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Vascular responses to endothelin-1 following inhibition of nitric oxide synthesis in the conscious rat

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1 The objectives of the present experiments were to assess the role of endogenous nitric oxide (NO) in mediating and/or modulating the effects of endothelin-1 (ET-1) on blood pressure and microvascular permeability in conscious rats.

2 Intravenous administration of the NO synthesis inhibitors, N^G-monomethyl-L-arginine (L-NMMA) or N^G-nitro-L-arginine methyl ester (L-NAME) at a dose (25 mg kg^{-1} or 2 mg kg^{-1}, respectively) which evoked maximum increase in mean arterial blood pressure (MABP) significantly attenuated (by about 40%) the vasodepressor response and potentiated (by 100–180%) the pressor response to ET-1 (1 nmol kg^{-1}, i.v.) compared to the effects of ET-1 in animals where the peripheral vasoconstrictor effects of L-arginine analogues were mimicked by an infusion of noradrenaline (620–820 ng kg^{-1} min^{-1}). Similar inhibition of the depressor and potentiation of the pressor actions of ET-1 were observed when the MABP which had been elevated by L-NMMA or L-NAME was titrated to normotensive levels with hydralazine or diazoxide before injection of ET-1.

3 L-NAME (2 mg kg^{-1}) increased the vascular permeability of the large airways, stomach, duodenum, pancreas, liver, kidney and spleen (up to 280%) as measured by the extravasation of Evans blue dye. The permeability of pulmonary parenchyma, skeletal muscle and skin was not affected significantly by L-NAME treatment. Elevation of MABP by noradrenaline infusion did not evoke protein extravasation in the vascular beds studied with the exception of the lung. In the large airways, tissue Evans blue content was similar following noradrenaline infusion and L-NAME.

4 Both the pressor and permeability effects of L-NAME (2 mg kg^{-1}) were effectively reversed by L-arginine (300 mg kg^{-1}) but not by D-arginine (300 mg kg^{-1}). The D-enantiomer of L-NAME, D-NAME (2 mg kg^{-1}) had no effect on the parameters studied.

5 Protein extravasation was significantly enhanced by ET-1 (1 nmol kg^{-1}) in the upper and lower bronchi, stomach, duodenum, kidney and spleen (up to 285%). This was potentiated by L-NAME (2 mg kg^{-1}), resulting in marked increases in tissue Evans blue accumulation (up to 550%) in these tissues. The effects of L-NAME and ET-1 were additive in the trachea, duodenum, pancreas and liver. Combined administration of L-NAME plus ET-1 significantly increased protein extravasation in the pulmonary parenchyma, where neither L-NAME nor ET-1 alone caused significant increases.

6 Noradrenaline infusion (620–820 ng kg^{-1} min^{-1}) potentiated the permeability action of ET-1 (1 nmol kg^{-1}) in the pulmonary circulation, whereas it did not modify ET-1-induced protein extravasation in the other vascular beds.

7 These results indicate that endogenous NO mediates, in part, the vasodepressor effect and attenuates the vasopressor action of ET-1 and modulates the effects of ET-1 on vascular permeability. These findings confirm the role of NO in the maintenance of blood pressure and suggest an important role for NO in the regulation of microvascular permeability.

Keywords: Endothelin-1; protein extravasation; vascular permeability; blood pressure; nitric oxide; L-arginine analogues

Introduction

Endothelial cells are capable of producing a variety of substances, including endothelin-1 (ET-1) (Yanagisawa *et al.*, 1988) and endothelium-derived relaxing factor/nitric oxide (NO) (Ignarro *et al.*, 1987; Palmer *et al.*, 1987) that play a role in the regulation of vascular tone, local blood flow and permeability. Once released, these substances may act on the same cells to modulate the synthesis and/or release of other substances. Thus, ET-1-induced NO release (De Nucci *et al.*, 1988) and inhibition of ET-1 release by NO have been described (Boulanger & Lüscher, 1990; Saijonmaa *et al.*, 1990).

The discovery that L-arginine is the physiological precursor for the formation of NO (Palmer *et al.*, 1988a,b; Sakuma *et al.*, 1988) led to the development and use of N^G-substituted analogues of L-arginine, such as N^G-monomethyl-L-arginine (L-NMMA) (Palmer *et al.*, 1988b; Sakuma *et al.*, 1988) and N^G-nitro-L-arginine methyl ester (L-NAME) (Moore *et al.*, 1990) as inhibitors of endothelial NO synthesis. The findings that intravenous injection of L-arginine analogues evoke large increases in arterial blood pressure in experimental animals (Aisaka *et al.*, 1989; Rees *et al.*, 1989; Whittle *et al.*, 1989; Gardiner *et al.*, 1990a; Hecker *et al.*, 1990; Vargas *et al.*, 1991) are considered to indicate that the resultant decrease in endothelial NO production is responsible for the elevation of blood pressure. The studies investigating the role of NO in mediating the vasodilator and vasodepressor responses to ET-1 resulted in conflicting results (cf. Fozard & Part, 1992) since both significant inhibition of the depressor action of ET-1 by L-NMMA (Whittle *et al.*, 1989) and failure of L-NMMA to attenuate the depressor response to ET-1 (Gardiner *et al.*, 1989) have been described in anaesthetized and conscious rats, respectively. Furthermore, L-NAME, a more potent inhibitor of NO synthesis than L-NMMA (Moore *et al.*, 1990; Rees *et al.*, 1990), was found to attenuate the

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vasodilator action of ET-1 in the conscious rat (Gardiner *et al.*, 1990b).

Recent evidence suggest that both ET-1 and NO could also affect vascular permeability. Administration of ET-1 evoked haemoconcentration in dogs (Goetz *et al.*, 1988) and rats (López-Farré *et al.*, 1989; Filep *et al.*, 1991), led to oedema formation in the forearm of man (Dahlöf *et al.*, 1990) and enhanced protein extravasation in the large airways, heart, stomach, duodenum, kidney and spleen in rats (Filep *et al.*, 1991; 1992; Zimmerman *et al.*, 1992). On the other hand, NO may inhibit vascular permeability to macromolecules, since L-NAME has recently been reported to increase protein extravasation in the cat mesenteric circulation (Kubes & Granger, 1992) and rat coronary circulation (Filep *et al.*, 1993).

The objective of the present experiments was to study whether or not inhibition of endogenous NO synthesis could modulate the effects of ET-1 on blood pressure and microvascular permeability in conscious rats. The experimental design incorporated animals where mean arterial blood pressure was adjusted to similar levels as observed following administration of L-arginine analogues.

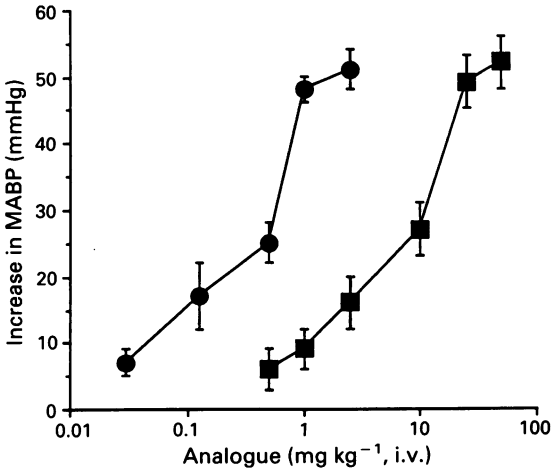


Figure 1 Peak pressor responses to intravenous injection of N^G-nitro-L-arginine methyl ester (L-NAME, ●) and N^G-monomethyl-L-arginine (L-NMMA, ■) in the conscious rat. Basal mean arterial blood pressure was 110 ± 1 mmHg ($n = 12$). Values are means ± s.e.means of 4–8 experiments.

