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European Journal of Pharmacology 528 (2005) 144–149
www.elsevier.com/locate/ejphar

Short communication

Role of voltage-sensitive calcium-channels in nitric oxide-mediated vasodilation in Spontaneously Hypertensive rats

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Received 8 August 2005; received in revised form 25 October 2005; accepted 28 October 2005

Abstract

This study demonstrates that the vasodilator potencies of nitric oxide (NO) donors such as sodium nitroprusside are increased in conscious Spontaneously Hypertensive (SH) as compared to Wistar Kyoto (WKY) rats. For example, the NO donors do not dilate hindlimb resistance arteries in WKY rats whereas they elicit pronounced vasodilator responses in SH rats. This study also demonstrates that the NO-mediated vasodilator responses in WKY and SH rats were markedly diminished after blockade of voltage-sensitive Ca^{2+}-channels (Ca^{2+}_{VS}-channels) with nifedipine, diltiazem or verapamil. These findings suggest that NO dilates resistance arteries in vivo via direct and/or hyperpolarization-induced closure of Ca^{2+}_{VS}-channels and that the increased potency of NO in SH rats may be due to the augmented Ca^{2+}_{VS}-channel activity reported in this strain.

Keywords: NO (nitric oxide); Voltage-sensitive Ca^{2+}-channel; Spontaneously Hypertensive rat

1. Introduction

Systemic injections of nitric oxide donors such as molsidomine and sodium nitroprusside elicit dose-dependent decreases in mean arterial blood pressure and mesenteric and renal vascular resistance in conscious normotensive rats whereas they have little effect on hindlimb vascular resistance (see Gardiner et al., 1990; Phillips et al., 1991; Davisson et al., 1996). Travis et al. (2000) demonstrated that the vasodilator responses elicited by systemic injections of nitric oxide (NO) donors in anesthetized rats were markedly attenuated after administration of the voltage-sensitive Ca^{2+}-channel (Ca^{2+}_{VS}-channel) blocker, nifedipine (see Opie, 1997) whereas the responses elicited by the endothelium-dependent vasodilator, acetylcholine (see Gardiner et al., 1990), and the endothelium-derived *S*-nitrosothiol, L-*S*-nitrosocysteine (see Myers et al., 1990; Rosenblum, 1992), were not affected. These in vivo findings support a wealth of in vitro evidence that a primary mechanism by which endothelium-derived nitric oxide dilates arteries is by the direct cGMP-dependent protein kinase-mediated and/or hyperpolarization-induced closure of Ca^{2+}_{VS}-channels in vascular smooth muscle (see Moncada et al., 1991). The above findings also support substantial evidence that L-*S*-nitrosocysteine and the endothelium-derived relaxing factor (EDRF) released by acetylcholine and bradykinin act by additional mechanisms including the activation of Ca^{2+}-dependent K^+ (K_{Ca}) channels (see Travis et al., 2000; Lang et al., 2003; Batenburg et al., 2004a,b).

There is direct evidence that Ca^{2+}_{VS}-channel activity in vascular smooth muscle of Spontaneously Hypertensive (SH) rats is increased compared to normotensive Wistar Kyoto (WKY) rats (Asano et al., 1988, 1993; Ohya et al., 1993, 1998; Cox and Lozinskaya, 1995; Matsuda et al., 1997; Kubo et al., 1998; Arii et al., 1999; Pratt et al., 2002). Exaggerated Ca^{2+}_{VS}-channel activity in resistance arteries of SH rats predicts that NO would be a more potent vasodilator in SH than in WKY rats. The aim of this study was to test this possibility by examining the responses to systemic injections of (i) the NO donors, (*Z*)-1-|*N*-methyl-*N*-[6(*N*-methylammoniohexyl)amino]|diazen-1-

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doi:10.1016/j.ejphar.2005.10.056

ium-1,2-diolate (MAHMA NONOate) and sodium nitroprusside (Travis et al., 2000), (ii) L-*S*-nitrosocysteine, and (iii) acetylcholine, in conscious SH and WKY rats before and after injection of one of three structurally different Ca^{2+}_{VS}-channel blockers, namely, the dihydropyridine, nifedipine; the benzothiazepine, diltiazem; and the phenylalkylamine, verapamil (Narita et al., 1983; Takata and Hutchinson, 1983; Nievelstein et al., 1985; Pruneau and Roy, 1987; Tasaka et al., 1987; Isshiki et al., 1988; Karasawa et al., 1988; Lee et al., 1988; Opie, 1997). Doses of diltiazem and verapamil were chosen that elicited similar responses to those of nifedipine in WKY and in SH rats.

