Amlodipine Attenuates Oxidative Stress-Induced Hypertension

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Background: Dihydropyridine Ca^{2+}-blockers, frequently used as antihypertensive and antianginal agents, have been found to exert potent antioxidant and cytoprotective activities against free radical-mediated vascular injury.

Methods: In the current study we examined the effect of amlodipine (AMLOD) on oxidative stress-induced hypertension in Sprague-Dawley rats administered buthionine-sulfoxine (BSO), a glutathione (GSH) synthase inhibitor, in the drinking water. The control animals received drug-free water. Blood pressure (BP) was measured by tail-cuff plethysmography. Plasma levels of total 8-isoprostane, thromboxane A_2, prostacyclin, nitric oxide, and aortic cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) were determined by enzyme immunoassay. Plasma, kidney, and heart GSH were analyzed by high-performance liquid chromatography.

Results: Administration of BSO significantly increased BP, isoprostane, and thromboxane A_2, whereas GSH, PGI_2, and cAMP were reduced. When given alone, AMLOD alone reduced BP and the plasma levels of isoprostane and thromboxane A_2, and elevated prostacyclin, nitric oxide, cGMP, and cAMP. When administered with BSO, AMLOD reversed the BSO-induced elevation of BP, isoprostane, and thromboxane A_2 as well as the reduction in prostacyclin, cAMP, and cardiac GSH levels.

Conclusions: The antihypertensive effect of amlodipine involves a reduction in oxidative stress, which appears to be mediated in part by the prostanoid endothelium-derived factors and nitric oxide. Am J Hypertens 2004;17:743–748 © 2004 American Journal of Hypertension, Ltd.

Key Words: Oxidative stress, glutathione, amlodipine, endothelial factors, signal transduction.

Amlodipine is a long acting dihydropyridine Ca^{2+} antagonist with vascular selectivity. In vitro studies have suggested that the dihydropyridine Ca^{2+} antagonists act as antioxidants by directly quenching several radical species.[1,2] The antioxidant activity of these Ca^{2+} antagonists probably contributes to their Ca^{2+} blocking and antihypertensive effects.[3]

Hypertension induced by oxidative stress has been demonstrated in normal rats after glutathione (GSH) depletion.[4–7] The pathogenesis of hypertension due to increased reactive oxygen species (ROS) has been attributed to endothelial dysfunction caused by inactivation of nitric oxide,[8] generation of vasoconstrictive isoprostanes from arachidonic acid peroxidation,[9] and a direct vasopressor or diminished vasodilator activity.[10] On the other hand, levels of free radical scavengers such as vitamin E,[11] glutathione,[12] and superoxide dismutase[13] have been reported

to be depressed in hypertensive patients and experimental animals.

Glutathione is the most abundant nonprotein intracellular thiol, with multiple roles as an antioxidant agent.[14] It functions as a scavenger of ROS and peroxynitrite. Induction of oxidative stress by GSH depletion has been demonstrated in rats after administration of buthionine sulfoximine (BSO), a selective inhibitor of γ-glutamyl cysteine synthetase, an enzyme in the GSH biosynthetic pathway.[15] This study investigates the cardiovascular effects of the dihydropyridine Ca^{2+} antagonist, amlodipine, on oxidative stress induced by GSH depletion and its effect on endothelium-derived factors and associated second-messenger signaling mechanisms, specifically, the changes in aortic cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP).

Methods
Experimental Design

Male Sprague-Dawley rats (Harlan Sprague-Dawley, Indianapolis, IN), 4 to 5 weeks of age, were grouped five per cage in the animal facility that has 12-h light/dark cycles with the temperature controlled at 21° to 23°C. Rodent Lab Chow (Purina Mills, St. Louis, MO) and water were made available ad libitum. After 1 week of acclimatization, the animals were individually housed and divided into ($n = 6$) receiving the following in the drinking water: 1) BSO, 30 mmol/L/day; 2) amlodipine, 3 mg/kg/day; 3) BSO + amlodipine; or 4) regular tap water. Before treatment and after 1 wk of treatment, indirect blood pressure (BP) and heart rate were measured. After these measurements, each animal was anesthetized (using 70 mg/kg ketamine and 10 mg/kg xylazine, intramuscularly), and the carotid and jugular vein were cannulated using PE 50 tubing containing heparin (20 IU/mL) in 0.9% NaCl. The cannulae were externalized in the posterior cervical region and occluded with a metal plug, and flushed with heparinized saline every 12 h.