Methods

The experiments were performed on conscious, chronically catheterized male Wistar rats weighing 220–310 g. The animals were kept in individual metabolic cages and were prepared as described previously (Filep *et al.*, 1987). Briefly, under anaesthesia (ketamine, 75 mg kg^{-1} and sodium pentobarbitone, 15 mg kg^{-1}) catheters were implanted into the abdominal aorta and vena cava through the central tail artery and left femoral vein, respectively. The venous catheter was led subcutaneously to the root of the tail. The catheters emerging from the tail were protected by an acrylic cuff-metal spiral device and were fed through the top of the metabolic cage. The animals were allowed to recover completely for at least 4 days following the surgical procedures. During the experiments the rats could move freely and had free access to food and water. Mean arterial blood pressure (MABP) was continuously monitored by an electromanometer using a Statham P23 dB pressure transducer.

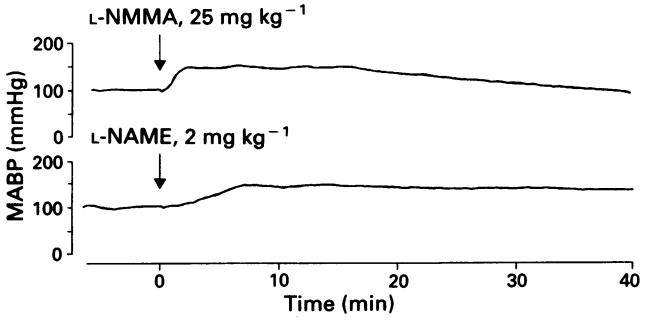


Figure 2 Time-course of changes in mean arterial blood pressure (MABP) following intravenous injection of N^G-nitro-L-arginine methyl ester (L-NAME, 2 mg kg^{-1}) and N^G-monomethyl-L-arginine (L-NMMA, 25 mg kg^{-1}) in conscious rats. These traces are representative for 8 and 4 experiments, respectively.

Experimental protocols

Initial experiments were designed to compare the pressor potency of L-arginine analogues. A 4 to 6 point dose-response curve was generated in each animal. On any one day each animal received only L-NAME or L-NMMA at 40–60 min intervals. In the second series of studies, the blood pressure responses to ET-1 (1 nmol kg^{-1}) were compared in the absence and presence of L-arginine analogues. The animals were pretreated with L-NAME (2 mg kg^{-1}) or L-NMMA (25 mg kg^{-1}) for 10 and 2 min, respectively before injection of ET-1. On any one day each animal received only one injection of ET-1. In order to compare directly the blood pressure responses to ET-1, in some animals MABP was either elevated by infusion of noradrenaline (620–820 ng kg^{-1} min^{-1}) to levels observed following L-NAME-treatment or L-NAME-induced elevation in MABP was titrated to normotensive levels with diazoxide (90 μmol kg^{-1}) or hydralazine (1.2–1.5 μmol kg^{-1}) before injection of ET-1. Both diazoxide and hydralazine cause direct (endothelium-independent) relaxation of the arterial smooth muscle. In another set of experiments, the ability of L-arginine analogues to inhibit the hypotensive effect of acetylcholine, which causes endothelium-dependent vasorelaxations *in vitro*, was studied in control rats and in animals receiving L-arginine analogues, noradrenaline infusion, L-NAME plus diazoxide or L-NAME plus hydralazine. At least 3 days were allowed to elapse between different experiments on the same rat.

In subsequent experiments protein extravasation was quantitated by measuring the extravasation of Evans blue dye, which binds to plasma albumin (Rawson, 1943). In this series of experiments, Evans blue dye (20 mg kg^{-1}, 25 mg ml^{-1} in 0.9% NaCl) was injected intravenously together with vehicle or ET-1 (1 nmol kg^{-1}) into animals pretreated with L-NAME (2 mg kg^{-1}) or during noradrenaline infusion (620–820 ng kg^{-1} min^{-1}). An additional group of animals received L-arginine (300 mg kg^{-1}, i.v.) or D-arginine (300 mg kg^{-1}, i.v.) 5 min after injection of L-NAME (2 mg kg^{-1}). Four animals were treated with N^G-nitro-D-arginine methyl ester (D-NAME, 2 mg kg^{-1}) for 10 min before injection of Evans blue dye. Ten min after injection of Evans blue dye, the animals were anaesthetized (sodium pentobarbitone, 50 mg kg^{-1}) and were perfused with 50 ml 0.9% NaCl through a catheter inserted into the abdominal aorta to remove the excess of intravascular dye. Then portions of the trachea, upper bronchi (airways extending from the bifurcation of the trachea to its entry to parenchyma), lower bronchi (defined as major airways surrounded by pulmonary parenchyma that can be easily dissected without magnification), pulmonary parenchyma, liver, spleen, pancreas, kidney, stomach, duodenum, skeletal muscle (right quadriceps) and dorsal skin were excised and weighed. Tissue Evans blue dye content was measured by spectrophotometry following extraction with formamide as described previously (Filep *et al.*, 1991). Evans

Table 1 Effects of L-arginine and D-arginine on N^G-nitro-L-arginine methyl ester (L-NAME)-induced changes in arterial blood pressurre and protein extravasation in conscious rats

	Vehicle ($n = 6$)	*L-NAME* ($n = 6$)	*D-Arginine plus L-NAME* ($n = 4$)	*L-Arginine plus L-NAME* ($n = 4$)	*D-NAME* ($n = 4$)
Basal MABP (mmHg)	106 ± 3	105 ± 4	104 ± 5	104 ± 3	107 ± 2
Peak ΔMABP (mmHg)	2 ± 2	43 ± 3**	39 ± 4*	3 ± 2†	2 ± 1†
Evans blue dye (μg mg^{-1} tissue weight)					
Trachea	62 ± 6	108 ± 12*	88 ± 7*	55 ± 6†	60 ± 5†
Upper Bronchi	52 ± 3	104 ± 9**	89 ± 14*	46 ± 8†	45 ± 7†
Lower Bronchi	49 ± 5	112 ± 10**	104 ± 12*	53 ± 7†	51 ± 6†
Pulmonary parenchyma	81 ± 7	110 ± 8	106 ± 13	97 ± 10	81 ± 18
Liver	53 ± 5	111 ± 10**	102 ± 11*	54 ± 18†	43 ± 8†
Spleen	118 ± 8	195 ± 23*	160 ± 21	90 ± 5†	111 ± 7†
Pancreas	38 ± 5	100 ± 11*	90 ± 15*	32 ± 6†	33 ± 7†
Kidney	57 ± 9	136 ± 20*	107 ± 14*	53 ± 13†	51 ± 7†
Stomach	71 ± 5	155 ± 25*	126 ± 16*	61 ± 8†	69 ± 13†
Duodenum	90 ± 9	252 ± 21**	205 ± 41*	86 ± 13†	99 ± 13†

Values are means ± s.e.mean. The animals were given L-arginine (300 mg kg^{-1}) or D-arginine (300 mg kg^{-1}) 5 min after injection of L-NAME (2 mg kg^{-1}). Ten min after administration of L-NAME, D-NAME (2 mg kg^{-1}) or their vehicle (0.9% NaCl, control), Evans blue dye (20 mg kg^{-1}) was injected i.v. The rats were killed 10 min after injection of the dye.
*$P<0.05$; **$P<0.01$ compared to controls; †$P<0.05$ compared to L-NAME-treated animals by Dunn's multiple contrast hypothesis test.

blue content of each sample was expressed as μg dye g^{-1} dry tissue weight to avoid underestimation of changes due to plasma fluid extravasation.