2. Materials and methods

2.1. Rats and surgical procedures

All studies were performed according to the NIH guide for the care and use of laboratory animals (NIH Publications No. 8023, revised 1978) and approved by the University of Iowa Animal Care and Use Committee. Male SH and WKY rats (14–16 weeks of age) were anesthetized with pentobarbital sodium (50 mg/kg, i.p.) and a catheter was placed into a carotid artery to record mean arterial blood pressure. A catheter was also placed in a jugular vein to give drugs. Pulsed Doppler flow probes were placed around an iliac artery and the superior mesenteric artery to measure blood flow velocities and to determine hindlimb and mesenteric vascular resistances, respectively (see Davisson et al., 1996; Travis et al., 2000). The rats were allowed 4 days to recover from surgery.

2.2. Protocols

Bolus injections of MAHMA NONOate (5–50 nmol/kg), sodium nitroprusside (1–8 μg/kg), L-*S*-nitrosocysteine (100–400 nmol/kg) and acetylcholine (0.1–1 μg/kg) were given before and beginning 30 min after injection of (i) vehicle, (ii) nifedipine (500 nmol/kg, $n=12$), (iii) diltiazem (1 μmol/kg, i.v., $n=12$), or (iv) verapamil (2.5 μmol/kg, i.v., $n=12$). The responses elicited by each injection of the test agents were allowed to subside completely before another injection was given.

2.3. Drugs

All drugs were from Sigma (St. Louis, MO, USA) except MAHMA NONOate (Alexis Biochemicals, San Diego, CA, USA) and sodium nitroprusside (Abbott, Chicago, IL, USA). Nifedipine, diltiazem and verapamil were dissolved in 1% dimethylsulfoxide in saline (vehicle). L-*S*-Nitrosocysteine was prepared as described previously (Davisson et al., 1996; Travis et al., 2000).

2.4. Statistics

All values mean±S.E.M. The data were analyzed by repeated measures analysis of variance and Student's modified -test with Bonferroni corrections for multiple comparisons between means (see Davisson et al., 1996; Travis et al., 2000). A value of $P<0.05$ denotes statistical significance.

3. Results

3.1. Effects of vehicle and Ca^{2+}_{VS}-channel blockers on resting hemodynamic parameters

The administration of vehicle did not affect resting parameters in WKY or SH rats or the responses elicited by the test agents ($P>0.05$, for all comparisons, data not shown). The effects of nifedipine on resting parameters are summarized in Table 1. Nifedipine elicited prompt reductions in mean arterial blood pressure and hindlimb and mesenteric resistances in WKY and SH rats. The maximal responses occurred between 3 and 7 min. These responses gradually subsided over the next 20–25 min and obtained plateau levels (see "Recovery" columns) that were sustained over the rest of the experiments. In WKY rats, plateau mean arterial pressure and hindlimb resistance values were not different from pre-injection values whereas resting mesenteric resistances were higher than pre-injection levels. In SH rats, plateau mean arterial pressure and hindlimb resistance values were less than pre-injection values whereas mesenteric resistances were higher than pre-injection values.

The responses elicited by 1 μmol/kg of diltiazem were similar to those of 500 nmol/kg nifedipine ($P>0.05$, for all comparisons). The maximal falls in mean arterial pressure and hindlimb and mesenteric resistances were; in WKY rats, −27±4%, −26±5% and −43±5%, respectively ($P<0.05$, for all responses); and SH rats, −28±3%, −41±5% and −39±4%, respectively ($P<0.05$, for all responses). Recovery Phase mean arterial pressure and hindlimb and mesenteric resistance values expressed as %change from pre-injection values in WKY rats were, +3±3% ($P>0.05$), −3±3% ($P>0.05$), and +24±4% ($P<0.05$). In SH rats, these values were, −14±3% ($P<0.05$),

Table 1
Effects of nifedipine on resting hemodynamic parameters in WKY and SH rats

