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Change in endothelial function in mesenteric arteries of Sprague-Dawley rats fed a high salt diet

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A high salt diet in some species results in elevated arterial blood pressure and alterations in vascular smooth muscle responses to agonists. Weanling male Sprague-Dawley rats were given either a high salt diet containing 8 % or a low salt diet of 0.4 % sodium chloride for a period of 4 weeks. At the end of the feeding period, tail systolic pressure was higher in the high salt than in low salt rats. The rats were then killed and the intestines removed. Vascular smooth muscle (VSM) responses were estimated from the changes in lumenal diameter of pressurised second order mesenteric resistance arteries. High salt diet resulted in enhanced VSM responses to noradrenaline. The vessels dilated in response both to acetylcholine and to sodium nitroprusside and the responses were similar in vessels from both high and low salt rats. However, vessels from high salt rats were resistant to the blocking of endothelium derived nitric oxide (EDNO) with L-NAME and the responses were instead abolished by blocking endothelium derived hyperpolarising factor (EDHF) with apamin and charybdotoxin. These results show that in Sprague-Dawley rats, a high salt diet enhances the vasoconstriction in response to noradrenaline. The vasodilatory responses to acetylcholine were not significantly changed. However, they appeared to be mediated mainly by EDHF rather than by EDNO as in the low salt animals.

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There is considerable epidemiological evidence in humans linking the ingestion of large quantities of salt to the development of hypertension (Intersalt, 1988; Stamler *et al.* 1991). A similar association has been reported from studies of experimental animals fed high levels of salt. This is particularly marked in the genetically selected Dahl salt sensitive rats (Dahl *et al.* 1962), but it also occurs to a smaller extent in non-selected animals including weanling Sprague-Dawley (SD) rats (Miyajima & Bunag, 1985; Nwaigwe & Sofola, 1989; Obiefuna *et al.* 1991*a*), adult SD rats (Giardina *et al.* 2001), Wistar rats (Huang & Johns, 2000), dogs (Vogel, 1966) and chimpanzees (Denton *et al.* 1995).

The mechanisms linking high salt intake to hypertension appear to be complex and to involve alterations in both reflex function (Ferrari & Mark, 1987) and in the contractile properties of the vascular smooth muscle (Mulvany *et al.* 1978; Adegunloye & Sofola, 1997; Nishida *et al.* 1998).

It is now well established that endothelial factors are of importance in the mediation and modulation of both vasoconstriction and vasodilatation, although the effects of different levels of salt intake on these factors are much less clear. Acetylcholine induces relaxation of vascular smooth muscle mediated largely through release of endothelium-dependent relaxing factors, mainly nitric oxide (EDNO). The acetylcholine-mediated relaxation has been shown to be impaired in both spontaneously hypertensive rats (Wu *et al.* 1998; Izzard & Heagerty, 1999) and in Dahl salt-sensitive (DS) rats (Nishida *et al.* 1998). However, in unselected Sprague-Dawley rats the effects of salt loading have been inconsistent (Obiefuna *et al.* 1991*b*; Lenda *et al.* 2000). Part of the inconsistency may have been due to the different vessels studied: aortic rings by Obiefuna; spino-trapezius vessels by Lenda. Also in mesenteric vessels, which are known to be of major importance in blood pressure control, there is some evidence that, in addition to endothelial derived nitric oxide, endothelium-derived hyperpolarising factor (EDHF) may also be of importance (Ebeigbe *et al.* 1990; Adeagbo & Triggle, 1993). It is not known, however, whether the actions mediated by EDNO or by EDHF are affected by the level of salt intake.

The present study was therefore undertaken using normal Sprague-Dawley rats, to examine the effects of salt loading on the contractile and relaxation properties of mesenteric vascular smooth muscle. We particularly wished to examine the relative roles of EDNO and EDHF in any changes in relaxation responses.

METHODS

Weanling Sprague-Dawley (SD) rats aged 5 weeks and weighing 160–170 g were allocated either to a diet of normal chow with 0.4 % NaCl (low) or a high salt chow with 8 % NaCl. The high salt chow was prepared by mixing 76 g of NaCl with 924 g of chow. The rats were fed on these diets for 4 weeks with water given *ad libitum*. Tail systolic blood pressures were determined in some of the rats before and at the end of the feeding period, by means of a cuff and pressure detecting equipment (Life Sciences Equipment, CA, USA) as described by Bunag & Teravainen (1991). All experimental procedures complied with the Animals (Scientific Procedures) Act 1986.