Drugs and chemicals

ET-1 was synthesized in our laboratories by solid-phase methodology. The purity of the preparation was greater than 97%. ET-1 was dissolved in distilled water and stored at − 20°C. On the day of the experiments an aliquot was removed and diluted further in 0.9% NaCl. Evans blue dye, L-arginine hydrochloride, D-arginine hydrochloride, noradrenaline hydrochloride, acetylcholine chloride, diazoxide, hydralazine hydrochloride and L-NAME were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A.; L-NMMA was obtained from Calbiochem, San Diego, CA, U.S.A.; D-NAME was purchased from Research Biochemicals International, Natick, MA, U.S.A. All drugs were dissolved in 0.9% NaCl immediately before use.

Statistical analysis

Results are expressed as means ± s.e.mean. Statistical evaluation of the data was performed by Dunn's multiple contrast hypothesis test (Dunn, 1964) when various treatments were compared to the same control or by the Wilcoxon signed rank test and Mann-Whitney U test for paired and unpaired observations, respectively. A $P<0.05$ level was considered significant for all tests.

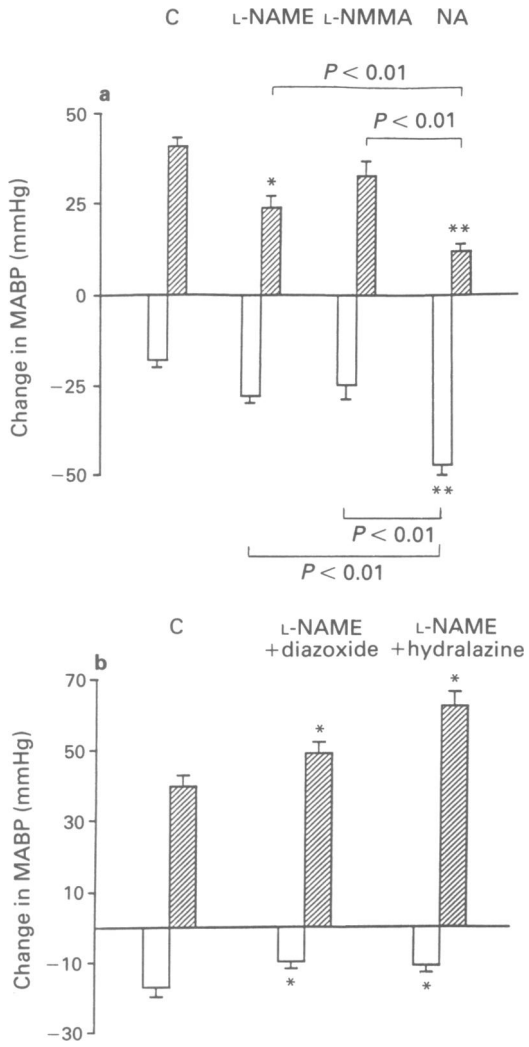
Results

Effects of L-arginine analogues on blood pressure responses to ET-1

Intravenous injection of L-NMMA and L-NAME produced dose-dependent increases in MABP in conscious rats (Figure 1). The maximum increase in MABP that could be evoked by these L-arginine analogues was similar (ΔMABP were 52 ± 4 mmHg and 51 ± 3 mmHg, respectively). Estimated ED_{50} values for L-NMMA and L-NAME were 7.8 and 0.5 mg kg^{-1}, respectively, indicating that L-NAME was approximately 16 times more potent than L-NMMA. The time course of the pressor action of the L-arginine analogues differed considerably (Figure 2). L-NMMA produced an immediate increase in MABP with a peak effect occurring within 1–3 min after the injection. In contrast, the pressor effect of L-NAME developed slowly, reaching the maximum change in MABP between 5 and 9 min (Figure 2). The duration of the pressor responses to L-NMMA was shorter than those of L-NAME; MABP returned to preinjection levels by 10–45 min depending on the dose of L-NMMA. The nitro analogue, L-NAME, however, evoked prolonged increases in MABP. For example, MABP remained stable at 145 mmHg for 50–60 min following administration of 2 mg kg^{-1} L-NAME (Figure 2). L-NAME (2 mg kg^{-1})-induced increase in MABP was effectively reversed by L-arginine (300 mg kg^{-1}), but not by D-arginine (300 mg kg^{-1}) (Table 1). At the dose of 2 mg kg^{-1}, D-NAME did not affect MABP (Table 1).

In subsequent experiments, L-NMMA and L-NAME were used at doses that evoked maximum increase in MABP, i.e. at 25 mg kg^{-1} and 2 mg kg^{-1}, respectively. As well established, ET-1 (1 nmol kg^{-1}) produced a sustained pressor effect preceded by a transient depressor action (Figure 3). Elevation of MABP by L-NAME, but not L-NMMA, significantly decreased the pressor response to ET-1, whereas the hypotensive response to ET-1 was not affected significantly (Figure 3a). In some animals, MABP was either elevated by an infusion of noradrenaline to levels comparable to those seen after injection of L-arginine analogues or was titrated to control levels by hydralazine or diazoxide and then the responses to ET-1 were tested. L-NMMA and L-NAME caused on average 40 and 47% inhibition of the depressor response to ET-1 and a 2 and 2.8 fold increase in the pressor action of ET-1 compared to the effects of ET-1 in animals receiving noradrenaline infusion (Figure 3a). Similarly, an approximately 40% inhibition of the depressor action and a 1.2–1.5 fold increase in the pressor effect of ET-1 were detected in animals treated with L-NAME plus hydralazine or L-NAME plus diazoxide compared to the effects of ET-1 in control (normotensive) animals (Figure 3b). These changes were statistically significant.

In another series of experiments, the ability of L-arginine analogues to inhibit the hypotensive effect of acetylcholine was tested. When the effects of acetylcholine in L-NMMA or L-NAME-treated animals and during noradrenaline infusion (i.e. when MABP values were similar before injection of acetylcholine) were compared, 40% inhibition of the depressor response to acetylcholine by L-arginine analogues was detected (Figure 4a). Similarly, the hypotensive effects of



