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# Effect of clenbuterol on non-endothelial nitric oxide release in rat mesenteric arteries and the involvement of $\beta$-adrenoceptors

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**1** The aim of the present study was to explore the contribution of adrenergic, sensory and nitrergic innervations to the inhibitory effects of the $\beta_2$-adrenoceptor agonist clenbuterol on responses to electrical field stimulation (EFS, 200 mA, 0.3 ms, 1–16 Hz, for 30 s, at 1 min interval) in rat mesenteric artery segments without endothelium and the possible involvement of adrenergic, sensory and nitrergic innervations.

**2** Clenbuterol (1 $\mu$M) reduced EFS-induced contractile responses, and this effect was reversed by the $\beta$-antagonist propranolol (1 $\mu$M) (contraction at 16 Hz expressed as % of 75 mM $K^+$-induced contraction was: control, $69\pm9$, clenbuterol, $31\pm6$, $n=13$, $P<0.001$; control, $83\pm5$, clenbuterol + propranolol $70\pm7$, $n=11$, $P>0.05$).

**3** In arteries preincubated with [$^3$H]-noradrenaline (NA), clenbuterol did not modify the tritium overflow evoked by EFS (200 mA, 0.3 ms, 4 Hz, for 60 s; ratio between tritium release in the second and first stimuli was: control, $0.80\pm0.05$ and clenbuterol added before second stimulus, $0.91\pm0.11$, $n=5$, $P>0.05$).

**4** The nitric oxide (NO) synthase inhibitors $N^G$-monomethyl-L-arginine (L-NMMA) and $N^G$-nitro-L-arginine methyl ester (L-NAME) (10 and 100 $\mu$M), and the guanylate cyclase inhibitor methylene blue (10 $\mu$M) increased the contractions caused by EFS (% contraction at 16 Hz, control, $81\pm7$, $n=26$; 10 $\mu$M L-NMMA, $109\pm12$, $n=8$, $P<0.05$; methylene blue, $119\pm6$, $n=6$, $P<0.05$). However, these contractions were decreased by the NO synthase substrate L-arginine 10 $\mu$M ($14\pm6\%$, $n=6$, $P<0.001$), but not modified by either the sensory neurones toxin capsaicin (0.5 $\mu$M, $75\pm6\%$, $n=6$, $P>0.05$) or the protein synthesis inhibitor cycloheximide (10 $\mu$M, $83\pm6\%$, $n=8$, $P>0.05$). None of these drugs altered the concentration-response curves to exogenous NA ($n=7$).

**5** Pretreatment with capsaicin or cycloheximide did not modify the reduction of the EFS-evoked contraction provoked by clenbuterol. However the presence of L-NMMA (or L-NAME) or methylene blue did decrease the effect of clenbuterol (% contraction at 16 Hz, clenbuterol, $31\pm6$, $n=13$; clenbuterol + 10 $\mu$M L-NMMA, $93\pm11$, $n=8$, $P<0.05$; clenbuterol + methylene blue, $90\pm7$, $n=6$, $P<0.05$).

**6** These results suggest that the reduction caused by clenbuterol in the contraction induced by EFS in rat mesenteric arteries seems to be mediated by NO release, through the activation of $\beta_2$-adrenoceptors probably present on nitrergic nerves.

