

## INFLUENCE OF ENDOTHELIUM REMOVAL AND L-NAME ON RESPONSES OF RAT COMMON CAROTID ARTERY TO $\alpha$ -ADRENOCEPTOR AGONISTS

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### ABSTRACT

In this study we investigated the effects of endothelium removal and L-NAME on responses to  $\alpha$ -adrenoceptor agonists. Male Wistar rats were killed by overdose with pentobarbitone sodium, after which the left and right common carotid arteries were removed. Rings of arteries 3-4 mm in length were cut from each vessel and then mounted in 10 mL isolated organ bath, bathed in Krebs maintained at 37°C and gassed with 95% O<sub>2</sub> plus 5% CO<sub>2</sub>. The preparations were allowed to equilibrate for an hour. L-NAME was added approximately 10-15 min prior to the onset of cumulative concentration-response curves (CCRC) to an agonist. In some preparations the endothelial layer was removed mechanically by gently rolling the tissue around a thin wire. Removal of the endothelium was confirmed pharmacologically by a lack of relaxant response to the potent endothelium-dependent vasodilator acetylcholine. Inhibition of NO synthesis by L-NAME results in significant vasoconstriction. L-NAME prevented the relaxation of rat carotid artery by acetylcholine, suggesting that both basal and stimulated release of nitric oxide can regulate vascular tone in this artery. Mechanical disruption of the vascular endothelium reduced, but did not abolish, the ability of L-NAME to produce contraction. This suggests an extra-endothelial site for nitric oxide synthesis in rat common carotid artery. Inhibition of nitric oxide synthase with L-NAME potentiated responses to phenylephrine and UK-14304 but not to noradrenaline. Mechanical disruption of the vascular endothelium potentiated responses to UK-14304, phenylephrine and noradrenaline. We suggest that constitutive NO activity has substantial inhibitory influence on vasoconstrictor responses to phenylephrine and UK-14304 but not to noradrenaline.

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### INTRODUCTION

Prostacyclin (PGI<sub>2</sub>), a potent vasorelaxant, was the first endothelium-derived vasoactive substance discovered in

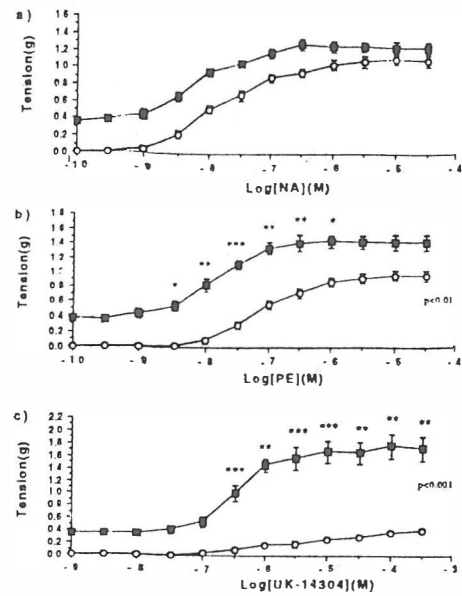
the late 1970s.<sup>1</sup> Later it was demonstrated that the vascular relaxation induced by acetylcholine was dependent on the presence of the endothelium.<sup>2</sup> Furchgott and Zawadzki observed that acetylcholine relaxed rings or strips of rabbit