Indirect BP Measurement in Conscious Rats

Tail-cuff plethysmography (Rat Tail Blood Pressure Monitor and Universal Oscillograph; Harvard Apparatus, Holliston, MA) was used to measure indirect BP. Heart rate was measured from the arterial pulse wave at the same time.

Collection and Storage of Blood Samples

Blood samples for plasma nitric oxide, thromboxane, and prostacyclin measurements were collected by free flow via the polyethylene cannula in the carotid artery into heparinized and indomethacin (100 mmol/L)-rinsed (for prostaglandin samples) tubes.

To assess plasma glutathione and isoprostane concentrations, blood samples were withdrawn via cardiac puncture from all animals (under ketamine/xylazine anesthesia administered intravenously) before they were killed; the samples were centrifuged at 3000 g for 25 min at 4°C. For the isoprostane assay, butylated hydroxytoluene was added to 1.0 mL of plasma to give a final concentration of 0.005% (v/v); aliquots were frozen and stored at -80°C until assayed. For the glutathione assay, 1.0 mL of plasma was combined with 1.0 mL of metaphosphoric acid, incubated for 5 min at room temperature, and then centrifuged at 3000 g for 3 min. The aliquots of the supernatant were collected, frozen, and stored at -80°C until assayed.

Tissue Harvesting for in Vitro Studies

Immediately after cardiac puncture, the heart and the kidneys were harvested, frozen in liquid nitrogen, and stored at -80°C. The aortic arch was put into HEPES-buffered Earl''s balanced salt solution (EBSS; Sigma Chemical, St. Louis, MO) containing 100 μmol/L indomethacin (a nonselective cyclooxygenase inhibitor) on ice before cAMP and cGMP incubations.

Total Plasma, Heart, and Kidney Glutathione Assay

Before homogenization, heart and kidney tissues were mixed 1:1.5 (w/v) with cold phosphate buffered saline. After centrifugation (3500 g for 30 min at 4°C), the supernatant was collected and frozen at -80°C until assayed. Protein was determined by the BioRad method.[16] Total GSH levels in plasma, heart, and kidney were measured by HPLC after derivatization according to the protocols of Abukhalaf et al.[17]

Measurement of Plasma Prostacyclin, Thromboxane B_2, Total 8-Isoprostane, and Nitric Oxide

After purification according to the manufacturer's instructions, plasma levels (pg/mL) of prostacyclin (as 6-keto-PGF$_{1\alpha}$), thromboxane A_2 (as TXB$_2$), and total 8-isoprostane (free plus esterified in lipoproteins) were measured using enzyme immunoassay kits (Cayman Chemical, Ann Arbor, MI). Plasma nitric oxide (as nitrates + nitrites) levels (μmol/L) were quantitated by a colorimetric microplate assay kit that uses the Greiss reagent (Cayman Chemical, Ann Arbor, MI).

Determination of Aortic Cyclic Guanosine-Monophosphate and Cyclic Adenosine Monophosphate

Aortic tissue pieces 7 mm in length were incubated for 30 min in HEPES-buffered EBSS containing 100 μmol/L indomethacin at 37°C. After 20 min in the presence of 100 μmol/L zaprinast (a cyclic guanosine-monophosphate [cGMP] phosphodiesterase inhibitor), the tissue was frozen in liquid nitrogen and stored at -80°C. After deproteinization, cGMP was determined by enzyme immunoassay (Cayman Chemical) and expressed as fmoL cGMP/mg protein. For cyclic adenosine monophosphate (cAMP), the protocol was identical to that of cGMP, except that 100 μmol/L isobutylmethylxanthine replaced 100 μmol/L zaprinast, and a cAMP enzyme immunoassay kit was used (Cayman Chemical).

Statistical Analysis

Values are reported as mean $\pm$ standard error (SEM), where n refers to the number of rats used. Statistical significance ($P < .05$) was evaluated using either the Student t test or, for multiple groups, analysis of variance followed by the Tukey-Kramer multiple comparison test.