**Keywords:** Rat mesenteric arteries; electrical field stimulation; clenbuterol; nitrergic innervation

## Introduction

Vascular tone is regulated by several mechanisms in which the vessel innervation plays a more or less important role depending on the type of the vessel (Vanhoutte *et al*., 1981; Marco *et al*., 1985; Kawasaki *et al*., 1988). Sympathetic activity has a role in the modulation of this tone (Vanhoutte *et al*., 1981). Electrical field stimulation (EFS) of vessels induces noradrenaline (NA) release from sympathetic nerves. Released NA can activate postjunctional $\alpha$-adrenoceptors to stimulate vasoconstriction or may activate postjunctional $\beta$-adrenoceptors to relax the blood vessels (Vanhoutte *et al*., 1981). NA release is essentially modulated by prejunctional $\alpha$- and $\beta$-receptors that, respectively, inhibit and facilitate this release (Misu & Kubo, 1986; Starke, 1987). The capacity of $\beta$-adrenoceptor agonists to potentiate the pressor response induced by EFS, by increasing NA release, has been shown in some vessels (Misu & Kubo, 1986). Nevertheless, the role of prejunctional $\beta$-adrenoceptors in the regulation of sympathetic neurotransmitter release is still not established in many vascular beds (Sadeghi & Eikenburg, 1993).

We have recently demonstrated that the $\beta_2$-adrenoceptor agonist clenbuterol, used for the treatment of asthma, increases the vasoconstrictor response induced by EFS in the rat tail artery by increasing adrenergic neurosecretion (Encabo *et al*., 1996).

Low and high concentrations, of the $\beta$-agonist isoprenaline, respectively, potentiate or inhibit vasoconstrictor responses to electrical stimulation in the rat mesenteric vascular bed (Kawasaki *et al*., 1982). These authors also suggest that the inhibition may result from a dominant postjunctional-induced relaxation or a weaker presynaptic effect, although the participation of other vasodilator transmitters cannot be ruled out. Indeed, calcitonin gene-related peptide (CGRP), widely distributed in the cardiovascular system and the main transmitter in rat mesenteric sensory nerves, seems to participate in vasomotor modulation (Kawasaki *et al*., 1988). In addition, nitric oxide (NO) formed in endothelial (Palmer *et al*., 1988), and other cells (González & Estrada, 1991) can modulate vascular tone. Recent studies have demonstrated the presence of local NO-containing nerve fibres in the walls of

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cerebral and peripheral vessels (Estrada *et al*., 1993; Yu *et al*., 1993), suggesting that these nitrergic fibres may release NO which could participate in vascular tone and blood flow regulation.

The objective of this study was to examine how clenbuterol can modulate the vasomotor response to EFS in the rat mesenteric artery and the possible roles of adrenergic, sensory and nitrergic innervation in this modulation.

## Methods

### *Tissue preparation*

Male Wistar rats (200–250 g) were killed by $CO_2$ inhalation, and then the first branch of the mesenteric artery was carefully dissected out, cleaned of connective tissue and kept in Krebs-Henseleit solution (KHS) at 4°C. Each artery was divided in two parts: one was used for reactivity and the other for tritium overflow experiments.

### *Tritium overflow*

Rat mesenteric artery segments of 2 mm in length were set up in a nylon net and immersed for 30 min in 10 ml of KHS at 37°C continuously gassed with 95% $O_2$–5% $CO_2$ mixture (stabilization period). Thereafter, segments were incubated for 60 min in 1 ml oxygenated KHS at 37°C containing (±)-[$^3$H]-NA (0.5 $\mu$M, 5 $\mu$Ci ml$^{-1}$, sp. act. 12.8 Ci mmol$^{-1}$). Afterwards, the arteries were transferred into a superfusion chamber with two parallel platinum electrodes, 0.5 cm apart, connected to a stimulator (Cibertec model CS9, Madrid, Spain, modified to supply the adequate current strength) for EFS. The arteries were superfused at a rate of 2 ml min$^{-1}$ with oxygenated KHS at 37°C for 100 min during which the steady-state level of basal tritium efflux was reached. Then, two electrical stimulation periods of 60 s (200 mA, 0.3 ms, 4 Hz, $S_1$ and $S_2$) were applied to the arteries at a 60 min interval, and the superfusate was collected in vials (10 in total) at 30 s intervals. These vials were distributed in the following manner: 2 before stimulation, to determine the basal level of tritium efflux, 2 during and 6 after the stimulation; these 6 vials were enough to recover basal level of tritium efflux. Ready-Protein solution (Beckman, Fullerton, CA, U.S.A.) was added to the vials and the radioactivity measured in a scintillation counter (Beckman LS 5000 TD). Cocaine (10 $\mu$M) and normetanephrine (10 $\mu$M) were added to the superfusion fluid after the incubation period, and maintained throughout the experiment to block neuronal and extraneuronal uptake of NA, respectively.