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aorta *in vitro*, but only if endothelium cells were present in the preparation.<sup>2</sup> Later Furchgott (1983) provided evidence that this effect was mediated by a labile non-prostanoid humoral factor, known as EDRF (endothelium-derived relaxing factor).<sup>3</sup> Based on the similarities in the pharmacological behaviour of EDRF and NO generated from acidified NO<sub>2</sub>, it was suggested in 1987 that EDRF may be NO.<sup>4</sup> Endothelium-dependent relaxation occurs in response to a variety of substances including platelet-derived products, such as adenosine nucleotides (ATP, ADP). Other numerous compounds, such as substance P and histamine can release EDRF.<sup>5,6</sup> In blood vessels, two major classes of NO synthase (NOS) activities have been described. One isoform is constitutively expressed in the endothelium under basal conditions and involved in the endothelium-dependent vasodilating response. The other NOS isoform is inducibly expressed, and it has been found in cytokine-treated vessels. Whereas activation of constitutive NOS generates small amounts of NO for short periods of time, inducible NOS stimulation results in a delayed and prolonged release of large amounts of NO. The vascular endothelial cells possess both the constitutive and inducible NO synthases.<sup>7</sup> The production of NO can be stimulated by several agonists acting on different cell-surface receptors and using distinct intracellular signal transduction pathways. NO is not stored and diffuses freely from its site of formation, whereas classical mediators are frequently stored in granules and released specifically. It is rapidly metabolized to nitrite and nitrate in the presence of oxygen with a half-life of approximately 3-5 seconds; the half-life is shortened by the presence of free radicals such as superoxide anion and is, thus, prolonged by free radical scavengers such as superoxide dismutase. It is soluble both in water (up to 2 mmol/L at 20°C and one atmosphere) and lipid.<sup>8</sup> Several analogues of L-arginine are inhibitors of vascular nitric oxide (NO) synthase. They inhibit NO synthase in an enantiomerically specific manner and act as competitive inhibitors of all three isozymes of NOS and, thus, have been used extensively in investigations of NO metabolism. NG-monomethyl-L-arginine (L-NMMA), N-iminoethyl-L-ornithine (L-NIO) and N<sup>ω</sup>-nitro-L-arginine methylester (L-NAME) are inhibitors of NO synthase in the vascular endothelium *in vitro* and *in vivo*.<sup>9,10</sup>

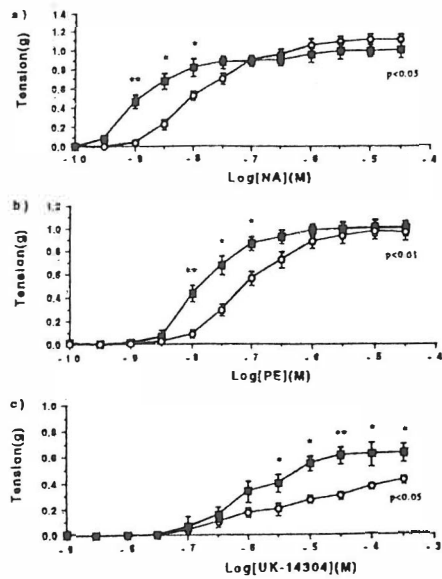
### MATERIALS AND METHODS

Common carotid arteries (700 μm in lumen diameter) were obtained from male Wistar rats, weighing 320-400g, which were killed by overdose with pentobarbitone sodium (i.p. injection). Although the carotid artery is smaller than the aorta, a pair of common carotid arteries were easily dissected out and were placed in cold, oxygenated modified Krebs-Henseleit solution (Krebs). The arteries were cleaned of any extraneous connective tissue using fine scissors. Each preparation was cut transversely in to 3-4 mm rings



**Fig. 1.** Effects of L-NAME (100 μM) (■) in the preparations with intact endothelium on control (O) CCRC to α-adrenoceptor agonists; a) noradrenaline (NA), b) phenylephrine (PE), c) UK-14304 in the rat isolated common carotid artery. Results are expressed as tension (g). Each point represents mean ± s.e. mean (n=10)