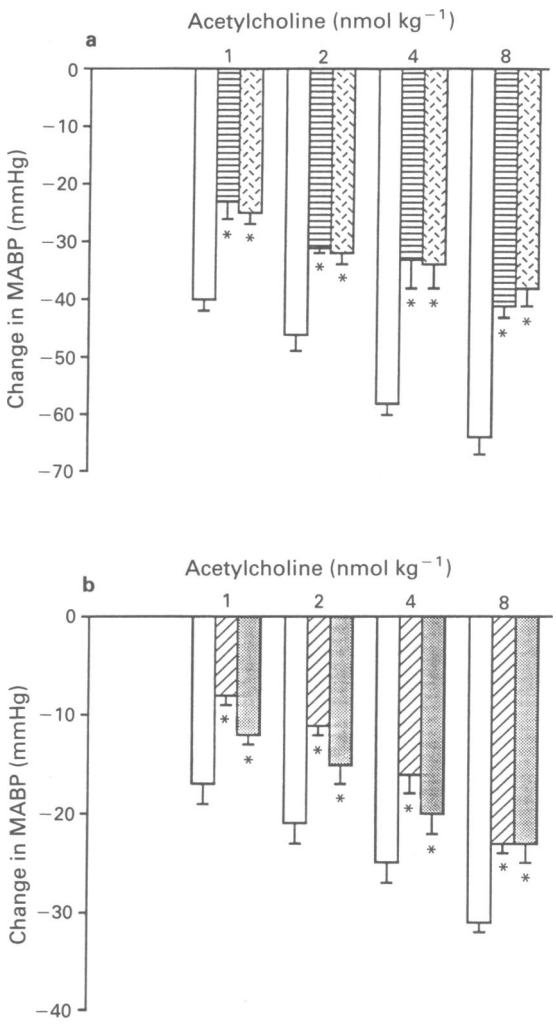


Figure 3 Endothelin-1-induced maximum decrease (open columns) and peak increase (hatched columns) in mean arterial blood pressure (MABP) in conscious rats. (a) The animals were pretreated with N^G-nitro-L-arginine methyl ester (L-NAME, 2 mg kg^{-1}) for 10 min, N^G-monomethyl-L-arginine (L-NMMA, 25 mg kg^{-1}) for 2 min, noradrenaline (NA, 620–820 ng kg^{-1} min^{-1}) for 10 min or 0.9% NaCl (control, C) before bolus i.v. injection of endothelin-1 (ET-1, 1 nmol kg^{-1}). MABP were 110 ± 3 mmHg ($n = 8$), 152 ± 5 mmHg ($n = 8$), 149 ± 4 mmHg ($n = 6$) and 152 ± 3 mmHg ($n = 6$) in control, L-NAME, L-NMMA and noradrenaline-treated animals, respectively, before injection of ET-1. (b) Following injection of L-NAME (2 mg kg^{-1}), the elevated MABP was restored to normotensive levels with diazoxide (90 μmol kg^{-1}) or hydralazine (1.2–1.5 μmol kg^{-1}) before bolus i.v. injection of ET-1 (1 nmol kg^{-1}). MABP were 110 ± 3 mmHg ($n = 6$), 107 ± 2 mmHg ($n = 6$) and 109 ± 5 mmHg ($n = 5$) in control animals receiving vehicle of L-NAME and diazoxide (C) and in rats treated with L-NAME plus diazoxide or L-NAME plus hydralazine, respectively, before injection of ET-1. Values are means with s.e.mean. $*P < 0.05$; $**P < 0.01$ (compared to control by Dunn's multiple contrast hypothesis test).

Figure 4 Hypotensive responses to acetylcholine in conscious rats following treatment with N^G-nitro-L-arginine methyl ester (L-NAME) or N^G-monomethyl-L-arginine (L-NMMA). (a) In control animals, mean arterial blood pressure (MABP) was elevated by an infusion of noradrenaline (620–820 ng kg^{-1} min^{-1}) (open columns) to levels observed following injection of L-NAME (2 mg kg^{-1}) (▤) and L-NMMA (25 mg kg^{-1}) (▩) before i.v. bolus injections of acetylcholine. MABP values were 156 ± 5 mmHg ($n = 10$), 159 ± 4 mmHg ($n = 8$) and 151 ± 8 mmHg ($n = 6$) in noradrenaline, L-NAME and L-NMMA-treated animals, respectively, before injection of the first dose of acetylcholine. (b) In animals treated with L-NAME (2 mg kg^{-1}), the elevated MABP was restored to normotensive levels with diazoxide (90 μmol kg^{-1}) (▨) or hydralazine (1.2–1.5 μmol kg^{-1}) (▩). Control animals (open columns) were given vehicle of L-NAME and diazoxide. MABP values were 111 ± 2 mmHg ($n = 6$), 107 ± 1 mmHg ($n = 4$) and 111 ± 2 mmHg ($n = 6$) in control animals and following L-NAME plus diazoxide and L-NAME plus hydralazine, respectively, before injection of the first dose of acetylcholine. Values are means with s.e.mean. $*P < 0.05$ compared to the control group by Dunn's multiple contrast hypothesis test.

acetylcholine were reduced by 30–50% in animals treated with L-NAME plus hydralazine or L-NAME plus diazoxide relative to the hypotensive effects of acetylcholine in control (untreated) animals (Figure 4b).

Effects of L-NAME on endothelin-1-induced protein extravasation

In agreement with our previous studies, injection of 1 nmol kg^{-1} ET-1 increased tissue Evans blue content in the upper and lower bronchi (Figure 5), stomach, duodenum, kidney and spleen (Figure 6), whereas no significant changes were detected in the trachea and pulmonary parenchyma (Figure 5), pancreas, liver, skeletal muscle and skin (Figures 6 and 7).

Treatment of the animals with L-NAME (2 mg kg^{-1}) resulted in enhanced tissue Evans blue accumulation in all vascular beds studied. The highest increases (up to 260–280%) were detected in the duodenum and pancreas (Figure 6). Tissue Evans blue content increased by 139, 129, 121, 118, 109, 74 and 65% in the kidney, upper and lower bronchi, stomach, liver, trachea and spleen, respectively

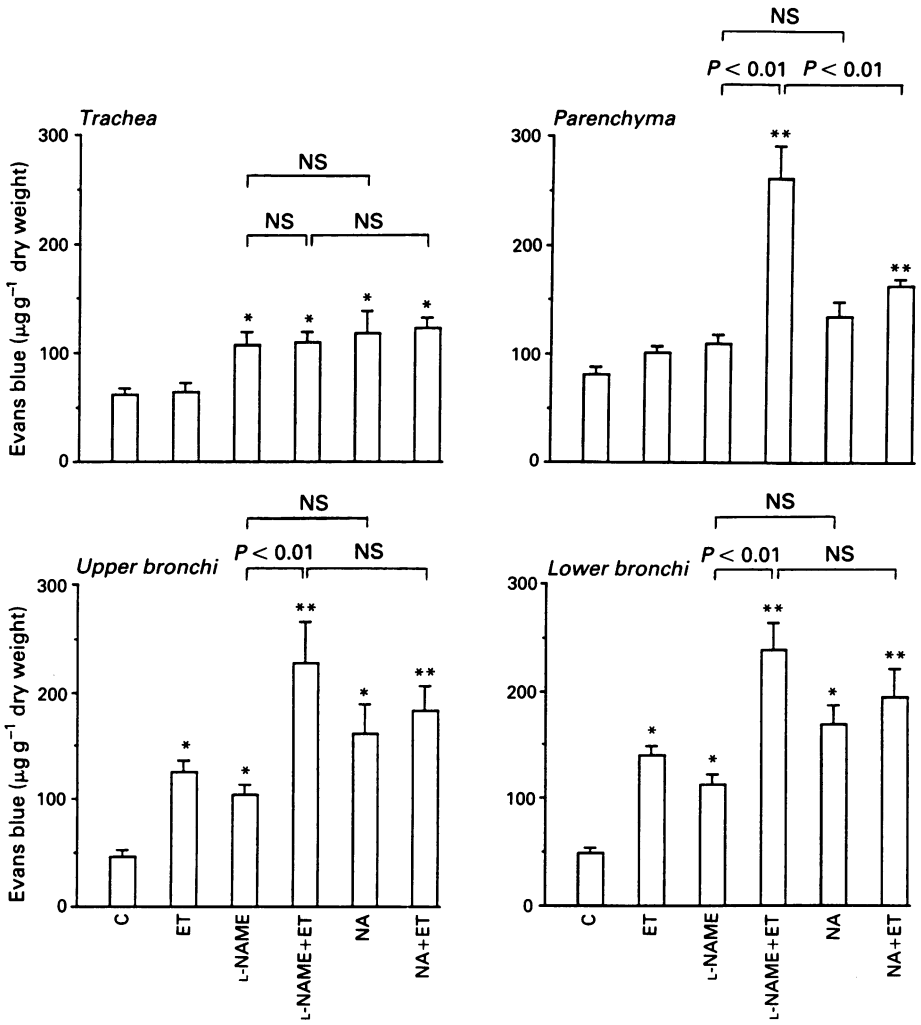


Figure 5 Effects of N^G-nitro-L-arginine methyl ester (L-NAME) and noradrenaline on endothelin-1-induced protein extravasation in rat airways. The animals were pretreated with L-NAME (2 mg kg^{-1}), noradrenaline (NA, 620–820 ng kg^{-1} min^{-1}) or 0.9% NaCl (control, C) for 10 min before bolus i.v. injection of endothelin-1 (ET, 1 nmol kg^{-1}) plus Evans blue dye (20 mg kg^{-1}). The rats were killed 10 min after injection of endothelin-1. Values are means with s.e.mean. $n = 5$ for L-NAME plus endothelin-1, $n = 6$ for all other groups. $^*P<0.05$; $^{**}P<0.01$ (compared to control by Dunn's multiple contrast hypothesis test). NS, not significant.