Results
Heart Rate and BP

Administration of BSO for 1 week increased systolic BP, whereas treatment with amlodipine alone for the same
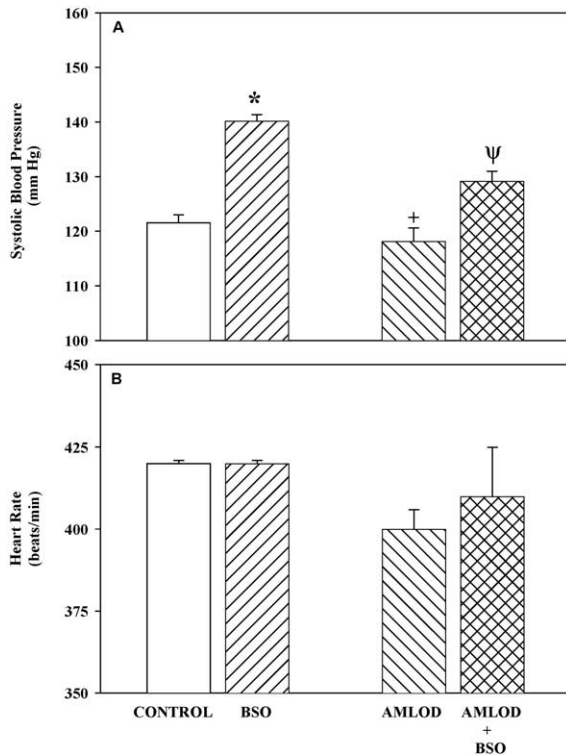
FIG. 1. Effect of buthionine-sulfoximine (BSO) and amlodipine (AMLOD) on systolic blood pressure (**A**) and heart rate (**B**) in Sprague-Dawley rats. Data are presented as mean $\pm$ SEM for six animals per group. Significant differences ($P < .05$) are denoted by: an asterisk (*) for all comparisons with the control animals; a plus (+) for amlodipine versus BSO; and a trident (ψ) for BSO + amlodipine versus BSO.

FIG. 2. Effect of buthionine-sulfoximine (BSO) and amlodipine (AMLOD) on plasma (**A**), heart (**B**), and kidney (**C**) glutathione (GSH) in Sprague-Dawley rats. Data are presented as mean $\pm$ SEM for six animals per group. Significant differences ($P < .05$) are denoted by an asterisk (*) for all comparisons with the control and by a diamond ($\blacklozenge$) for BSO + amlodipine versus amlodipine.

period did not significantly alter BP. When combined with BSO, amlodipine attenuated the BSO-induced elevation in BP, without altering heart rate (Fig. 1).

Plasma and Tissue Total Glutathione

The BSO-treated animals exhibited reductions in total glutathione (GSH) in plasma, heart, and kidney. Amlodipine alone did not significantly alter GSH levels in the plasma and tissues. When BSO and amlodipine were administered together, there was no significant alteration of the BSO-induced reduction in the plasma, kidney, and heart GSH levels (Fig. 2).

Plasma Levels of Total 8-Isoprostane and Thromboxane B$_2$

As shown in Fig. 3, BSO elevated plasma total 8-isoprostane and thromboxane B$_2$. Amlodipine alone reduced plasma total 8-isoprostane and thromboxane B$_2$ and reversed the BSO-induced effect.