and suspended between thick wire supports. During the preparation of the arterial ring segments, any contact with the luminal surfaces was avoided to preserve endothelial integrity. In some preparations the endothelial layer was removed mechanically by gently rolling the tissue around a thin wire. Removal of the endothelium was confirmed pharmacologically by a lack of relaxant response to the potent endothelium-dependent vasodilator acetylcholine. This ensured that endothelium removal had been successful. Fluorescent dyes were also used to examine histologically the structure of rubbed endothelial cells in some random preparations.<sup>11</sup> Each ring was suspended horizontally by means of two stainless-steel L-shaped hooks carefully passed through the lumen. The upper support was connected by cotton to an isometric transducer while the lower support was connected to a glass tissue holder. The arterial rings were mounted in 10 mL isolated organ bath, bathed in Krebs maintained at 37°C and gassed with 95% O<sub>2</sub> plus 5% CO<sub>2</sub>. The rings were then placed under resting tension at 2.5-3g for each group of arterial rings of carotid artery. Isometric contractions were measured by a Grass FTO3 transducer connected to a Linseis (TYP 7208) pen recorder. In all experiments, tissues were left to equilibrate for a 60 min period, during which time the tension was re-adjusted to a set value which was maintained constant throughout the rest of the experimental day. Each preparation was then exposed to noradrenaline (1 μM) and allowed to contract for 5-10 min. This first contraction to an agonist minimizes changes



**Fig. 2.** Effects of removing the endothelium (■) on cumulative concentration-response curves to  $\alpha$ -adrenoceptor agonists in preparations with endothelium (○); a) noradrenaline (NA), b) phenylephrine (PE), c) UK-14304 in the rat isolated common carotid artery. Results are expressed as tension (g). Each point represents mean  $\pm$  s.e. mean ( $n=10$ ). Statistically significant differences are represented by \*  $p<0.05$  and \*\*  $0.001 < p<0.01$  (unpaired Student's t-test). Value of  $p$  shows comparison of two curves by one way ANOVA.

in the sensitivity of preparations to further addition of agonists. Following complete washout, an additional one hour equilibration period was allowed before commencement of any other experimental procedure. Cumulative concentration-response curves (CCRC) were constructed in a cumulative manner by increasing the concentration of the agonists in half-log increments. An initial control CCRC, to

**Table I.** The effect of N<sup>o</sup>-nitro-L-arginine methylester (L-NAME) on responses to  $\alpha$ -adrenoceptor agonists in the rat isolated common carotid artery.

Agonist	Effect of L-NAME	$pD_2$	n	Maximum response(g)
Noradrenaline	Control	$7.89 \pm 0.11$	12	$1.13 \pm 0.035$
	+L-NAME	$8.07 \pm 0.1$	12	$0.88 \pm 0.024$
Phenylephrine	Control	$7.03 \pm 0.09$	10	$0.98 \pm 0.021$
	+L-NAME	$8.02 \pm 0.1^*$	10	$1.15 \pm 0.014$
UK-14304	Control	$5.06 \pm 0.08$	12	$0.44 \pm 0.018$
	+L-NAME	$6.63 \pm 0.12$	12	$1.45 \pm 0.08$

$pD_2$  is expressed as the log of the  $EC_{50}$  (concentration producing 50% of the maximum response). n Shows the number of animals. Data are expressed as mean  $\pm$  s.e. mean. Statistical comparisons with controls were carried out using unpaired Student's t test. \* $p<0.05$ .

any given agonist, was obtained in each preparation. Following attainment of the maximal control contraction, preparations were washed until complete relaxation was effected. The preparations were then left for a further period of 45-60 min before re-exposure to the agonist. L-NAME was added approximately 10-15 min prior to the onset of CCRC to an agonist. In order to examine the effect of L-arginine (300  $\mu$ M) it was added nearly 20-30 min prior to the onset of CCRC to an agonist. Results are expressed as mean  $\pm$  standard error of mean. Comparisons between the two groups were performed using the paired or unpaired Student's t-test with values as follow: \*  $p<0.05$ , \*\*  $0.001 < p<0.01$ , \*\*\*  $p<0.001$ . Comparisons among several groups were performed using one-way analysis of variance. A value of  $p<0.05$  was taken as statistically significant.

