannin, LY294002, PD98059, SB203580, L-JNKI, superoxide dismutase mimetic MnTMPyP were from Alexis Chemicals (Läufelfingen, Switzerland). 4-amino-5-(4-chlorophenyl)-7-(t-butyl) pyrazolo[3,4-d]pyrimidine (PP2) was obtained from Calbiochem (San Diego, Calif., USA).

Statistical Methods
Values are expressed as means ± SEM. Statistical evaluation was performed with Student’s t test for paired data or ANOVA followed by Fisher’s protected least significant difference test where appropriate. Values of p < 0.05 were considered statistically significant.

Results
GSE Induces Endothelium-Dependent Relaxations in Isolated Coronary Arteries
In the presence of indomethacin (10 μM), GSE induced concentration-dependent relaxations in rings with endothelium starting at concentrations greater than 10 μg/ml and reaching a near maximal relaxation at 100 μg/ml, whereas in rings without endothelium no such effect was observed (fig. 1a, b). Relaxations to GSE

Fig. 1. a Original tracings showing endothelium-dependent relaxations to GSE in isolated porcine coronary arteries. b Effect of N⁶-glyco-L-arginine (L-NA, 100 μM), apamin (100 nM) plus charybdotoxin (100 nM), and the combination of L-NA and apamin plus charybdotoxin on GSE induced relaxation in coronary artery rings with endothelium. The effect of GSE in rings without endothelium is also shown. All experiments were performed in the presence of indomethacin (10 μM). Results are shown as means ± SEM of 6 different experiments. * p < 0.05 for inhibitory effect.
were significantly reduced by L-NA, an inhibitor eNOS, minimally affected by charybdotoxin plus apamin, markedly decreased by the combination of L-NA with charybdotoxin plus apamin and by the combination of ODQ with charybdotoxin plus apamin (relaxations induced by 300 μg/ml GSE were 97.0 ± 8.7% and 1.8 ± 1.7% in the absence and presence of ODQ 3 μM, n = 4). These findings indicate that GSE causes endothelium-dependent relaxations involving predominantly NO and also, to some extent, EDHF. To characterize the mechanisms involved in the endothelium-dependent NO-mediated relaxation to GSE, all further experiments were performed in the presence of charybdotoxin (100 nM), apamin (100 nM) and indomethacin (10 μM). Results are shown as the means ± SEM of 6 different experiments. * p < 0.05 for inhibitory effect; # = different from control (p < 0.05).

Fig. 2. Role of reactive oxygen species in endothelium-dependent NO-mediated relaxations to GSE in intact coronary artery rings. Artery rings were incubated with either superoxide dismutase (SOD) or MnTMPyP (a) and either catalase or PEG-catalase (b) for 30 min before the addition of U46619. All experiments were performed in the presence of charybdotoxin (100 nM), apamin (100 nM) and indomethacin (10 μM). Results are shown as the means ± SEM of 6 different experiments. * p < 0.05 for inhibitory effect; # = different from control (p < 0.05).

Fig. 3. Role of several enzymatic sources of ROS on endothelium-dependent NO-mediated relaxations to GSE in intact coronary artery rings. Artery rings with endothelium were incubated with an inhibitor of NADPH oxidase (apocynin) or of xanthine oxidase (allopurinol) or of cytochromes P450 (sulfaphenozol) or of the mitochondrial respiratory chain (rotenone) for 30 min before the addition of U46619. All experiments were performed in the presence of charybdotoxin (100 nM), apamin (100 nM) and indomethacin (10 μM). Results are shown as the means ± SEM of 5 different experiments.