(Figures 5 and 6). No significant changes were detected in the pulmonary parenchyma, skeletal muscle and skin (Figures 5 and 7). Administration of L-arginine, but not D-arginine (300 mg kg^{-1}) prevented the L-NAME-induced increases in protein extravasation (Table 1). Neither L-arginine nor D-arginine alone affected protein extravasation (data not shown). D-NAME (2 mg kg^{-1}) had no significant effect on Evans blue accumulation in the vascular beds studied (Table 1). Elevation of MABP by infusion of noradrenaline to levels seen following L-NAME did not affect Evans blue accumulation in all organs studied with the exception of the lung (Figures 5, 6 and 7). Noradrenaline infusion and L-NAME treatment evoked similar increases in Evans blue content in the large airways and pulmonary parenchyma (Figure 5).

When ET-1 was administered to rats treated with L-NAME, protein extravasation was markedly enhanced. L-NAME potentiated (up to 550%) the permeability effect of ET-1 in the upper and lower bronchi, stomach and kidney (Figures 5 and 6). A significant increase in protein extravasation was observed in the pulmonary parenchyma following L-NAME plus ET-1, where neither ET-1 nor L-NAME alone affected significantly Evans blue content (Figure 5). In the trachea, duodenum, pancreas and liver, the effects of ET-1 and L-NAME were additive (Figures 5 and 6). No significant changes were detected in the skeletal muscle and skin (Figure 7). Noradrenaline infusion did not modify ET-1-induced Evans blue accumulation in the organs studied with the exception of the lung (Figures 6 and 7). In the upper and lower bronchi, combined administration of noradrenaline plus ET-1 markedly enhanced Evans blue accumulation, albeit tissue Evans blue content was somewhat lower than that seen following L-NAME plus ET-1 (Figure 5). Although combined administration of noradrenaline and ET-1 resulted in a 100% increase in protein extravasation in the parenchyma, tissue Evans blue content was significantly lower than that observed following L-NAME plus ET-1 (Figure 5).

Discussion

The present results show that treatment of the animals with L-arginine analogues attenuates the depressor and potentiates the pressor response to ET-1 and markedly enhances protein extravasation elicited by ET-1 in the airways, kidney and gastrointestinal tract. The findings that the pressor and permeability responses to L-NAME can be reversed by L-arginine, but not D-arginine and the failure of D-NAME to affect blood pressure and protein extravasation are consistent with the notion that the effects of L-NAME result from inhibitory actions on NO synthesis.

In confirmation of earlier observations (Aisaka *et al.*, 1989; Rees *et al.*, 1989; Whittle *et al.*, 1989; Gardiner *et al.*, 1990a;

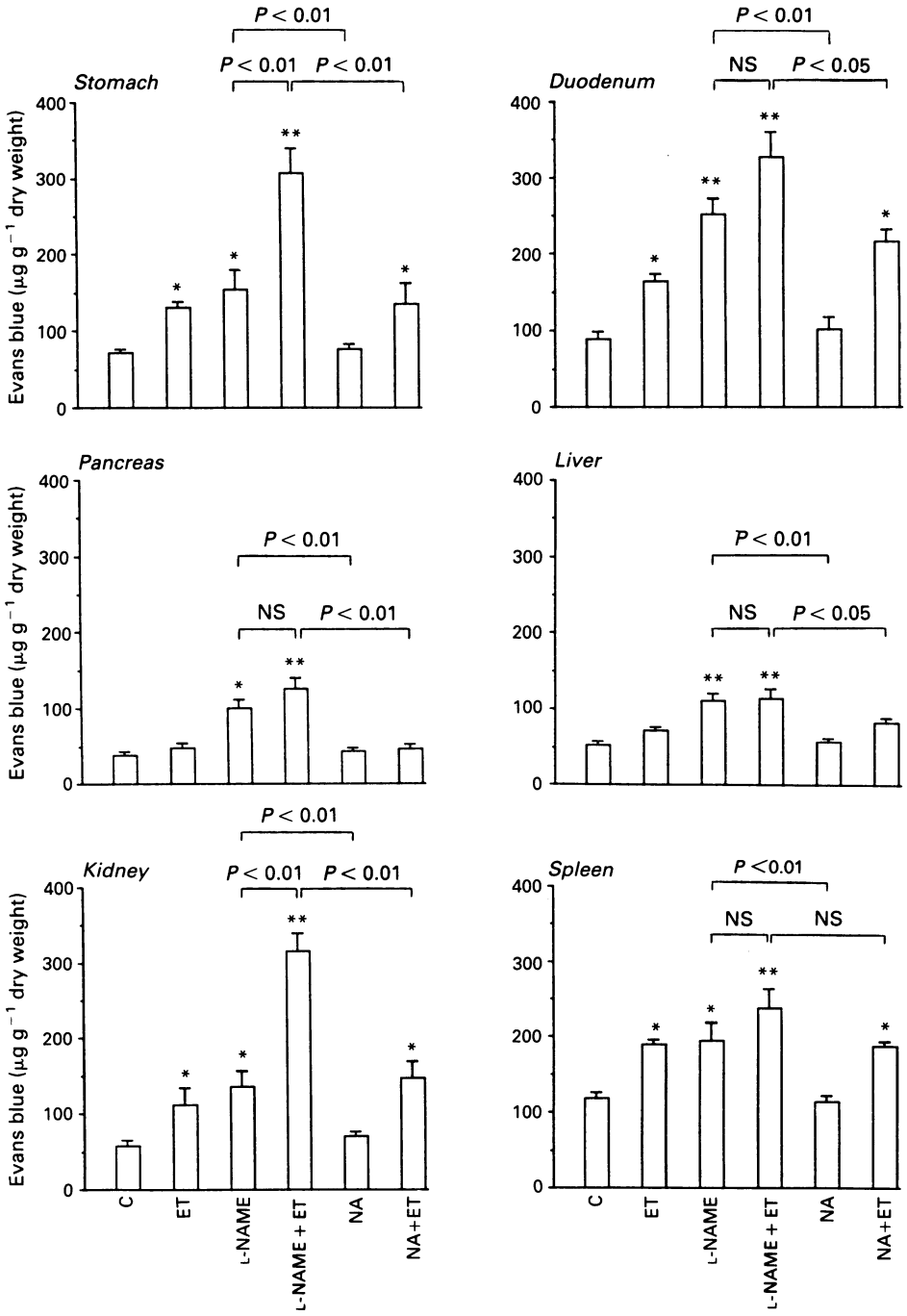


Figure 6 Effects of N^G-nitro-L-arginine methyl ester (L-NAME) and noradrenaline on endothelin-1-induced protein extravasation in rat stomach, duodenum, pancreas, liver, kidney and spleen. The animals were pretreated with L-NAME (2 mg kg^{-1}), noradrenaline (NA, 620–820 ng kg^{-1} min^{-1}) or 0.9% NaCl (control, C) for 10 min before bolus i.v. injection of endothelin-1 (ET, 1 nmol kg^{-1}) plus Evans blue dye (20 mg kg^{-1}). The animals were killed 10 min after injection of endothelin-1. Values are means with s.e.mean. $n = 5$ for L-NAME plus endothelin-1, $n = 6$ for all other groups. $^*P < 0.05$; $^{**}P < 0.01$ (compared to control by Dunn's multiple contrast hypothesis test). NS, not significant.