At the end of the feeding period, the rats were killed with carbon dioxide, the abdomen was opened and, after tying off the mesenteric vessels, the intestine was removed and placed in cold physiological salt solution (PSS) at 4 °C. The PSS consisted of (mM): NaCl 119, $NaHCO_3$ 25, KCl 4.7, $CaCl_2$ 1.6, $MgSO_4$ $7H_2O$ 1.17, KH_2PO_4 1.18, sodium EDTA 0.026 and glucose 5.5. From the mesentery, second order mesenteric arteries of about 5 mm in length were dissected out and mounted between two glass cannulae in an arteriograph (Living Systems Instrumentation, Burlington, VT, USA). The vessel was perfused and superfused with PSS at 37 °C and pH 7.4 which was gassed with 5 % CO_2 in oxygen. The PSS also contained 10^{-5} M indomethacin to inhibit prostaglandin synthesis. The vessels, of diameter about 250 μm, were pressurised to 70 mmHg via a servo-controlled pressure system. The internal diameter of the vessel was determined by an inverted microscope-video tracking system (Living Systems Instrumentation, USA) and displayed on a monitor. The values of vessel diameter were also recorded using a heated stylus recorder (Devices Instruments Ltd, Hertfordshire, UK).

The vessel was allowed to equilibrate for 60–90 min, with two challenges of 10^{-6} M noradrenaline (NA) being applied. At the end of the equilibration period, the vessel was subjected to the following procedures. (1) Cumulative doses of NA were added to the PSS to obtain concentrations of 10^{-8}–10^{-5} M and the concentration–response curves (CRCs) were determined. (2) Following preconstriction with NA to 50 % of the resting diameter, CRCs were determined for acetylcholine (ACh) at concentrations of 10^{-8}–10^{-5} M. Tests were repeated after addition of 10^{-4} M N^{ω}-L-nitro-L-arginine methyl ester (L-NAME). (3) Following preconstriction with NA to 50 % of the resting diameter, CRCs were determined for sodium nitroprusside at concentrations of 10^{-8}–10^{-5} M. (4) Effects on the responses to ACh were determined after administration of L-NAME (10^{-4} M), followed by apamin (Ap, 1 μM) and charybdotoxin (ChTX, 1 μM) to block effects of EDHF as described by Doughty *et al.* (1999).

All the drugs used were obtained from Sigma (Poole, Dorset, UK). Indomethacin was dissolved in 2.5 % sodium carbonate to make a stock solution of 10^{-2} M. Fresh preparations of L-NAME and indomethacin were made on each experimental day.

Concentration–response curves were determined for NA and ACh before and after blockade of EDNO as well as after blocking EDHF. Significance tests were carried out using one-way ANOVA and unpaired Student's *t* test for intergroup analyses. *P* values less than 0.05 were taken as being significant.

RESULTS

At the end of the experimental feeding period, the weights of the control (low salt, CR) and salt-loaded rats (SR) were 273 ± 3 g (n = 13) and 225 ± 2 g (n = 12), respectively. The tail systolic blood pressure (SBP) in the 4 weeks increased from 101 ± 4 to 121 ± 3 mmHg in control rats (n = 6) and from 99 ± 4 to 155 ± 4 mmHg in the salt rats (n = 5). The terminal SBP in the salt rats was significantly higher than in control rats ($P < 0.01$).

Responses to noradrenaline

The concentration–lumenal diameter response curves (CRCs) of pressurised mesenteric arteries to noradrenaline were studied in eight low salt and eight high salt rats. The contractile responses of the high salt rats were greater than

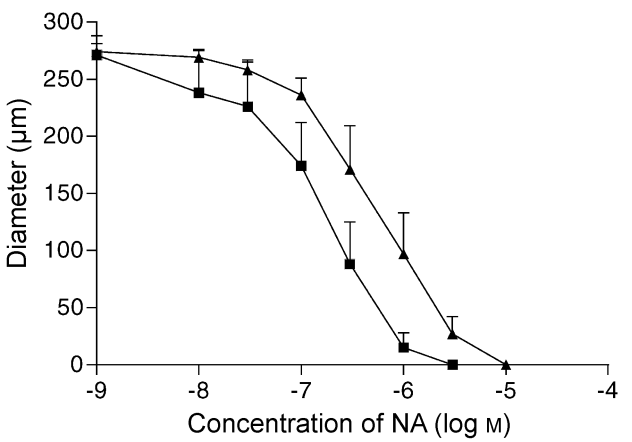

Figure 1. Responses to noradrenaline

Concentration–response curves of intralumenal diameter *vs.* noradrenaline in low salt (CR ▲, n = 8) and high salt (SR ■, n = 8) rats.