Role of ROS in GSE-Induced NO-Mediated Relaxation

Previous studies have indicated that red wine polyphenols are able to induce NO- and EDHF-mediated relaxations and hyperpolarization in porcine coronary arteries that are strictly dependent upon the intracellular formation of superoxide anions in endothelial cells [12–14]. Therefore, experiments were performed to determine
the role of ROS in GSE-induced NO-mediated relaxations. NO-mediated relaxations to GSE were abolished by the membrane permeable superoxide dismutase mimetic, MnTMPyP, and the membrane permeable catalase, PEG-catalase, whereas native catalase was without effect, and native superoxide dismutase slightly but significantly potentiated relaxations (fig. 2a, b). Relaxations to GSE were not significantly affected by the antioxidant and NADPH oxidase inhibitor apocynin, the xanthine oxidase inhibitor allopurinol, the cytochrome P450 oxidase inhibitor sulfaphenazol and by the mitochondrial chain respiration inhibitor rotenone (fig. 3).

Role of Redox-Sensitive Protein Kinases in GSE-Induced NO-Mediated Relaxation

ROS such as superoxide anions have important signaling function in vascular cells [21]. In particular, numerous protein kinases are redox-sensitive such as mitogen-activated protein kinases (MAPK) p38, extracellular-related kinases 1 and 2 (ERK1/2), c-Jun N-terminal kinase (JNK), Src kinase and phosphoinositide 3-kinase (PI3-kinase). Therefore, the role of these redox-sensitive protein kinases in GSE-induced NO-mediated relaxation was clarified. Relaxations to GSE were significantly inhibited by PP2 and wortmannin (inhibitors of Src kinase and PI3-kinase, respectively), whereas they were not affected by L-JNKI (an inhibitor of JNK), PD98059 (an inhibitor of ERK1/2 kinase) and SB203580 (an inhibitor of p38 MAPK) (fig. 4a, b). In contrast to GSE, PP2 did not affect NO-mediated relaxations to bradykinin (fig. 4c). PP2 and wortmannin did not significantly affect the level of contraction induced by U46619 (the values were 17.0 ± 1.0 and 15.1 ± 1.2 g in the absence and presence of PP2, and 12.6 ± 1.3 and 11.2 ± 1.1 g in the absence and presence of wortmannin).

GSE Stimulates the Formation of ROS in Cultured and Native Endothelial Cells

The ability of GSE to stimulate the formation of ROS was examined in cultured coronary artery endothelial cells using DHE staining, and in coronary artery sections using 2',7'-dichlorodihydrofluorescein diacetate. GSE increased DHE fluorescence in cultured endothelial cells, and MnTMPyP abolished this effect (fig. 5a, b). GSE also consistently increased the low basal fluorescence signal in coronary artery sections with endothelium (fig. 5c). The increased fluorescence signal was predominantly located at the luminal surface of intact sections and also to some extent in the adventitia, whereas no such increase was observed in the vascular smooth muscle (fig. 5c). The GSE-increased fluorescence signal in the endothelium was abolished by MnTMPyP (fig. 5d).

GSE Induces a Redox-Sensitive Phosphorylation of Src, Akt and eNOS in Endothelial Cells

Numerous studies have indicated that the PI3-kinase pathway mediates activation of eNOS through the Akt-dependent phosphorylation of eNOS at Ser1177 in re-
response to several physiological stimuli, including shear stress, estrogens, vascular endothelial growth factor, H$_2$O$_2$ and high density lipoprotein [22–27]. In order to determine whether such a mechanism is involved in the endothelial formation of NO in response to GSE, the phosphorylation level of Akt and eNOS was determined by immunoblotting. Unstimulated endothelial cells had a low level of phosphorylated Akt at Ser473 (fig. 6a) and of phosphorylated eNOS at Ser1177 (fig. 6b). Exposure of endothelial cells to GSE caused the appearance within 5–10 min of a pronounced phosphorylation of Akt at Ser473 and eNOS at Ser1177. Thereafter, the signal persisted for at least 120 min for Akt whereas the phosphorylation level of eNOS returned to baseline at 30 min (fig. 6a, b). In addition, GSE also significantly enhanced the phosphorylation level of Src in endothelial cells (fig. 7a). GSE-induced phosphorylation of Src, Akt and eNOS was abolished by pretreatment of cells with MnTMPyP and that of Akt and eNOS by PEG-catalase (fig. 7a–c). In addition, inhibition of Src kinase (by PP2) and PI3-kinase (by wortmannin, LY294002) prevented the stimulatory effect of GSE on Akt and eNOS (fig. 8a, b).