Tetrodotoxin (0.1 $\mu$M) and clenbuterol (1 $\mu$M) were used to interfere with tritium overflow, and were administered 20 min and 40 min before $S_2$, respectively.

The stimulation-induced tritium overflow was calculated by subtracting the basal tritium release from that evoked by electrical stimulation. Thereafter, the ratios of the net tritium overflow between $S_2$ and $S_1$ were calculated to eliminate differences between the arteries. The actions of the drugs on the evoked overflow were expressed as their effects on these ratios. The amount of radioactivity released is indicated in d.p.m. mg$^{-1}$ of tissue.

### *Contractile responses*

For isometric tension recording, each artery was divided into segments of 2 mm in length. Each segment was set up in an organ bath (Nielsen & Owman, 1971), containing 5 ml of KHS at 37°C continuously bubbled with a 95% $O_2$–5% $CO_2$ mixture (pH of 7.4). Two horizontally arranged stainless steel pins, 75 $\mu$m in diameter were passed through the lumen of the vascular cylinder. One pin was fixed to the organ bath wall, while the other one was vertically connected to a strain gauge for isometric tension recording. The isometric contraction was recorded through a force-displacement transducer (Grass FTO3C; Quincy, Mass., U.S.A.) connected to a polygraph (Grass, model 7D). For EFS experiments, the segments were mounted between two platinum electrodes 0.5 cm apart, connected to a stimulator (Cibertec model CS9) modified to supply the adequate current strength. The segments were subjected to a tension of 0.5 g, which was readjusted every 15 min during a 90 min equilibration period before drug administration. The vessels were exposed to 75 mM $K^+$ to check their functional integrity.

Frequency-response curves to EFS or concentration-response curves to NA (10 nM–10 $\mu$M) were performed. The parameters used for the electrical stimulation experiments were 200 mA, 0.3 ms, 1–16 Hz, for 30 s with an interval of 1 min between each stimulus; this time was required to recover basal tone. A washout period of at least 1 h was necessary to avoid desensitization between consecutive curves. When the effect of 0.1 $\mu$M tetrodotoxin on the contraction elicited by electrical stimulation was assessed, it was added to the bath 20 min in advance.

The effect of 1 $\mu$M clenbuterol on frequency-response curves was not modified by endothelium removal. However, as there was a possibility that the action of the other drugs used could be to some extent, endothelium-dependent and thereby alter the results, all experiments were performed in the absence of endothelium. Endothelium was removed by gentle rubbing of the luminal surface of segments with a wooden probe. The absence of endothelium was tested by the inability of 10 $\mu$M acetylcholine to relax segments precontracted with 1 $\mu$M NA. Endothelium removal did not alter the contractions elicited by 75 nM $K^+$.

To determine the effects of 1 $\mu$M clenbuterol, 1 $\mu$M propranolol, 10 and 100 $\mu$M $N^G$-nitro-L-arginine methyl ester (L-NAME) and $N^G$-monomethyl-L-arginine (L-NMMA), 10 $\mu$M L-arginine, 10 $\mu$M methylene blue and 0.5 $\mu$M capsaicin on NA or EFS-induced responses, drugs were added to the bath 40 min before a second concentration-response curve to NA or frequency-response curve, except for capsaicin, which was added 1 h earlier. The effect of these substances was analysed by comparing the curve obtained in their presence, with the previous control curve (paired experiments). To analyse the inhibitory effect of propranolol on the clenbuterol-induced effect, three successive EFS-response curves were performed at 1 h intervals. The first curve was the control, propranolol and propranolol plus clenbuterol were respectively added 40 min before the second and the third curves (paired experiments).

To assess the possible role of inducible NO synthase on NA and the EFS-response curves, the arteries were incubated from the time of removal from the animal until the end of the experiment with KSH containing 10 $\mu$M cycloheximide.