Strain	Parameter	Actual values			% Change from pre	
		Pre	Maximum	Recovery	Maximum	Recovery
WKY	MAP (mm Hg)	116±3	81±4	118±3	−29±5[a]	+2±3
	HLR (mm Hg/kHz)	167±18	129±15	164±12	−23±6[a]	−2±6
	MR (mm Hg/kHz)	45±4	28±3	53±6	−38±7[a]	+18±5[a]
SH	MAP (mm Hg)	159±5[b]	72±4	143±3	−23±4[a]	−11±3[a]
	HLR (mm Hg/kHz)	279±25[b]	209±14	235±26	−38±7[a]	−16±5[a]
	MR (mm Hg/kHz)	63±6[b]	43±3	82±10	−32±5[a]	+27±6[a]

The values are mean±S.E.M. WKY=Wistar Kyoto rat. SH=Spontaneously Hypertensive rat. MAP=mean arterial blood pressure. HLR=hindlimb vascular resistance. MR=mesenteric vascular resistance. There were 10 rats in each group. The dose of nifedipine was 500 nmol/kg.

[a] $P<0.05$, post-nifedipine versus pre.

[b] $P<0.05$, SH pre-injection versus WKY pre-injection.

−22±4% ($P<0.05$), and +24±4% ($P<0.05$). The maximal falls in mean arterial pressure elicited by 2.5 μmol/kg verapamil in WKY (−43±4%) and SH rats (−36±4%) were greater than those elicited by nifedipine or diltiazem ($P<0.05$ for both comparisons). However, the maximal falls in hindlimb resistance in WKY (−27±3%, $P<0.05$) and SH rats (−43±4%, $P<0.05$) and the maximal falls in mesenteric resistance in WKY (−43±5%, $P<0.05$) and SH (−36±4%, $P<0.05$) rats were similar to those elicited by nifedipine and diltiazem ($P>0.05$ for all comparisons). Recovery Phase values after injection of verapamil were similar to those after administration of nifedipine or diltiazem ($P>0.05$ for both comparisons). Recovery Phase mean arterial pressure and hindlimb and mesenteric resistance values expressed as %change from pre-injection values in WKY rats were, +1±4% ($P>0.05$), −4±3% ($P>0.05$), and +34±3% ($P<0.05$). In SH rats, these values were, −17±3% ($P<0.05$), −26±4% ($P<0.05$), and +28±4% ($P<0.05$).

3.2. Effects of nifedipine, diltiazem and verapamil on the responses elicited by the test agents

The pattern of responses elicited by the NO-donors, sodium nitroprusside and MAHMA NONOate, was similar in that they caused dose-dependent falls in mean arterial blood pressure and mesenteric resistance but no changes in hindlimb resistance in WKY rats whereas they elicited more pronounced falls in mean arterial blood pressure and mesenteric resistance and substantial falls in hindlimb resistance in SH rats. These responses were markedly attenuated by nifedipine. The responses elicited by selected doses of sodium nitroprusside and MAHMA NONOate before and after injection of nifedipine are summarized in Table 2. As can be seen, these doses of the NO-donors were more potent vasodilators in SH than in WKY rats (note especially the falls in hindlimb resistance) and these responses were markedly smaller after injection of nifedipine. The pattern of responses elicited by L-*S*-nitrosocysteine and acetylcholine was similar in that they caused dose-dependent falls in mean arterial blood pressure and mesenteric and hindlimb resistances in WKY rats and much smaller responses in SH rats. None of these responses were affected by nifedipine. The responses elicited by selected doses of L-*S*-nitrosocysteine and acetylcholine before and after injection of nifedipine are summarized in Table 2. L-*S*-Nitrosocysteine and acetylcholine were more potent vasodilators in WKY than in SH rats (in direct contrast to the NO-donors) and these responses were not affected by nifedipine.

Table 2
Vasodilator responses in conscious WKY and SH rats before and after administration of nifedipine