Hecker *et al.*, 1990; Vargas *et al.*, 1991), the present study also shows a profound increase in MABP following administration of L-NMMA and L-NAME. L-NAME appeared to be approximately 16 times more potent than L-NMMA. The pressor action of L-NMMA was rapid in onset and shorter in duration relative to L-NAME. This might be attributed to rapid metabolization of L-NMMA to L-citrulline and L-arginine by endothelial cells (Hecker *et al.*, 1990). On the other hand, the pressor action of L-NAME developed slowly, but the elevation in MABP remained stable for more than 40 min.

Since an elevation of basal MABP may augment the apparent vasodepressor activity of various substances, such effects of L-arginine analogues on basal MABP may lead to an underestimation of the degree of inhibition of vasodepressor responses. In order to circumvent MABP differences between control and L-NMMA or L-NAME-treated animals, MABP was either elevated in the control group by an infusion of noradrenaline to comparable levels as seen after NO blockade or the elevated MABP was titrated to control (normotensive) levels by diazoxide or hydralazine before injection of ET-1. Under these paradigms, significant attenuation, but not complete inhibition, of ET-1-induced hypotension could be demonstrated. Similar attenuation of the hypotensive effect of ET-1 has been reported in the presence of L-NAME when glyceryl trinitrate (an endo-

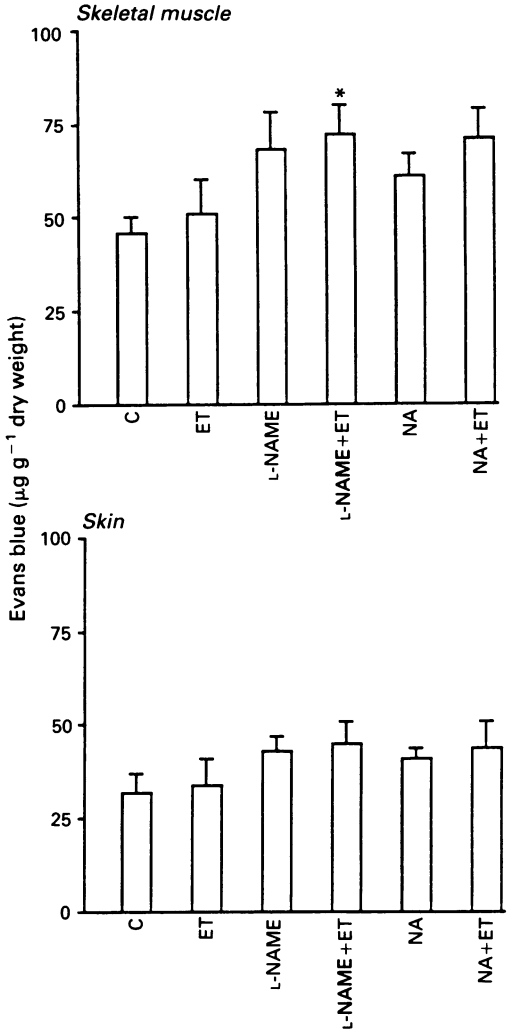


Figure 7 Effects of N^G-nitro-L-arginine methyl ester (L-NAME) and noradrenaline on endothelin-1-induced protein extravasation in rat skeletal muscle and dorsal skin. The animals were pretreated with L-NAME (2 mg kg^{-1}), noradrenaline (NA, 620–820 ng kg^{-1} min^{-1}) or 0.9% NaCl (control, C) for 10 min before bolus i.v. injection of endothelin-1 (ET, 1 nmol kg^{-1}) plus Evans blue dye (20 mg kg^{-1}). The animals were killed 10 min after injection of endothelin-1. Values are means with s.e.mean. $n = 5$ for L-NAME plus endothelin-1, $n = 6$ for all other groups. $^*P < 0.05$ (compared to control by Dunn's multiple contrast hypothesis test).

thelium-independent vasodilator) was used as a reference (Gardiner *et al.*, 1990b). The present study also shows that NO blockade significantly potentiated the pressor response to ET-1 under conditions when the confounding influence of varying basal MABP was avoided. In contrast, L-NMMA-treatment did not lead to a consistent appearance of a vasopressor response to ET-1 in the anaesthetized rat (Whittle *et al.*, 1989). It is not known at present whether this apparent discrepancy might be attributed to the fact that in these latter experiments ET-1 was used at doses which did not evoke a pressor action or to the transient action of L-NMMA (see above). The present findings provide further support to the hypothesis that the vasopressor action of ET-1 is attenuated by NO (De Nucci *et al.*, 1988).

Although *in vitro* experiments suggest unambiguously that NO mediates the vasorelaxant action of acetylcholine (Ignarro *et al.*, 1987; Palmer *et al.*, 1987), studies which sought to correlate the *in vivo* hypotensive action of acetylcholine with NO have been controversial. L-NMMA has been reported to attenuate (Vargas *et al.*, 1991) or to inhibit (Whittle *et al.*, 1989) the hypotensive response to acetylcholine, whereas others did not detect any inhibition (Aisaka *et al.*, 1989; Van Gelderen *et al.*, 1991). Furthermore, L-NAME did not affect the decrease in MABP elicited by acetylcholine relative to glyceryl trinitrate (Gardiner *et al.*, 1990b). However, the different preinjection levels of MABP might also have affected the responses to glyceryl trinitrate. In the present study, attenuation of the hypotensive effects of acetylcholine by both L-NMMA and L-NAME was observed when the effects of acetylcholine were compared either at elevated MABP (i.e. following L-NMMA or L-NAME treatment versus noradrenaline infusion) or at normal MABP (i.e. control animals vs. animals receiving L-NAME plus diazoxide or hydralazine). Since the degree of inhibition was similar under these conditions and never exceeded 50%, it is feasible that a substantial part of the hypotensive response to acetylcholine is independent of NO.

Based on its potency and long duration of action, L-NAME was chosen for the vascular permeability studies. As anticipated, ET-1 (1 nmol kg^{-1}) did evoke significant increases in tissue Evans blue content in the upper and lower bronchi, stomach, duodenum, kidney and spleen. These effects of ET-1 were markedly potentiated by L-NAME (2 mg kg^{-1}), despite the fact that L-NAME by itself also promoted Evans blue accumulation in these tissues. Furthermore, a significant increase in protein extravasation was detected in the pulmonary parenchyma following L-NAME plus ET-1, where both L-NAME and ET-1 alone evoked only a slight, statistically non-significant increase in Evans blue content. The present data suggest that the effects of L-NAME on permeability are not simply a consequence of changes in systemic blood pressure as noradrenaline infusion neither mimicked the effects of L-NAME nor potentiated Evans blue accumulation elicited by ET-1 with the exception of the lung. These findings are consistent with the notion that changes in systemic blood pressure and/or an elevation in capillary hydrostatic pressure are not major determinants of protein extravasation (Grega *et al.*, 1986). Indeed, mediator-stimulated protein extravasation can be primarily attributed to an increase in the hydraulic conductivity of the microvascular membrane secondary to formation of interendothelial cell gaps in the venules (Grega *et al.*, 1986). In the pulmonary circulation, capillary filtration rate increases dramatically when left atrial pressure exceeds 40 cmH_2O (Nicolaysen *et al.*, 1979; Rippe *et al.*, 1984). During the acute phase of generalized vasoconstriction elicited by noradrenaline or L-NAME, there may be significant increases in left atrial end diastolic pressure. Rapid increases in pulmonary perfusion pressure have been reported to cause structural changes (disruption and widening of the endothelial junctions) in the capillaries leading to increases in permeability (Tsukimoto *et al.*, 1990). Large doses of catecholamines (Theodore & Rabin, 1976) or stimulation of sympathetic nerves (Hakim *et al.*, 1981; Sakakibara *et al.*, 1992) produce severe pulmonary oedema with protein-rich oedema fluid. However, the findings that tissue Evans blue content in the pulmonary parenchyma was significantly higher following administration of L-NAME plus ET-1 than following noradrenaline plus ET-1, suggest that an increase in pulmonary pressure might not be the sole mechanism responsible for the permeability enhancing effect of L-NAME. L-NAME treatment did not result in an increase in protein extravasation in the skeletal muscle and skin. One possible explanation is that oedema formation could probably be masked by arterial vasoconstriction elicited by L-NAME. Alternatively, the different susceptibility of different vascular beds to L-NAME may either reflect differences in local NO production or differences in the activity of NO in different regions.