Plasma Levels of Nitric Oxide and Prostacyclin

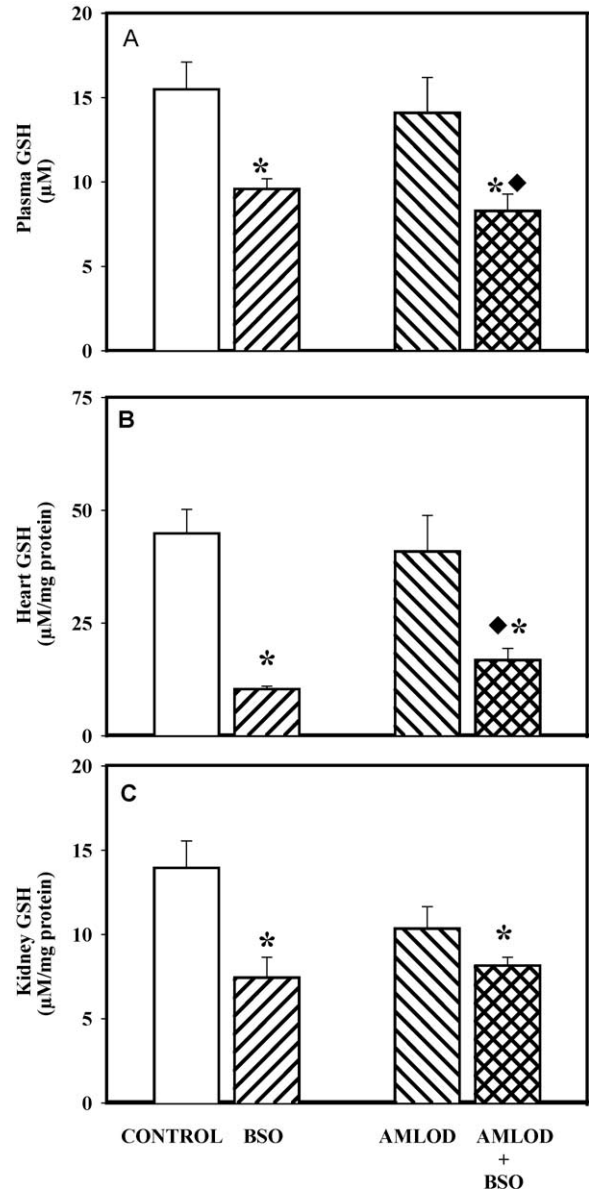As shown in Fig. 4, amlodipine elevated plasma NO, but this effect was blunted in the presence of BSO. Plasma

prostacyclin was reduced by BSO, both alone and in combination with amlodipine.

Aortic Tissue cAMP and cGMP Levels

Treatment with BSO reduced aortic cAMP and there was a tendency for decreased levels of cGMP. Amlodipine alone raised aortic levels of both cAMP and cGMP. When administered concurrently with BSO, amlodipine prevented the reductions in cAMP and cGMP induced by BSO (Fig. 5).
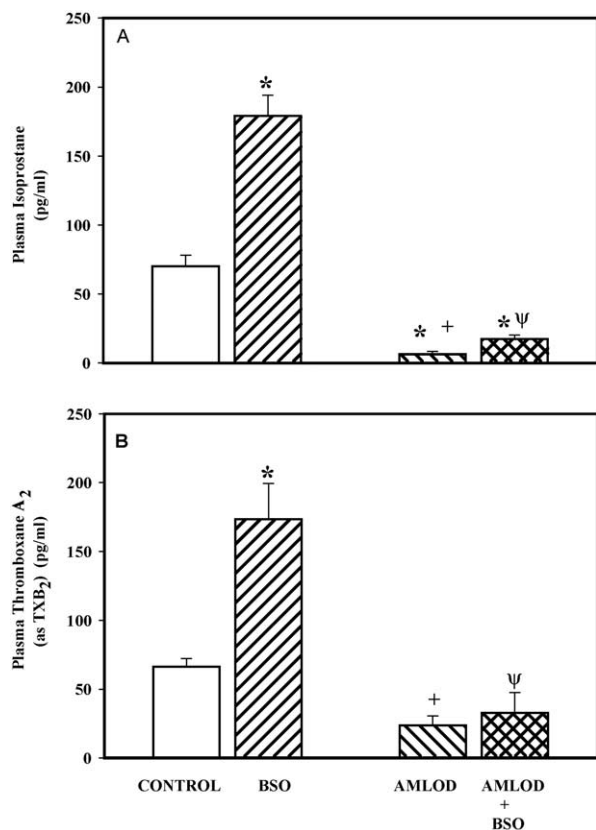
FIG. 3. Effect of buthionine-sulfoximine (BSO) and amlodipine (AMLOD) on plasma isoprostane (**A**) and thromboxane (**B**) levels in Sprague-Dawley rats. Data are presented as mean ± SEM for six animals per group. Significant differences ($P < .05$) are denoted as: an asterisk (*) for all comparisons with the control; a plus (+) for amlodipine versus BSO; and a trident (ψ) for BSO + amlodipine versus BSO.
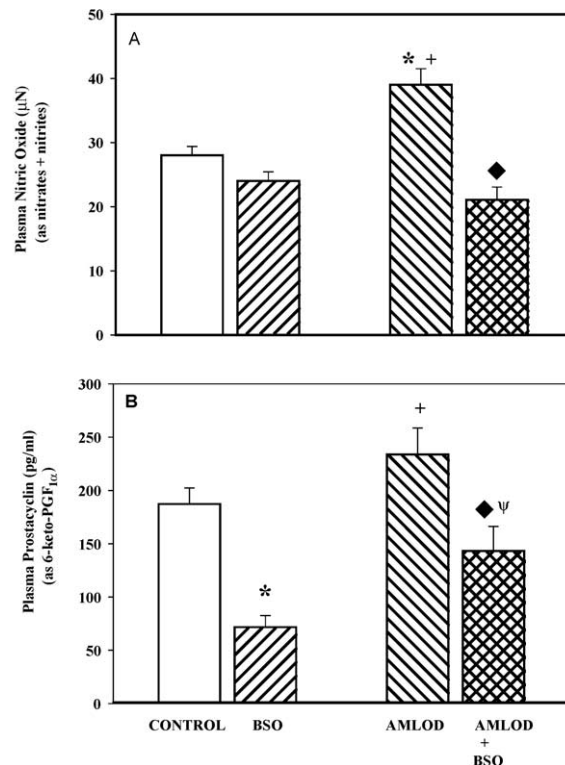