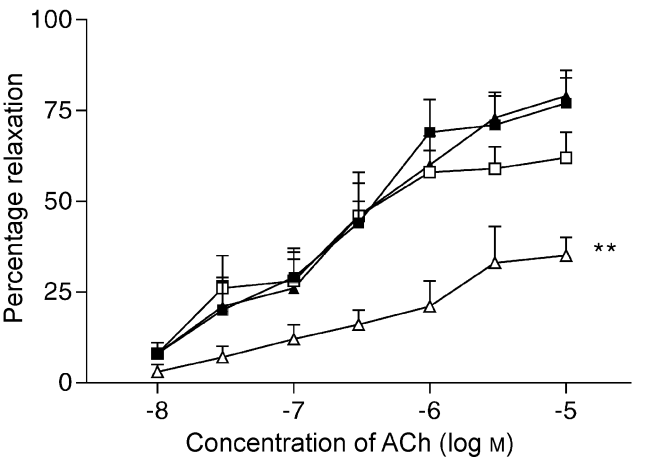

Figure 2. Responses to acetylcholine

Concentration–response curves of percentage relaxation *vs.* acetylcholine in low salt rats (CR, n = 10), before (▲) and after (△) L-NAME (dose 10^{-4} M) and in high salt rats (SR, n = 10) before (■) and after (□) L-NAME. Relaxation curves before L-NAME were similar in both groups but the low salt group showed significantly depressed response after L-NAME (** $P < 0.03$).

those of the low salt animals (Fig. 1). The EC_{50} for the high salt rats was $(2.45 \pm 0.83) \times 10^{-7}$ M while that for the low salt rats was $(5.77 \pm 2.6) \times 10^{-7}$ M ($P < 0.02$). In addition, the dose of NA eliciting maximum constrictor response, E_{max}, was siginificantly lower ($P < 0.03$) in the high salt than in the low salt rats ($(1.43 \pm 0.57) \times 10^{-6}$ M *vs.* $(5.43 \pm 1.6) \times 10^{-6}$ M, $P < 0.03$).

Responses to acetylcholine

Acetylcholine induced a dose-dependent relaxation of the preconstricted vessels in both groups of rats. Vessels were preconstricted from 267 ± 12 to 140 ± 13 μm in CR and from 251 ± 15 to 131 ± 12 μm in SR. The responses, shown in Fig. 2, were not significantly different between the groups prior to L-NAME ($P > 0.05$). The IC_{30} (concentration of acetylcholine causing a 30 % increase in diameter) values for the high and low salt rats were, respectively, 2.12 ± 0.99 and $2.54 \pm 1.73 \times 10^{-7}$ M ($P > 0.05$).

The effects on the relaxation responses of L-NAME are shown in Fig. 2. CR vessels were preconstricted from 260 ± 10 to 126 ± 12 μm and SR vessels from 269 ± 16 to 134 +18 μm. L-NAME significantly reduced the relaxation to ACh in the low salt rats but had no significant effect in the high salt rats. For example, at the highest dose of ACh tested (10^{-5} M) in low salt rats L-NAME reduced the relaxation from 79.2 ± 9.3 to 35.1 ± 5.2 % ($P < 0.001$), whereas in high salt rats the relaxations before and after L-NAME were, respectively, 77.3 ± 8.7 and 62.4 ± 7.3 % ($P > 0.05$).

In two experiments in each group in which the endothelium was removed by passing air bubbles through the vessels, the relaxation responses to acetylcholine were completely abolished.