Our results also show that the potentiating effect of NO synthesis inhibition upon ET-1-induced protein extravasation is not due to the haemodynamic effect of L-NAME alone. Furthermore, the enhanced vascular permeability by L-NAME cannot be attributed to an increase in capillary surface area for protein filtration, since vasoconstrictors like

L-NAME generally cause derecruitment of capillaries (Granger *et al.*, 1989). It is likely, however, that inhibition of NO synthesis would lead to endothelial dysfunction (Lieberthal *et al.*, 1989; Hutcheson *et al.*, 1990; Lefer & Ma, 1991). Since NO may scavenge small amounts of superoxide released by endothelial cells (Zweier *et al.*, 1988) and inhibit adhesion of platelets and neutrophil granulocytes to the vascular endothelium (Radomski *et al.*, 1987; Kubes *et al.*, 1991), decreased NO production would result in local accumulation of free radicals and/or adhesion of platelets and neutrophil granulocytes to endothelial cells, which, in turn, would lead to an increase in microvascular permeability (Del Maestro *et al.*, 1981; Kubes & Granger, 1992).

The present observations may have relevance in pathological conditions where plasma and local tissue levels of ET-1 are elevated and endothelial NO production is decreased. In addition to vascular spasm, ET-1-induced plasma extravasation with local oedema formation would induce or predispose to tissue damage. When NO synthesis is decreased, the effects of ET-1 appear to become more dominant, leading to an imbalance of endothelium-dependent regulation of microvascular protein permeability. The findings that NO donors can decrease intestinal tissue damage and attenuate endothelial dysfunction in splanchnic artery occlusion-induced shock (Aoki *et al.*, 1990) lend further support to this hypothesis.

In conclusion, the present data demonstrate that endogenous NO mediates, in part, the vasodepressor effect and attenuates the vasopressor action of ET-1 and counteracts the vascular permeability effect of ET-1. These findings suggest an important role for NO in the regulation of vascular tone and microvascular permeability in conscious rats.

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References

AISAKA, K., GROSS, S.S., GRIFFITH, O.W. & LEVI, R. (1989). L-arginine availability determines the duration of acetylcholine-induced systemic vasodilation in vivo. *Biochem. Biophys. Res. Commun.*, **163**, 710–717.

AOKI, N., JOHNSON, G.III & LEFER, A.M. (1990). Beneficial effects of two forms of NO administration in feline splanchnic artery occlusion shock. *Am. J. Physiol.*, **258**, G275–G281.

BOULANGER, C.M. & LÜSCHER, T.F. (1990). Release of endothelin from the porcine aorta: inhibition by endothelium-derived nitric oxide. *J. Clin. Invest.*, **85**, 587–590.

DAHLÖF, B., GUSTAFFSSON, D., HEDNER, T., JERN, S. & HANSSON, L. (1990). Regional haemodynamic effects of endothelin-1 in rat and man: unexpected adverse reactions. *J. Hypertension*, **8**, 811–817.

DE NUCCI, G., THOMAS, R., D'ORLEANS-JUSTE, P., ANTUNES, E., WALDER, C., WARNER, T.D. & VANE, J.R. (1988). Pressor effects of circulating endothelin are limited by its removal in the pulmonary circulation and by the release of prostacyclin and endothelium-derived relaxing factor. *Proc. Natl. Acad. Sci. U.S.A.*, **85**, 9797–9800.

DEL MAESTRO, R.F., BJORK, J. & ARFORS, K.E. (1981). Increase in microvascular permeability induced by enzymatically generated free radicals. I. In vivo study. *Microvasc. Res.*, **22**, 239–254.

DUNN, O.J. (1964). Multiple comparisons using rank sums. *Technometrics*, **6**, 241–252.

FILEP, J., FÖLDES-FILEP, E. & FRÖLICH, J.C. (1987). Vascular responses to leukotrienes B_4, C_4 and D_4 following FPL 55712, indomethacin, saralasin, phentolamine and verapamil in the conscious rat. *Br. J. Pharmacol.*, **90**, 431–439.

FILEP, J.G., FÖLDES-FILEP, E., ROUSSEAU, A., FOURNIER, A., SIROIS, P. & YANO, M. (1992). Endothelin-1 enhances vascular permeability in the rat heart through the ET_A receptor. *Eur. J. Pharmacol.*, **219**, 343–344.

FILEP, J.G., FÖLDES-FILEP, E. & SIROIS, P. (1993). Nitric oxide modulates vascular permeability in the rat coronary circulation. *Br. J. Pharmacol.*, **108**, 323–326.

FILEP, J.G., SIROIS, M.G., ROUSSEAU, A., FOURNIER, A. & SIROIS, P. (1991). Effects of endothelin-1 on vascular permeability in the conscious rat: interactions with platelet-activating factor. *Br. J. Pharmacol.*, **104**, 797–804.

FOZARD, J.R. & PART, M.L. (1992). The role of nitric oxide in the regional vasodilator effects of endothelin-1 in the rat. *Br. J. Pharmacol.*, **105**, 744–750.

FURCHGOTT, R.F. (1984). The role of endothelium in the responses of vascular smooth muscle to drugs. *Annu. Rev. Pharmacol. Toxicol.*, **24**, 175–197.

FURCHGOTT, R.F. & VANHOUTTE, P.M. (1989). Endothelium-derived relaxing and contracting factors. *FASEB J.*, **3**, 2007–2018.

GARDINER, S.M., COMPTON, A.M., BENNETT, T., PALMER, R.M.J. & MONCADA, S. (1989). N^G-monomethyl-L-arginine does not inhibit the hindquarters vasodilator action of endothelin-1 in conscious rats. *Eur. J. Pharmacol.*, **171**, 237–240.

GARDINER, S.M., COMPTON, A.M., BENNETT, T., PALMER, R.M.J. & MONCADA, S. (1990a). Control of regional blood flow by endothelium-derived nitric oxide. *Hypertension*, **15**, 486–492.

GARDINER, S.M., COMPTON, A.M., KEMP, P.A. & BENNETT, T. (1990b). Regional and cardiac haemodynamic responses to glycerol trinitrate, acetylcholine, bradykinin and endothelin-1 in conscious rats: effects of N^G-nitro-L-arginine methyl ester. *Br. J. Pharmacol.*, **101**, 632–639.

GOETZ, K.L., WANG, B., MADWED, J.B., ZHU, J.L. & LEADLEY, R.J.Jr. (1988). Cardiovascular, renal and endocrine responses to intravenous endothelin in conscious dogs. *Am. J. Physiol.*, **255**, R1064–R1068.

GRANGER, D.N., KVIETYS, P.R., KORTHUIS, R.J. & PREMEN, A.J. (1989). Microcirculation of the intestinal mucosa. In *Handbook of Physiology. The Gastrointestinal System, Motility and Circulation.* Vol. 1 pp. 1405–1474, Bethesda, M.D.: The Am. Physiol. Soc.

GREGA, G.J., ADAMSKI, S.W. & DOBBINS, D.E. (1986). Physiological and pharmacological evidence for the regulation of permeability. *Fed. Proc.*, **45**, 96–100.