FIG. 4. Effect of buthionine-sulfoximine (BSO) and amlodipine (AMLOD) on plasma nitric oxide (**A**) and prostacyclin (**B**) levels in Sprague-Dawley rats. Data are presented as mean ± SEM for six animals per group. Significant differences ($P < .05$) are denoted as: an asterisk (*) for all comparisons with the control; a plus (+) for amlodipine versus BSO; a trident (ψ) for BSO + amlodipine versus BSO; and a diamond (♦) for BSO + amlodipine versus amlodipine.

Discussion

The aim of this study was to evaluate the role of endothelium-derived factors, along with their associated signal transduction second messengers, in the antioxidant and antihypertensive effect of amlodipine, a dihydropyridine calcium channel blocker. Several studies have demonstrated that amlodipine displays antioxidant and cytoprotective activities against free radical-mediated vascular endothelial injury both in vivo and in vitro.[18–20]

In these experiments, oxidative stress was induced by the administration of BSO, which caused significant reductions in GSH levels in the plasma, heart, and kidney after 1 week. This pattern of change is consistent with previous studies that demonstrated the GSH-reducing effect of BSO and its interorgan translocation, turnover, and metabolism.[21] The reduction in GSH was associated with a significant increase in BP. Likewise, Vaziri et al demonstrated that administration of BSO caused GSH depletion, oxidative stress, and severe hypertension in normotensive rats.[4] In addition, BSO elevated plasma 8-isoprostane, a prostaglandin-like product formed by free

radical peroxidation of arachidonic acid in membranes, which represents a reliable and specific in vivo marker of oxidative stress and lipid peroxidation.[22,23]

Amlodipine did not significantly alter GSH levels, but it did decrease plasma isoprostane and attenuated the BSO-induced effect. The effect of amlodipine on GSH metabolism is not well understood. Studies that examined the effects of other calcium antagonists including nicardipine, nifedipine, verapamil, and diltiazem on free radical injury in cultured endothelial cells reported that the protective mechanisms of calcium channel blockers against free radical injury may be mediated by their lipid antiperoxidative activities, which prevent the glutathione decrease induced by oxidant stress.[24]