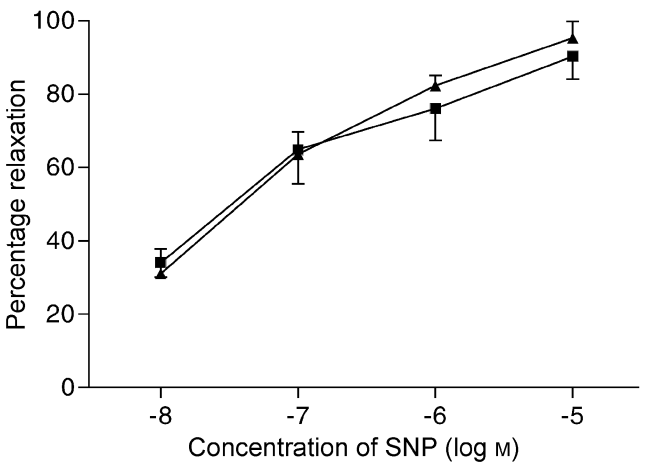

Figure 3. Responses to sodium nitroprusside

Concentration–response curves for sodium nitroprusside (SNP) in CR (▲, $n = 4$) and SR (■, $n = 4$). The relaxation responses were similar in both groups of rats.

Responses to sodium nitroprusside

These experiments were carried out on four rats from each group. After precontraction of the vessels with NA (from 242 ± 12 to 127 ± 20 μm in CR and from 275 ± 17 to 135 ± 14 μm in SR), and in presence of indomethacin and L-NAME, the relaxation responses to the NO donor were found to be similar in the two groups of rats (Fig. 3).

Responses to acetylcholine after L-NAME, apamin and charybdotoxin

The results of experiments, carried out on four rats in the low salt group and five rats in the high salt group are shown in Fig. 4. Vessels were preconstricted from 255 ± 20 to 126 ± 21 μm in CR and from 269 ± 21 to 129 ± 27 μm in SR. In low salt rats (Fig. 4*A*) relaxation responses to ACh were significantly inhibited by L-NAME ($P < 0.03$) and with the addition of apamin (Ap) and charybdotoxin (ChTx), the residual relaxation responses were abolished. In the high salt rats (Fig. 4*B*), the relaxation responses to

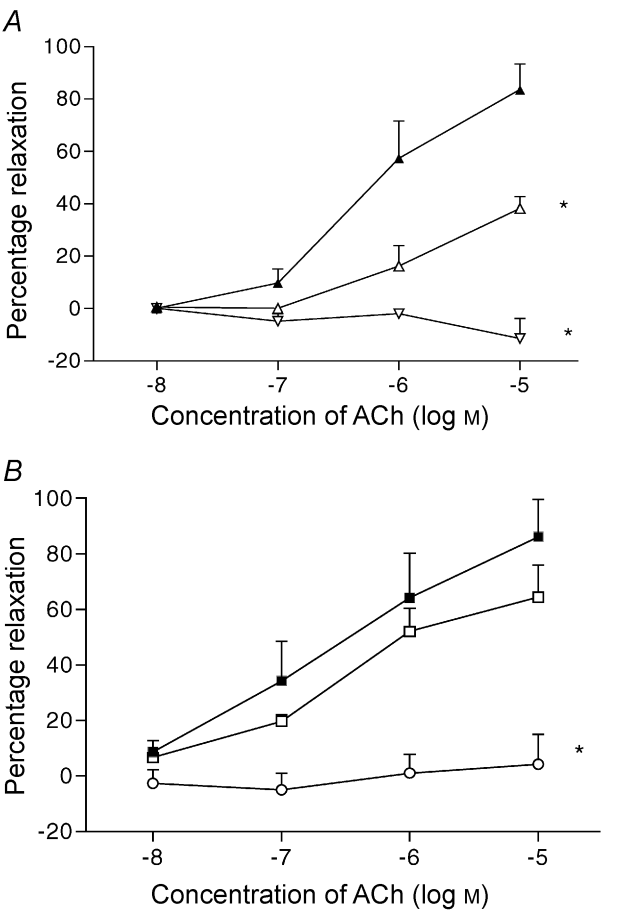

Figure 4. Responses to acetylcholine after L-NAME, apamin and charybdotoxin

A, concentration–response relaxation curves in rats fed a low salt diet ($n = 4$) before blockers (▲), after L-NAME (dose 10^{-4} M, △), and followed by apamin (Ap, 1 μM) and charybdotoxin (ChTx, 1 μM) (▽). *B*, same procedure in rats fed a high salt diet ($n = 5$), before blockers (■), after L-NAME (□), and followed by Ap and ChTx (○). * $P < 0.01$ *vs.* response curve prior to blockers.