The possible participation of NO in the effect of clenbuterol was analysed in three or four successive frequency-response curves. The first curve was the control and the following curves were achieved in the presence of L-NMMA (or L-NAME), clenbuterol, L-arginine alone or a combination of two or three of these drugs. When two or more drugs were administered, they were added simultaneously to the bath.

To investigate the possible participation of guanylate cyclase in the effect induced by clenbuterol, another set of

experiments was performed. In this case, the second and the third curves were performed in the presence of methylene blue and methylene blue plus clenbuterol, respectively.

In segments precontracted with 1 $\mu$M NA, two concentration-response curves to clenbuterol (10 nM – 10 $\mu$M) were performed; one in the absence and the other in the presence of propranolol.

The responses are expressed as a percentage of the contraction induced by 75 mM $K^+$.

### *Solutions, drugs and statistical analysis*

The composition of KHS was as follows (mM): NaCl 115, $CaCl_2$ 2.5, KCl 4.6, $KH_2PO_4$ 1.2, $MgSO_4 \cdot 7H_2O$ 1.2, $NaHCO_3$ 25, glucose 11.1 and $Na_2$ EDTA 0.03 (to prevent the oxidation of unstable substances). Drugs used were: (−)-NA hydrochloride, acetylcholine chloride, propranolol hydrochloride, tetrodotoxin, clenbuterol hydrochloride, L-NAME, capsaicin, L-arginine, cycloheximide and methylene blue (Sigma; St. Louis, MO, U.S.A.), cocaine hydrochloride (Depósito de Estupefacientes, Ministerio de Sanidad y Consumo, Madrid, Spain), (±)-[$^3$H]-NA hydrochloride (New England Nuclear, Boston, MA, U.S.A.), and L-NMMA (Wellcome Research Laboratories, Beckenham, U.K.). Stock solutions (10 mM) of drugs were made in distilled water, except for NA which was dissolved in a NaCl (0.9%)-ascorbic acid (0.01% w/v) solution. These solutions were kept at −20°C and appropriate dilutions were made in KHS on the day of the experiment.

Results are given as mean ± s.e.mean. Statistical analysis was carried out by means of Student's *t* test for paired and unpaired experiments. A *P* value of less than 0.05 was considered significant.

## Results

### *[$^3$H]-NA release experiments*

EFS induced a tritium overflow in rat mesenteric arteries preincubated with [$^3$H]-NA. Two successive electrical pulses induced tritium-overflows: $S_1 = 833 \pm 82$ and $S_2 = 669 \pm 78$ d.p.m. $mg^{-1}$, $S_2/S_1 = 0.80 \pm 0.05$ ($n = 5$). The evoked tritium overflow was markedly reduced by 20 min pre-incubation with 0.1 $\mu$M tetrodotoxin ($S_2/S_1 = 0.04 \pm 0.01$, $n = 4$, $P < 0.001$).

Addition of 1 $\mu$M clenbuterol 40 min before $S_2$ did not modify the basal or stimulated tritium overflow in control arteries (control, $S_2/S_1 = 0.80 \pm 0.05$, $n = 5$; with clenbuterol, $S_2/S_1 = 0.91 \pm 0.11$, $n = 5$, $P > 0.05$).

### *Contractile responses*

Successive frequency (1, 2, 4, 8 and 16 Hz)-response curves applied to endothelium-denuded segments were similar. EFS-induced contractions that were practically abolished by 0.1 $\mu$M tetrodotoxin and significantly reduced by 1 $\mu$M phentolamine (Table 1).

The presence of 1 $\mu$M clenbuterol decreased the contractile response to EFS (Figure 1a). The $\beta$-antagonist propranolol (1 $\mu$M) did not modify the contractile response to electrical stimulation, but it did prevent the inhibitory effect of clenbuterol (Figure 1b).