Consistent with the BP data, administration of BSO significantly altered the levels of endothelium-derived factors, evidenced by elevations in thromboxane A_2 and reductions in prostacyclin and nitric oxide. Amlodipine reduced plasma TXA_2 and significantly attenuated the increases induced by BSO. It has been demonstrated in spontaneously hypertensive rats that oxygen-derived thromboxane A_2 and prostaglandin H_2 release in smooth muscles causes free radical-induced vasoconstriction in the rat aorta.[25] An imbalance between thromboxane and prostacyclin has been demonstrated in oxidative stress, in
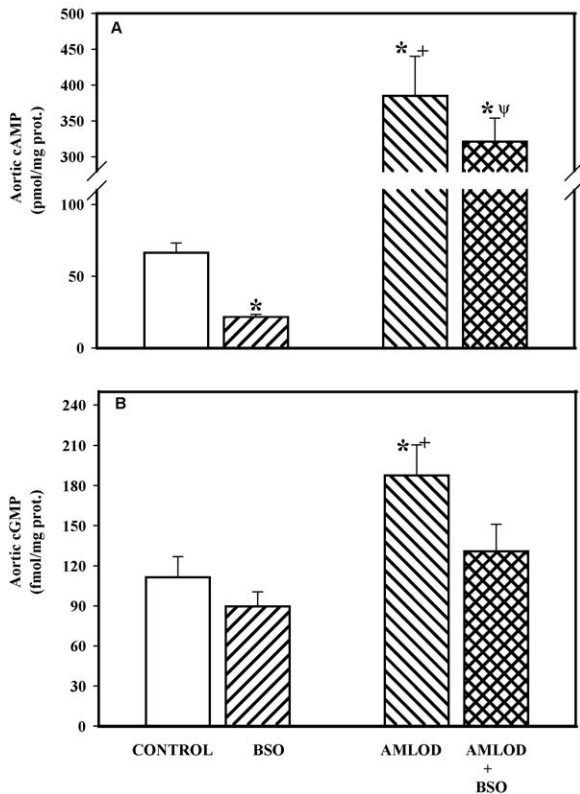
FIG. 5. Effect of buthionine-sulfoximine (BSO) and amlodipine (AMLOD) on cyclic adenosine monophosphate (cAMP) (**A**) and cyclic guanosine monophosphate (cGMP) (**B**) levels in aortic tissue of Sprague-Dawley rats. Data are presented as mean ± SEM for six animals per group. Significant differences ($P < .05$) are denoted as: an asterisk (*) for all comparisons with the control; a plus (+) for BSO versus amlodipine; and a trident (ψ) for BSO versus amlodipine + BSO.

that lipid peroxides activate the cyclooxygenase enzyme to increase thromboxane synthesis while at the same time inhibiting prostacyclin synthase to decrease prostacyclin synthesis.[26] In this study, we found that calcium channel blockade with amlodipine prevented oxidative stress-induced impairment of prostacyclin production in vivo, which is consistent with previous in vitro studies using rat aortic smooth muscle cells.[27] Consistent with the prostacyclin data, amlodipine raised cAMP and attenuated the reductions on cAMP induced by BSO. Cyclic AMP is known to act as a second messenger for vasorelaxation by hyperpolarization and may provide an explanation for the PGI_2 mechanism of action on reducing BP.[28]

Amlodipine significantly elevated plasma NO accompanied by elevation of cyclic GMP levels in the aorta. These results suggest that the decrease in TXA_2 and increase in prostacyclin formation by amlodipine may be associated with the release of NO. It is known that nitric oxide diffuses out of endothelial cells, where it is synthesized, and stimulates guanylate cyclase in vascular smooth muscle cells, causing vascular relaxation.[29] Several studies have shown that amlodipine enhances NO production via

a kinin-dependent mechanism involving either altered local production or activity of kinins.[30–32] During oxidative stress, nitric oxide and superoxide react to form peroxynitrite, a potent cytotoxic oxidant, thus reducing the bioavailability of NO.[33] It has also been shown that, by inactivation of NO, superoxide production appears to be an essential mechanism for endothelial dysfunction.[34] Treatment with amlodipine alone increased plasma levels of nitric oxide; however, in the presence of BSO this effect was blocked, implying that oxidative stress may impair the beneficial effect of amlodipine in terms of nitric oxide availability. Consistently, it has been demonstrated that in vivo thiol depletion results in endothelial dysfunction and a reduced receptor-mediated vascular relaxation. This effect is caused by reduced endothelial NO formation.[35] Generally, oxidant stress has been shown to regulate both nitric oxide and prostacyclin synthase, because peroxynitrite formed by simultaneous generation of nitric oxide and superoxide selectively inhibit prostacyclin synthase.[36] Hink et al also demonstrated that vascular peroxynitrite formation inhibits the activity of prostacyclin synthase as well as NO and cGMP signaling, which may contribute to vascular dysfunction.[37]

In conclusion, these findings suggest that amlodipine attenuates BSO-induced hypertension, which appears to be mediated in part by the prostanoid endothelium-derived factors and nitric oxide.

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