ACh were little affected by L-NAME, but the relaxation responses after application of L-NAME, Ap and ChTx were also abolished. The comparisons of the effects of the inhibitors on the relaxation responses in control and salt-loaded rats to the highest dose of ACh used, i.e. 10^{-5} M, are shown in Fig. 5.

DISCUSSION

Dietary salt loading to various strains of rats is known to result in increases in arterial blood pressure. This effect is greatest in the genetically selected Dahl salt-sensitive animals (Dahl *et al.* 1962) but it also occurs in other animals including Sprague-Dawley rats (Miyajima & Bunag, 1985; Torii, 1980; Obiefuna *et al.* 1991*a*; Giardina *et al.* 2001) and to a lesser extent in Wistar rats (Huang & Johns, 2000; Kagota *et al.* 2001). The results from our study, using Sprague-Dawley rats, confirm this earlier work; systolic blood pressure was on average 34 mmHg higher in the high salt group.

Previous work has demonstrated that vessels from salt-loaded Sprague-Dawley rats constricted more powerfully in response to the application of noradrenaline (Obiefuna *et al.* 1991*a*,*b*; Adegunloye & Sofola, 1997). However, these investigators had used aortic ring preparations rather than the perfused small artery preparation used in this study. The perfused vessel preparations is more physiological as it is perfused along its length at a normal distending pressure (Halpern & Kelley, 1991; Buus *et al.* 1994; Dunn *et al.* 1994) Also, physiologically, it is the small arteries which are of importance in regulation of vascular resistance.

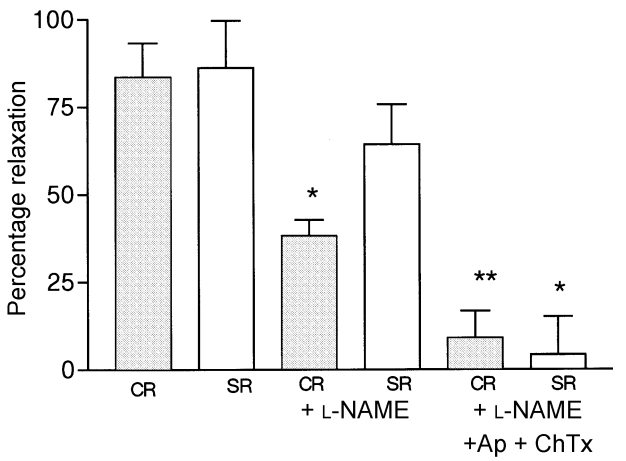

Figure 5. Comparison of maximum relaxation responses to ACh in the presence of inhibitors

Bar diagram showing comparison of responses at the maximum dose of ACh used (10^{-5} M), in low salt (CR) and high salt rats (SR), before inhibitors (left panels), after L-NAME (10^{-4} M) (middle panels) and after apamin (Ap 1 μM) and charybdotoxin (ChTx 1 μM) in addition to L-NAME. * $P < 0.01$, ** $P < 0.001$ between corresponding values prior to addition of blockers.

Previous work has shown that perfused vessels may give responses that are not seen in ring preparations (Dunn *et al.* 1994; Falloon *et al.* 1995). Thus our work showing the enhanced responses to noradrenaline in the salt-loaded rats confirms previous findings, but using a more physiological technique. It seems quite possible that the enhanced response to this physiological vasoconstrictor would contribute to the elevated blood pressure following salt loading. This observation may also explain the enhanced vascular responsiveness in salt-sensitive essential hypertension (Bragulat *et al.* 2001), even under the same sympathetic tone.