### Solutions and drugs

The composition of the modified Krebs-Henselite solution was as follows (in mM): NaCl 118.4, NaHCO<sub>3</sub> 25, KCl 4.7, KH<sub>2</sub>PO<sub>4</sub> 1.6, MgSO<sub>4</sub> 0.6, CaCl<sub>2</sub> 2.5 and glucose 11. Na<sub>2</sub>EDTA (23  $\mu$ M) was also included in the Krebs solution in all experiments to prevent degradative oxidation of NA, and propranolol (1  $\mu$ M) and cocaine hydrochloride (10  $\mu$ M) were also included to inhibit  $\beta$ -adrenoceptors and neuronal uptake of NA, respectively. The following compounds were used: UK-14304 (Pfizer); (-)-phenylephrine HCl (Sigma); (-)-noradrenaline bitartrate (Sigma); propranolol HCl (Sigma); cocaine HCl (MacCarthys); L-NAME (Sigma); L-arginine (Sigma). All drugs were dissolved in distilled water. All concentrations of the drugs used are expressed as final concentration in the organ bath.

### RESULTS

L-NAME itself in unrubbed preparations caused a large sustained increase in vascular tone that was  $58 \pm 9.5$  % of the contraction to noradrenaline (NA) (1  $\mu$ M). L-Arginine (300  $\mu$ M) totally reversed the effects of L-NAME but had no effect on its own. Adding L-NAME (100  $\mu$ M) resulted in a small reduction of the "maximum" response to NA.  $pD_2$  was not changed significantly, 8.07 compared with 7.89 (Table I, Fig.1). Adding L-NAME (100  $\mu$ M) resulted in increase of the maximum response and sensitivity to phenylephrine (PE).  $pD_2$  was significantly increased to 8.02 compared with 7.03 ( $p<0.05$ ) (Table I, Fig. 1). L-NAME increased the response and the potency of UK-14304 as well as increasing the maximum tension that it was able to achieve in these vessels by 336% of control absolute maximum tensions.  $pD_2$  increased to 6.63 compared with 5.06 ( $p<0.01$ ) (Table I). Surprisingly, mechanical disruption of the vascular endothelium reduced, but did not abolish, the ability of L-NAME to produce contraction. Addition of L-NAME to rubbed preparations produced a significantly smaller

**Table II.** The effect of mechanical disruption of the vascular endothelium on responses to  $\alpha$ -adrenoceptor agonists in the rat isolated common carotid artery.

Agonist	Endothelium	pD <sub>2</sub>	n	Maximum response(g)
Noradrenaline	Present	7.85 ± 0.14	8	1.1 ± 0.01
	Absent	8.68 ± 0.1*	8	1 ± 0.03
Phenylephrine	Present	7.1 ± 0.06	8	0.96 ± 0.013
	Absent	7.83 ± 0.04*	8	1 ± 0.021
UK-14304	Present	5.18 ± 0.07	8	0.41 ± 0.03
	Absent	5.76 ± 0.09	8	0.62 ± 0.052

pD<sub>2</sub> is expressed as the log of the EC<sub>50</sub> (concentration producing 50% of the maximum response). n Shows the number of animals. Data are expressed as mean ± s.e. mean. Statistical comparisons with controls were carried out using unpaired Student's t test. \**p* < 0.05.

contraction than in unrubbed preparations (20 ± 6% of NA 1  $\mu$ M). In some preparations the endothelial layer was removed mechanically by gently rolling the tissue around a thin wire. Removing the endothelium increased responses to NA at low concentrations, but the maximum response was not changed. pD<sub>2</sub> increased to 8.68 compared with 7.85 (*p* < 0.05) (Table II). Removing the endothelium increased responses to PE at low concentrations, and pD<sub>2</sub> increased to 7.83 compared with 7.1 (*p* < 0.05) (Table II, Fig. 2). In preparations with rubbed endothelium the maximum response to UK-14304 increased. pD<sub>2</sub> increased to 5.76 compared with 5.06 (*p* < 0.05) (Table II, Fig. 2).