The presence of the protein synthesis inhibitor cycloheximide (10 $\mu$M) in KHS from the time the arteries were removed from the animal (Table 2), or preincubation with the neurotoxin, capsaicin (0.5 $\mu$M), 1 h before the second curve, did not modify the contractile responses induced by EFS

**Table 1** Effect of 0.1 $\mu$M tetrodotoxin and 1 $\mu$M phentolamine on the frequency-contraction curve in rat mesenteric artery segments

| | *Frequency* (Hz) | | | | |
|---|---|---|---|---|---|
| | *1* | *2* | *4* | *8* | *16* |
| Control | 9 ± 4 | 16 ± 5 | 32 ± 6 | 55 ± 7 | 69 ± 9 |
| Tetrodotoxin | 0* | 0* | 2 ± 1* | 4 ± 2* | 7 ± 3* |
| Phentolamine | 0* | 2 ± 1* | 4 ± 1* | 10 ± 2* | 18 ± 4* |

Results (means ± s.e.mean) are expressed as a percentage of the response elicited by 75 mM $K^+$; $n = 5$, paired experiments. *$P < 0.001$.

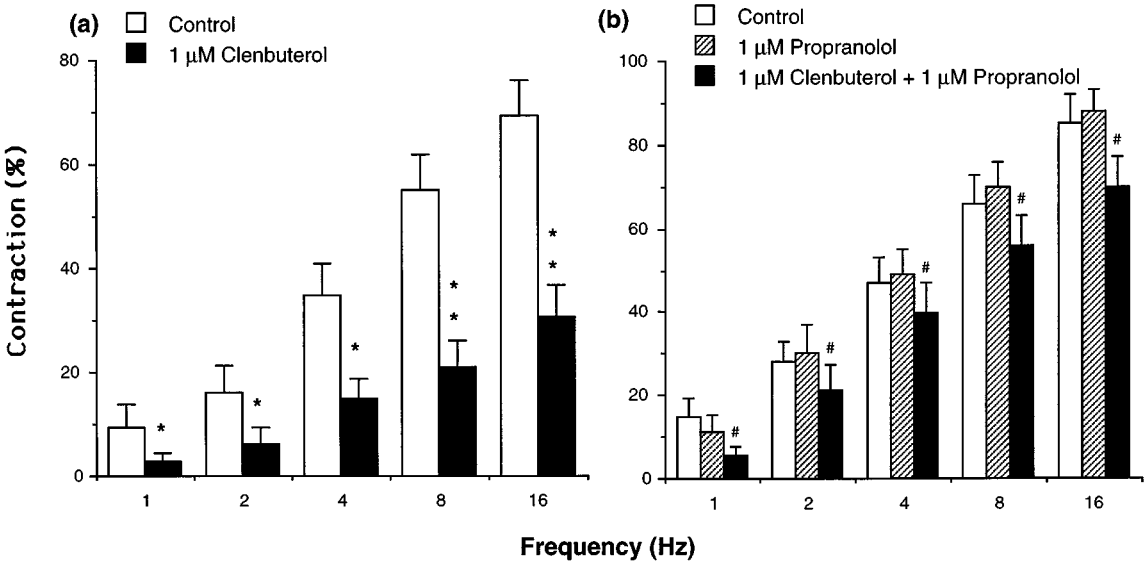


**Figure 1** (a) Effect of clenbuterol ($n = 13$), and (b) propranolol alone or associated with clenbuterol ($n = 11$), on the frequency-contraction curves achieved in rat mesenteric artery segments. Results (means ± s.e.mean) are expressed as a percentage of previous tone with 75 mM $K^+$ (601 ± 28 mg, $n = 24$); $n$ = number of paired experiments. *$P < 0.05$, **$P < 0.001$ vs control; $^{\#}P < 0.05$ vs propranolol.

(Figure 2). The contraction induced by EFS was significantly increased by preincubation with 10 $\mu$M L-NMMA or 10 $\mu$M methylene blue (Figure 2), and similar results were obtained with 100 $\mu$M L-NMMA or 10 and 100 $\mu$M L-NAME (data not shown). The presence of 10 $\mu$M L-arginine decreased the response to EFS (Figure 2). The presence of these drugs did not modify basal tone ($n=6-8$).