HAKIM, T.S., MINNEAR, F.L., VAN DER ZEE, H., BARIC, P.S. & MALIK, A.B. (1981). Adrenoceptor control of lung fluid and protein exchange. *J. Appl. Physiol.*, **51**, 68–72.

HECKER, M., MITCHELL, J.A., HARRIS, H.J., KATSURA, M., THIEMERMANN, C. & VANE, J.R. (1990). Endothelial cells metabolize N^G-methyl-L-arginine to L-citrulline and subsequently to L-arginine. *Biochem. Biophys. Res. Commun.*, **167**, 1037–1043.

HUTCHESON, I.R., WHITTLE, B.J.R. & BOUGHTON-SMITH, N.K. (1990). Role of nitric oxide in maintaining vascular integrity in endotoxin-induced acute intestinal damage in the rat. *Br. J. Pharmacol.*, **101**, 815–820.

IGNARRO, L.J., BUGA, G.M., WOOD, K.S., BYRNS, R.E. & CHAUDHURI, G. (1987). Endothelium-derived relaxing factor produced and released by artery and vein is nitric oxide. *Proc. Natl. Acad. Sci. U.S.A.*, **84**, 9265–9269.

KUBES, P. & GRANGER, D.N. (1992). Nitric oxide modulates microvascular permeability. *Am. J. Physiol.*, **262**, H611–H615.

KUBES, P., SUZUKI, N. & GRANGER, D.N. (1991). Nitric oxide: an endogenous modulator of leukocyte adhesion. *Proc. Natl. Acad. Sci. U.S.A.*, **88**, 4651–4655.

LEFER, A.M. & MA, X.L. (1991). Endothelial dysfunction in the splanchnic circulation following ischemia and reperfusion. *J. Cardiovasc. Pharmacol.*, **17** (Suppl. 3), S186–S190.

LIEBERTHAL, W., WOLF, E.F., RENNKE, H.G., VALERI, C.R. & LEVINSKY, N.G. (1989). Renal ischemia and reperfusion impair endothelium-dependent vascular relaxation. *Am. J. Physiol.*, **256**, F894–F900.

LÓPEZ-FARRÉ, A., MONTANES, I., MILLÁS, I. & LÓPEZ-NOVOA, J.M. (1989). Effect of endothelin on renal function in rats. *Eur. J. Pharmacol.*, **163**, 187–191.

MOORE, P.K., AL-SWAYEH, O.A., CHONG, N.W.S., EVANS, R.A. & GIBSON, A. (1990). N^G-nitro-L-arginine, a novel, L-arginine-reversible inhibitor of endothelium-dependent vasodilation *in vitro*. *Br. J. Pharmacol.*, **99**, 408–412.

NICOLAYSEN, G., WAALER, B.A. & AARSETH, P. (1979). On the existance of stretchable pores in the exchange vessels of the isolated rabbit lung preparation. *Lymphology*, **12**, 201–207.

PALMER, R.M.J., ASHTON, D.S. & MONCADA, S. (1988a). Vascular endothelial cells synthesize nitric oxide from L-arginine. *Nature*, **333**, 664–666.

PALMER, R.M.J., FERRIGE, A.G. & MONCADA, S. (1987). Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature*, **327**, 524–526.

PALMER, R.M.J., REES, D.P., ASHTON, D.D. & MONCADA, S. (1988b). L-arginine is the physiological precursor for the formation of nitric oxide in endothelium-dependent relaxation. *Biochem. Biophys. Res. Commun.*, **153**, 1251–1256.

RADOMSKI, M.W., PALMER, R.M.J. & MONCADA, S. (1987). Endogenous nitric oxide inhibits human platelet adhesion to vascular endothelium. *Lancet*, **ii**, 1057–1058.

RAWSON, R.A. (1943). The binding of T-1824 and structurally related diazo dyes by the plasma proteins. *Am. J. Physiol.*, **138**, 708–717.

REES, D.D., PALMER, R.M.J. & MONCADA, S. (1989). Role of endothelium-derived nitric oxide in the regulation of blood pressure. *Proc. Natl. Acad. Sci. U.S.A.*, **86**, 3375–3378.

REES, D.D., PALMER, R.M.J., SCHULZ, R., HODSON, H.F. & MONCADA, S. (1990). Characterization of three inhibitors of endothelial nitric oxide synthase *in vitro* and *in vivo*. *Br. J. Pharmacol.*, **101**, 746–752.

RIPPE, B., TOWNSLEY, M., THIGPEN, J., PARKER, J.C., KORTHUIS, R.J. & TAYLOR, A.E. (1984). Effects of vascular pressure on the pulmonary microvasculature in isolated dog lungs. *J. Appl. Physiol.*, **57**, 233–239.

SAIJONMAA, O., RISTIMAKI, A. & FYHRQUIST, F. (1990). Atrial natriuretic peptide, nitroglycerine, and nitroprusside reduce basal and stimulated endothelin production from cultured endothelial cells. *Biochem. Biophys. Res. Commun.*, **173**, 514–520.

SAKAKIBARA, H., HASHIBA, Y., TAKI, K., KAWANISHI, M., SHIMADA, Y. & ISHIKAWA, N. (1992). Effect of sympathetic nerve stimulation on lung vascular permeability in the rat. *Am. Rev. Respir. Dis.*, **145**, 685–692.

SAKUMA, I., STUEHR, D.J., GROSS, S.S., NATHAN, C. & LEVI, R. (1988). Identification of arginine as a precursor of endothelium-derived relaxing factor. *Proc. Natl. Acad. Sci. U.S.A.*, **85**, 8664–8667.

THEODORE, J. & ROBIN, E.D. (1976). Speculations on neurogenic pulmonary edema (NPE). *Am. Rev. Respir. Dis.*, **113**, 405–411.

TSUKIMOTO, K., MATHIEU-COSTELLO, D., PREDILETTO, R. & WEST, J.B. (1990). Structural basis of increased permeability of pulmonary capillaries with high transmural pressures. *Am. Rev. Respir. Dis.*, **141**, A297.

VAN GELDEREN, E.M., HEILIGERS, J.P.C. & SAXENA, P.R. (1991). Haemodynamic changes and acetylcholine-induced hypotensive responses after N^G-nitro-L-arginine methyl ester in rats and cats. *Br. J. Pharmacol.*, **103**, 1899–1904.

VARGAS, H.M., CUEVAS, J.M., IGNARRO, L.J. & CHAUDHURI, G. (1991). Comparison of the inhibitory potencies of N^G-methyl-, N^G-nitro- and N^G-amino-L-arginine on EDRF function in the rat: evidence for continuous basal EDRF release. *J. Pharmacol. Exp. Ther.*, **257**, 1208–1215.

WHITTLE, B.J.R., LOPEZ-BELMONTE, J. & REES, D.D. (1989). Modulation of the vasodepressor actions of acetylcholine, bradykinin, substance P and endothelin in the rat by a specific inhibitor of nitric oxide formation. *Br. J. Pharmacol.*, **98**, 646–652.

YANAGISAWA, M., KURIHARA, H., KIMURA, S., TOMOBE, Y., KOBAYASHI, M., MITSUI, Y., YAZAKI, Y., GOTO, K. & MASAKI, T. (1988). A novel potent vasoconstrictor peptide produced by vascular endothelial cells. *Nature*, **332**, 411–415.

ZIMMERMAN, R.S., MARTINEZ, A.J., MAYMIND, H. & BARBEE, R.W. (1992). Effect of endothelin on plasma volume and albumin escape. *Circ. Res.*, **70**, 1027–1034.

ZWEIER, J.L., KUPPUSAMY, P. & LUTTY, G.A. (1988). Measurement of endothelial cell free radical generation: evidence for a central mechanism of free radical injury in postischemic tissues. *Proc. Natl. Acad. Sci. U.S.A.*, **85**, 4046–4050.

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