Agent	Parameter (%)	WKY		SH	
		Pre	Post	Pre	Post
SNP	ΔMAP	−16±2[a]	−5±2[a,b]	−27±2[a,c]	−7±2[a,b]
	ΔHLR	−4±3	−2±3	−28±3[a,c]	−4±3[b]
	ΔMR	−23±3[a]	−4±3[b]	−45±4[a,c]	−14±3[a,b]
MN	ΔMAP	−21±2[a]	−7±2[b]	−37±4[a,c]	−8±3[a,b]
	ΔHLR	−3±3	−1±2	−31±3[a,c]	−7±2[a,b]
	ΔMR	−29±3[a]	−12±2[a,b]	−43±3[a,c]	−18±3[a, b]
L-SNC	ΔMAP	−33±3[a]	−39±4[a]	−12±4[a,c]	−14±2[a]
	ΔHLR	−38±3[a]	−44±4[a]	−11±3[a,c]	−16±3[a]
	ΔMR	−29±4[a]	−31±4[a]	−14±3[a,c]	−19±3[a]
ACh	ΔMAP	−22±3[a]	−26±3[a]	−12±2[a,c]	−14±3[a]
	ΔHLR	−42±4[a]	−46±2[a]	−16±3[a,c]	−18±3[a]
	ΔMR	−27±3[a]	−33±3[a]	−15±2[a,c]	−19±3[a]

Data are mean±S.E.M. WKY=Wistar Kyoto rat. SH=Spontaneously Hypertensive rat. MAP=mean arterial blood pressure. HLR=hindlimb vascular resistance. MR=mesenteric vascular resistance. The dose of nifedipine was 500 nmol/kg. MN=MAHMA NONOate (25 nmol/kg). SNP=sodium nitroprusside (2 μg/kg). L-SNC=L-*S*-nitrosocysteine (200 nmol/kg). ACh=acetylcholine (0.5 μg/kg). There were 12 rats in each group.

[a] *$P<0.05$, significant response.*

[b] $P<0.05$, post-nifedipine versus pre.

[c] $P<0.05$, pre-nifedipine responses in SH versus WKY rats.

The effects of diltiazem and verapamil on responses to the test agents were similar to those of nifedipine. For example, the falls in mesenteric resistance elicited by the 2 μg/kg dose of sodium nitroprusside before and after injection of diltiazem were; in WKY rats, −26±3% and −8±4 %, respectively ($P<0.05$, post-diltiazem versus Pre); and in SH rats (−41±3% and −19±4%, respectively ($P<0.05$, post-diltiazem versus Pre). In addition, the falls in mesenteric resistance elicited by the 2 μg/kg dose of sodium nitroprusside before and after injection of verapamil were; in WKY rats, −29±3% and −10±2%, respectively ($P<0.05$, post-diltiazem versus Pre); and in SH rats, −46±3% and −17±3%, respectively ($P<0.05$, post-verapamil versus Pre). The responses elicited by MAHMA NONOate in WKY and SH rats were also markedly attenuated after injection of diltiazem or verapamil whereas the responses elicited by L-*S*-nitrosocysteine and acetylcholine were not affected ($P>0.05$, for all responses, data not shown).

4. Discussion

One principal finding of this study was that the NO donors, sodium nitroprusside and MAHMA NONOate, were more potent vasodilators in conscious SH than WKY rats. In especial, the NO donors did not dilate hindlimb resistance arteries in WKY rats whereas they elicited robust responses in SH rats. The finding that the NO-mediated responses in WKY and SH rats were markedly attenuated by nifedipine, diltiazem and verapamil suggests that Ca^{2+}_{VS}-channels are the major target for NO-mediated signal transduction processes. Specifically, blockade of Ca^{2+}_{VS}-channel greatly reduces the ability of NO to relax vascular smooth muscle in resistance arteries in vivo. The findings with diltiazem and verapamil are important in that nifedipine has actions other than the blockade of Ca^{2+}_{VS}-channels (see Hirasawa and Pittman, 2003).

It is likely that NO elicits its effects on Ca^{2+}_{VS}-channels via generation of cGMP and subsequent cGMP-mediated activation of cGMP-dependent protein kinase (Moncada et al., 1991; Campbell et al., 1996). cGMP-dependent protein kinase may also reduce the activity of Ca^{2+}-activated Cl^- channels, which play a major role in the depolarization-induced activation of Ca^{2+}_{VS}-channels (Lamb et al., 2000). The exaggerated vasodilation elicited by the NO donors in SH rats could certainly be due to the loss of baroreceptor reflex function in these rats (see Widdop et al., 1990). However, the present findings are

also consistent with direct in vitro evidence that the expression and activity of Ca^{2+}_{VS}-channels are augmented in vascular smooth muscle of SH rats (Asano et al., 1988, 1993; Ohya et al., 1993, 1998; Cox and Lozinskaya, 1995; Matsuda et al., 1997; Kubo et al., 1998; Arii et al., 1999; Pratt et al., 2002). To our knowledge, the mechanisms underlying the increased expression/activity of Ca^{2+}_{VS}-channels in resistance arteries of SH rats are not known. However, since cGMP-dependent protein kinase down-regulates Ca^{2+}_{VS}-channel activity (see Campbell et al., 1996), it would follow that a loss of endothelium-derived NO and/or *S*-nitrosothiols, both of which generate cGMP in vascular smooth muscle (see Travis et al., 2000), would lead to the increased expression/activity of Ca^{2+}_{VS}-channels.