Pre-incubation with 10 $\mu$M L-LMMA or 10 $\mu$M methylene blue decreased the inhibitory effect of clenbuterol on the EFS-evoked contraction (Figure 3a,b). A similar reduction in the inhibitory effect of clenbuterol was produced by 100 $\mu$M L-LMMA or 10 and 100 $\mu$M L-NAME, while the effect of clenbuterol remained unchanged in the presence of capsaicin or treatment with cycloheximide (Table 2). The effect of clenbuterol was reversed with L-NMMA, methylene blue or L-NAME and, in some experiments, the subsequent addition of 10 $\mu$M L-arginine restored the inhibitory effect of clenbuterol (Figure 3a) shown in Figure 1a.

Two successive concentration-response curves to NA (10 nM – 10 mM), separated by a 60 min interval, were similar. The contractile responses elicited by NA were not modified by 1 $\mu$M clenbuterol (Figure 4). Similarly, 1 $\mu$M propranolol, 10 and 100 $\mu$M L-NAME or L-NMMA, 10 $\mu$M L-arginine, 0.5 $\mu$M

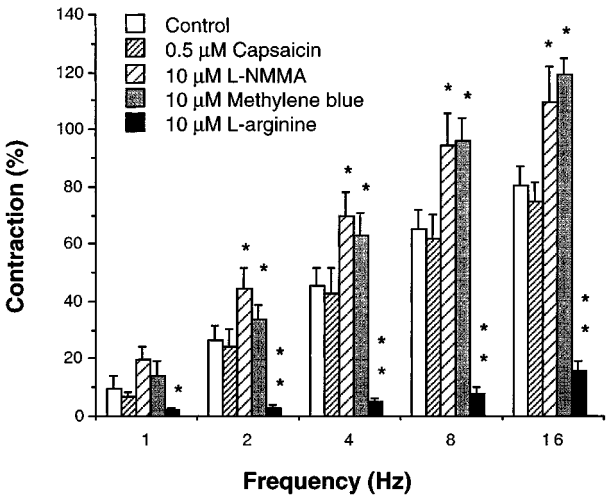


**Figure 2** Effect of capsaicin ($n=6$), L-NMMA ($n=8$), methylene blue ($n=6$) and L-arginine ($n=6$) on the frequency-contraction curves achieved in rat mesenteric artery segments (control, $n=26$). Results (means ± s.e.mean) are expressed as a percentage of response elicited by 75 mM $K^+$ (639 ± 32 mg). $n$ = number of unpaired experiments. *$P<0.05$; **$P<0.001$ vs control.

**Table 2** (a) Effect of 10 $\mu$M cycloheximide on the frequency-contraction curve in rat mesenteric artery segments and (b) effect of 0.5 $\mu$M capsaicin and 10 $\mu$M cycloheximide on the inhibitiory effect of clenbuterol on the frequency-contraction curve

| | *Frequency* (Hz) | | | | | |
|---|---|---|---|---|---|---|
| | *1* | *2* | *4* | *8* | *16* | n |
| (a) Control | 8 ± 2 | 23 ± 3 | 44 ± 6 | 66 ± 5 | 88 ± 8 | 7 |
| Cycloheximide | 9 ± 2 | 24 ± 2 | 43 ± 5 | 62 ± 5 | 82 ± 6 | 8 |
| (b) Clenbuterol | 3 ± 2 | 6 ± 3 | 15 ± 4 | 21 ± 5 | 31 ± 6 | 13 |
| + Capsaicin | 3 ± 1 | 6 ± 2 | 17 ± 5 | 28 ± 7 | 40 ± 6 | 6 |
| + Cycloheximide | 5 ± 2 | 7 ± 2 | 12 ± 2 | 23 ± 3 | 38 ± 5 | 5 |

Results (means ± s.e.mean) are expressed as a percentage of the response elicited by 75 mM $K^+$; unpaired experiments.