Another possible factor which could contribute to elevated blood pressure following salt loading could be a change in the vasodilator function of the resistance vessels. Dilatation may be effected be endothelial release of nitric oxide and this may be stimulated *in vitro* by acetylcholine. In fact a normal response to acetylcholine has been used as an index of normal endothelial function (Vanhoutte *et al.* 1995). The effects of salt loading on responses to acetylcholine have not been consistent. Obiefuna *et al.* (1991*b*) reported no difference in the responses of aortic ring preparations, from SD rats and Kagota *et al.* (2001) made similar observations in aortic rings from Wistar rats. Lenda *et al.* (2000), however, reported that salt loading depressed acetylcholine-induced vasodilatation of pial vessels. Our study, of the responses of perfused mesenteric vessels, did not show any significant difference in the responses of the two groups of rats to acetylcholine. However, the mechanism causing this dilatation does appear to be quite different. In the low salt rats the dilatation was greatly reduced following addition of L-NAME, indicating that EDNO was the major dilating agent. In the high salt rats, however, L-NAME had no significant effect, indicating that EDNO was not a significant mechanism in dilating those vessels. The difference between the two groups was not due a difference in the responsiveness of the vessels to nitric oxide, because addition of sodium nitroprusside, a nitric oxide donor had similar effects in both groups. However, the possibility still exists that alteration in nitric oxide production can modulate sympathetic neuro-effector mechanisms as reported in normal (Thomas & Victor, 1998) or DS rats (Nishida *et al.* 1998) and in man (Chavoshan *et al.* 2002).

We found that both the residual dilatation after L-NAME in the low salt rats and the much larger dilatation in the high salt animals were completely abolished by apamin and charybdotoxin. This implies that EDHF is responsible for causing dilatation. Earlier reports have shown that combined intraluminal administration of 1 μM apamin and 1 μM charybdotoxin, inhibitors of small and intermediate potassium-activated calcium channels, respectively, effectively abolished responses to EDHF (Doughty *et al.* 1999). This has also been further confirmed by

electrophysiological studies in which both agents when administered together cause inhibition of acetylcholine-induced hyperpolarisation and prevented EDHF-induced relaxation (Chen & Cheung, 1997; Coleman *et al.* 2001). Our present results suggest that, in the salt-loaded animals, EDHF is the vasodilatory mechanism of relatively greater importance and in combination with the enhanced vasoconstrictor response would result overall in an increased pressor effect.

The change in the mechanism causing vasodilatation following salt loading seems surprising, particularly in view of the observation that the overall response was not significantly affected. However, the involvement of EDHF has also previously been shown to be important in diabetic rats where the acetylcholine-induced relaxation was abolished by apamin and charybdotoxin (Wigg *et al.* 2001). Comparable results have been reported by Izzard & Heagerty (1999) who noted that the flow-related dilatation of pressurised mesenteric arteries of spontaneously hypertensive rats was reduced compared to normal rats and it was insensitive to inhibition with L-NAME.

It is becoming apparent that vasodilatation may be mediated through different mechanisms depending both on the vessels studied as well as on the condition. For example, Lenda *et al.* (2000) reported that a high salt diet resulted in reduced relaxation in vessels of spinotrapezius muscle and suggested that this was due to production of reactive oxygen radicals. Liu *et al.* (1999) suggested that, after L-NAME application, pial arteries of salt-loaded rats actually showed a small vasoconstriction in response to acetycholine. In Dahl salt-sensitive rats, Ruud *et al.* (1999) suggested the involvement of nitric oxide isoform II in the development of the hypertension.

Our observations are the first to implicate an increased role of EDHF in the relaxation occurring in response to ACh in salt-loaded animals. EDHF is known to cause significant vasodilatation in several blood vessels (Mombouli & Vanhoutte, 1997) and to be of particular importance in mesenteric resistance vessels of the rat (Ebeigbe *et al.* 1990; Nagao *et al.* 1992; Adeagbo & Triggle, 1993; Shimokawa *et al.* 1996). We can only speculate on the significance of our findings in relation to the development of hypertension. The overall relaxation in response to ACh was not different in our salt-loaded rats. However, ACh is not the normal physiological stimulus to the endothelium; shear stress is. It would, therefore, be of interest to examine the mechanisms of vasodilatation in response to flow in these animals.

In conclusion, we have demonstrated that a high salt diet in weanling Sprague-Dawley rats results in elevated blood pressure and this is associated with an enhanced vasoconstrictor response to noradrenaline. It does not alter vasorelaxant responses to a nitric oxide donor or to administration of acetylcholine. However, the mechanism of ACh-induced dilatation is different and appears to be associated with an enhanced production of EDHF by the vascular endothelium.

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