In contrast to the above findings, the vasodilator actions of L-*S*-nitrosocysteine and the EDRF released by acetylcholine were markedly attenuated in SH rats. The vasorelaxant actions of L-*S*-nitrosocysteine and the EDRF released by bradykinin in isolated porcine coronary arteries involve the activation of small and medium conductance K_{Ca} channels (Batenburg et al., 2004a,b). As such, the loss of response to L-*S*-nitrosocysteine and acetylcholine in SH rats may be due to the down-regulation of these channels. However, Asano et al. (1993) reported that K_{Ca} channel activity is increased in vascular smooth muscle of SH rats. The vasodilation resulting from activation of K_{Ca} channels is due primarily to hyperpolarization-induced closure of Ca^{2+}_{VS}-channels (see Asano et al., 1993). The lack of effect of nifedipine, diltiazem and verapamil on the vasodilator actions of L-*S*-nitrosocysteine and acetylcholine in WKY or SH rats suggests that L-*S*-nitrosocysteine and the EDRF released by acetylcholine must relax vascular smooth muscle by mechanisms in addition to closure of these channels. The loss of response to exogenous L-*S*-nitrosocysteine and endothelium-derived L-*S*-nitrosocysteine released by acetylcholine (see Myers et al., 1990; Rosenblum, 1992) may involve the down-regulation of stereoselective L-*S*-nitrosocysteine recognition sites (see Davisson et al., 1996; Lipton et al., 2001; Batenburg et al., 2004a,b) other than K_{Ca} channels, and/or reduced susceptibility of these sites to *S*-nitrosation by L-*S*-nitrosocysteine (see Stamler et al., 1992; Lipton et al., 1993; Lang et al., 2003). The present findings are consistent with the possibility that the reduced vasodilator potency of L-*S*-nitrosocysteine in SH rats contributes to diminished endothelium-dependent vasodilation in these rats. However, it is certainly possible that the reduced acetylcholine-induced vasodilation in SH rats is due in part to the diminished release of endothelium-derived nitrosyl factors including NO and *S*-nitrosothiols.

The changes in resting hemodynamic parameters elicited by nifedipine, diltiazem and verapamil deserve attention. As expected, administration of these Ca^{2+}_{VS}-channel antagonists elicited robust depressor and vasodilator responses in both WKY and SH rats (see Narita et al., 1983; Takata and Hutchinson, 1983; Nievelstein et al., 1985; Pruneau and Roy, 1987; Tasaka et al., 1987; Isshiki et al., 1988; Karasawa et al., 1988; Lee et al., 1988; Opie, 1997). In WKY rats, these responses subsided with 25–30 min such that mean arterial blood pressure and hindlimb vascular resistances were equal to pre-injection values. In SH rats, the vasodilator responses also subsided within 25–30 min such that mean arterial blood pressure and hindlimb resistances were sustained at levels slightly lower than pre-injection levels. On the basis that nifedipine, diltiazem and verapamil markedly attenuated the vasodilator actions of NO, it is evident that the Ca^{2+}_{VS}-channel antagonists were still in effect after 25–30 min. The mechanisms responsible for the recovery of mean arterial blood pressure and vascular resistances may involve baroreflex-mediated increases in sympathetic nerve activity (Huang and Leenen, 1999) that would promote neurogenic vasoconstriction via α_1-adrenoceptor-mediated activation of phospholipase C leading to increases in intracellular Ca^{2+} in vascular smooth muscle via release of endogenous stores of Ca^{2+} (Abdel-Latif, 1986; Lamb et al., 2000). Interestingly, the falls in mesenteric resistance in WKY and SH rats not only fully recovered after injection of Ca^{2+}_{VS}-channel antagonists, but were maintained at levels slightly *higher* than pre-injection levels. This suggests that endogenous EDRFs may not buffer sympathetic neurogenic (phospholipase C-mediated) vasoconstriction in the mesenteric bed as effectively as in the hindlimb bed. Moreover, on the basis that blockade of Ca^{2+}_{VS}-channels markedly diminished the vasodilator actions of NO donors, it is evident that endothelium-derived NO is an important regulator of vascular tone in the mesenteric bed.