**Effect of GSE on Endothelial Cell Viability**

The viability of endothelial cells was not affected following exposure to GSE (300 μg/ml) for 30 min, as assessed by CellTiter 96 MDSU Aqueous One Solution cell proliferation assay (Promega, 103.1 ± 1.4%, 2 different experiments performed in quadruplicate).

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Grape Skin Polyphenols and eNOS Activation

**Fig. 6.** GSE causes a time-dependent phosphorylation of Akt at Ser473 (**a**) and eNOS at Ser1177 (**b**) in cultured porcine coronary artery endothelial cells. All experiments were performed in the presence of charybdotoxin (100 nM), apamin (100 nM) and indomethacin (10 μM). Results are shown as the means ± SEM of 3 different experiments. * p < 0.05 for inhibitory effect.

**Fig. 7.** Role of intracellular ROS on GSE-induced phosphorylation of Src (**a**), Akt (**b**) and eNOS (**c**) in coronary artery endothelial cells. Endothelial cells were exposed either to solvent, MnTMPyP or PEG-catalase for 30 min before the addition of GSE for 10 min. Thereafter, the level of p-Src, p-Akt and p-eNOS was determined by Western blot analysis. Upper panels show representative immunoblots and lower panels show the corresponding cumulative data. All experiments were performed in the presence of charybdotoxin (100 nM), apamin (100 nM) and indomethacin (10 μM). Results are shown as the means ± SEM of 3 or 4 different experiments. * p < 0.05 vs. control; # p < 0.05 vs. GSE treatment alone.
The present findings indicate that an alcohol-free polyphenol-rich GSE from *V. labrusca* induces potent endothelium-dependent relaxations of porcine coronary arteries by increasing the formation of NO and also, to some extent, EDHF. They further indicate that the GSE-induced formation of NO is due to the redox-sensitive Src kinase/PI3-kinase/Akt-dependent activation of eNOS by phosphorylation.

GSE caused potent relaxations of intact coronary arteries, but only minor ones in rings without endothelium. The fact that relaxations to GSE were reduced by L-NA and not affected by the combination of charybdotoxin plus apamin indicates a major role for NO. However, since the addition of charybdotoxin plus apamin to L-NA further reduced relaxations to GSE, EDHF is also involved to some extent. The present findings are in agreement with previous ones indicating that GSE caused vasodilatation in the perfused rat mesenteric arterial bed by increasing the formation of NO and EDHF [17, 18]. Besides grape skin extracts, other grape-derived products, including red wine, red wine extracts, grape juices and grape seed extracts, have also been shown to cause endothelium-dependent relaxations involving NO and EDHF [9, 10, 13, 28, 29].

The present findings also indicate that GSE is able to cause the intracellular formation of ROS in cultured and native coronary artery endothelial cells, but not in native vascular smooth muscle. Moreover, they further indicate that the GSE-induced formation of ROS is a critical initial event in the signal transduction pathway leading to eNOS activation and subsequent relaxation. Indeed, GSE-induced relaxations were markedly reduced by membrane permeable analogues of superoxide dismutase and catalase, but not by native superoxide dismutase and catalase. Similar findings have been observed with red wine polyphenols and grape juice [12–14, 29]. Although the source of ROS in endothelial cells remains to be determined, endothelium-dependent relaxations to GSE and red wine polyphenols were not affected by inhibitors of xanthine oxidase, NADPH oxi-
dase, the mitochondrial chain respiration, cytochromes P450 or cyclooxygenases [present findings, 12, 14]. Potential sources include other types of ROS-generating enzymes and the polyphenolic compound itself [21, 30, 31]. In addition to polyphenols, several physiological activators of eNOS, such as vascular endothelial growth factor, shear stress, and bradykinin, have also been shown to induce the formation of ROS in endothelial cells [32–34].