## DISCUSSION

The role of NO in regulation of vascular tone in the rat common carotid artery has been demonstrated by showing that inhibition of its synthesis by L-NAME results in significant vasoconstriction. L-NAME prevented the relaxation of rat carotid artery by the endothelium dependent vasodilator acetylcholine. As reported by Rees and co-workers<sup>12</sup> inhibition of NO can induce an endothelium-dependent and enantiomerically specific contraction of the vascular rings, confirming that there is a continuous use for L-arginine for the basal release of NO. The magnitude of this basal release can be determined indirectly by measuring the degree of contraction.<sup>13</sup> In our study L-arginine (300  $\mu$ M), which is a substrate for EDRF,<sup>3</sup> totally reversed the effects of L-NAME but had no effect on its own. Mechanical disruption of the vascular endothelium reduced, but did not abolish, the ability of L-NAME to produce contraction. This suggests an extra-endothelial site for nitric oxide synthesis in rat common carotid artery. One site could be the vascular

smooth muscle cell, as already suggested for rabbit pulmonary artery.<sup>14</sup> In rabbit pulmonary artery, removal of the vascular endothelium increased the maximum contractile responses to electrical field-stimulation (EFS) but did not inhibit the ability of L-NAME to potentiate contractile responses to EFS. Other sites of synthesis could be cells in the adventitia.<sup>15</sup> However, the role of extraendothelial NO in regulation of tone in blood vessels is unclear. In the present study, the effect of UK-14304 was significantly enhanced in the presence of L-NAME. Mechanical disruption of the vascular endothelium mimicked the effect of L-NAME on contractile responses to UK-14304 and phenylephrine, consistent with L-NAME inhibiting endothelium-derived nitric oxide synthase. In agreement with this study, it was reported that removal of the endothelium either by balloon catheter or mechanically by a small metal wire in rat carotid artery results in an increase of the contractions evoked by phenylephrine.<sup>16</sup> The results show that inhibition of nitric oxide synthase with L-NAME potentiates responses to phenylephrine and UK-14304 in rat common carotid artery. In the case of noradrenaline, L-NAME did not increase sensitivity to NA, and mechanical disruption increases the potency of noradrenaline without effect on maximum response. L-NAME has been widely used as a specific NO synthesis inhibitor at the dose used in the present study.<sup>13,16</sup> The vascular endothelium produces and releases many other factors such as the relaxant prostacyclin and contractile factor endothelin.<sup>17,18</sup> As the response to noradrenaline was augmented by endothelium disruption, but not by L-NAME, this could imply that endothelial cell-derived vasoactive factors other than NO also exert effects on adrenoceptor activation. It should be noted that alkyl esters of L-arginine, such as L-NAME, are antagonists of muscarinic cholinergic receptors which may have some effects on other receptors.<sup>19</sup> We know that noradrenaline stimulates both postjunctional and prejunctional  $\alpha_1$ - and  $\alpha_2$ -adrenoceptors, but phenylephrine only stimulates postjunctional  $\alpha_1$ - and  $\alpha_2$ -adrenoceptors. UK-14304 is a full agonist of  $\alpha_2$ -adrenoceptors in various pharmacological preparations.<sup>20</sup> This effect of L-NAME on noradrenaline is in contrast to other reports.<sup>17,21</sup>

There are several explanations for the quantitative differences in the effectiveness of endothelium removal and NO inhibition in the isolated rat carotid artery on  $\alpha$ -adrenoceptor agonists. One main explanation is that each agonist used in this study stimulates different postjunctional  $\alpha$ -adrenoceptors<sup>20</sup> and inhibition of the basal release of nitric oxide by L-NAME or endothelium removal can have different effects. Another explanation is that differences in responses to  $\alpha$ -adrenoceptor agonists can arise from a variety of factors, e.g. a concomitant stimulatory action of these factors on smooth muscle, and the release of different endothelium-dependent vasoconstrictor and vasodilator substances.

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