The finding that MAHMA NONOate did not elicit dilator responses in the hindlimb beds of conscious WKY rats whereas the L-type Ca^{2+}_{VS}-channel blockers did reduce hindlimb vascular resistance in these rats would seem at odds with the contention that the major mechanism by which NO dilates resistance arteries in vivo is by inhibition of L-type-Ca^{2+}_{VS}-channel activity. One possible explanation is that cGMP-dependent protein kinase is unable to diminish Ca^{2+}_{VS}-channel activity under resting conditions because the configuration of the channels does not favor the binding of the kinase. Moreover, when Ca^{2+}_{VS}-channel activity is increased, these channels may assume a configuration that favors binding of the kinase, which is then able to phosphorylate/inhibit the activity of the Ca^{2+}_{VS}-channels. It is important to note that the present study does not provide direct evidence that NO dilates resistance arteries via direct and/or hyperpolarization-induced closure of Ca^{2+}_{VS}-channels. Accordingly, it is possible that the reduction in intracellular Ca^{2+} levels in vascular smooth muscle elicited by Ca^{2+}_{VS}-channel blockers may down-regulate the activity of the intracellular signaling cascades responsible for NO-mediated vasodilation.

Our findings suggest that a major mechanism by which NO relaxes vascular smooth muscle in vivo is the closure of L-type Ca^{2+}_{VS}-channels that are blocked by nifedipine, diltiazem and verapamil. The clinical relevance of these findings and in particular with respect to the treatment of coronary vasospasm must be addressed. More specifically, our findings suggest that L-type Ca^{2+}_{VS}-channel blockers would diminish the vasodilator potency of NO donors in the coronary bed. However, there is to our knowledge, no evidence that the anti-anginal efficacy of NO donors such as nitroglycerin or isosorbide dinitrate is hampered by L-type Ca^{2+}_{VS}-channel blockers. This apparent anomaly might be explained by evidence that the vasorelaxant actions of the NO donor, sodium nitroprusside, were markedly diminished in

coronary arteries from α_{1H} T-type Ca^{2+}_{VS}-channel knockout mice whereas nifedipine elicited robust vasorelaxant responses in these arteries (Chen et al., 2003). It is well known that T-type Ca^{2+}_{VS}-channels are insensitive to nifedipine, diltiazem and verapamil (see Opie, 1997). Taken together, it appears that the principal mechanism by which NO relaxes vascular smooth muscle in coronary arteries is via closure of α_{1H} T-type Ca^{2+}_{VS} channels. Accordingly, the lack of effect of nifedipine, diltiazem and verapamil on α_{1H} T-type Ca^{2+}_{VS}-channels would explain why these L-type Ca^{2+}_{VS}-channel blockers do not impair NO-mediated vasodilation in coronary arteries of mice and perhaps humans.

In summary, this study demonstrates that the vasodilator actions of NO are potentiated in SH as compared to WKY rats whereas the vasodilator actions of acetylcholine and the putative EDRF, L-*S*-nitrosocysteine (Myers et al., 1990; Rosenblum, 1992), were diminished. The finding that the vasodilator actions of NO were markedly attenuated by nifedipine, diltiazem and verapamil suggests that NO exerts its effects primarily via inhibition of Ca^{2+}_{VS}-channel activity and supports considerable in vitro evidence that the activity of these channels is increased in arterial smooth muscle in SH rats (see Pratt et al., 2002). L-*S*-Nitrosocysteine may serve as an EDRF (see Myers et al., 1990; Rosenblum et al., 1992) and an endothelium-derived hyperpolarizing factor (EDHF) (see Batenburg et al., 2004a,b). Accordingly, the loss of vasodilator potency of this *S*-nitrosothiol in SH rats may contribute to the exaggerated vasoconstriction in this rat strain. The possibility that the vasodilator potencies of other potential prostanoid and non-prostanoid EDHFs (Batenburg et al., 2004a,b) are diminished in SH rats remains to be determined.

Acknowledgments

This study was supported by National Institutes of Health grant HL-14388.

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