ROS are now widely recognized as important signaling molecules involved in the physiological control of cell function, in part by the activation of redox-sensitive protein kinases [21]. The present findings indicate a major role of the PI3-kinase/Akt pathway, under the control of ROS, in GSE-induced eNOS activation. Indeed, GSE caused a comparable time course for relaxation and phosphorylation of eNOS and Akt in endothelial cells. In addition, inhibition of the PI3-kinase prevented the GSE-induced phosphorylation of Akt and eNOS and relaxations. In contrast to PI3-kinase, inhibition of other redox-sensitive kinases, such as p38 MAPK, ERK1/2 and JNK, did not affect GSE-induced NO-mediated relaxations. Moreover, NO-mediated relaxations to bradykinin were affected by neither MnTMPyP nor wortmannin [13]. Furthermore, membrane permeable analogues of superoxide dismutase and catalase prevented the phosphorylation of Akt and eNOS in response to GSE. Altogether, these findings indicate that grape-derived polyphenols activate eNOS by using an original signaling pathway involving the redox-sensitive activation of the PI3-kinase/Akt pathway, leading to an enhanced formation of NO following phosphorylation of eNOS. Consistent with such a sequence of events, moderate concentrations of hydrogen peroxide increase the activity of eNOS through the PI3-kinase/Akt-dependent phosphorylation of eNOS at Ser1177 [35, 36].

The present findings also indicate that Src kinase is an upstream mediator of the PI3-kinase/Akt pathway leading to the GSE-induced phosphorylation of eNOS. Indeed, inhibition of Src kinase reduced GSE-induced phosphorylation of Akt and eNOS and relaxations. Moreover, GSE caused the phosphorylation of Src kinase; this response was prevented by the membrane-permeable analogue of superoxide dismutase, but not of catalase. These findings indicate that the initial event leading to NO formation in response to GSE is the intracellular formation of ROS. The moderate pro-oxidant signal, involving superoxide anions, triggers the phosphorylation of Src kinase leading to the activation of the PI3-kinase/Akt pathway that ultimately enhances eNOS activity by phosphorylation. In addition, the moderate pro-oxidant signal might also involve hydrogen peroxide, which in turn acts downstream of Src kinase to activate the PI3-kinase/Akt pathway resulting also in an increased eNOS activity.

Although polyphenols caused a modest pro-oxidant signal in endothelial cells, no such effect has been observed with other types of vascular cells. Indeed, polyphenols did not induce the formation of ROS in the vascular smooth muscle of coronary arteries [present findings] and they prevented growth factor-induced NADPH oxidase-dependent formation of ROS in vascular smooth muscle cells [37]. In addition, grape seed and skin extracts prevented the stimulation-induced release of superoxide anions in platelets [38]. These protective effects of polyphenols might be due to their ability to scavenge superoxide anions, peroxyl radicals, hydroxyl radicals and peroxynitrite [39–41]. In addition, the protective effect might also be due to their ability to prevent the expression of pro-oxidant enzymes, such as NADPH oxidase and xanthine oxidase, and to increase that of antioxidant enzymes such as catalase [42–44]. Indeed, an intake of red wine polyphenols prevented oxidative stress and the endothelial dysfunction in the aorta, which were induced by the infusion of angiotensin II to rats [44]. Altogether, the previous and present findings suggest that the beneficial effect of grape-derived polyphenols on the vascular system might be due to their ability to enhance the redox-sensitive formation of vasoprotective factors in endothelial cells and to prevent oxidative stress in the vascular smooth muscle and platelets.

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References


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