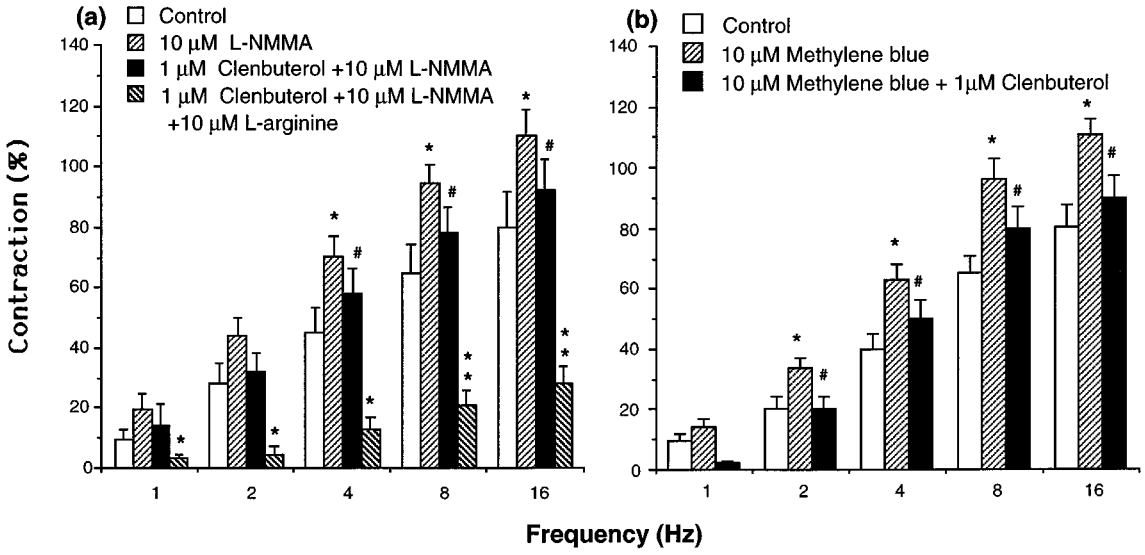


**Figure 3** (a) Effect of L-NMMA, clenbuterol + L-NMMA and clenbuterol + L-NMMA + L-arginine ($n=8$), and (b) effect of methylene blue and clenbuterol + methylene blue ($n=6$) on the frequency-contraction curves achieved in rat mesenteric artery segments. Results (means ± s.e.mean) are expressed as a percentage of contraction induced by 75 mM $K^+$ (638 ± 35 mg); $n$ = number of paired experiments. *$P<0.05$ vs control. $^{\#}P<0.05$ vs L-NMMA or methylene blue.

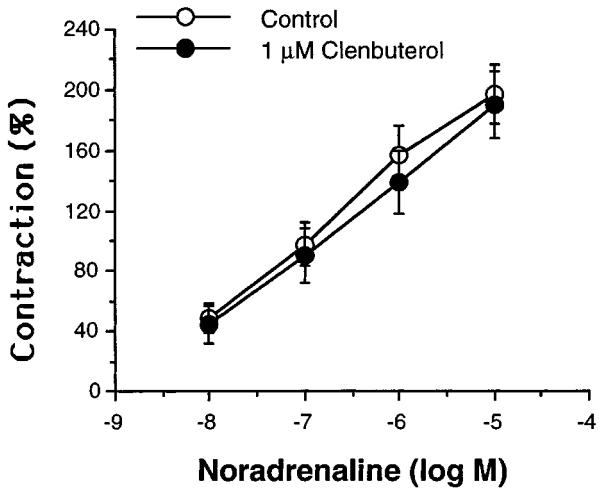


**Figure 4** Effect of clenbuterol on the contraction-response curve to NA in rat mesenteric artery segments ($n=7$). Results (means ± s.e.mean) are expressed as a percentage of the response elicited by 75 mM $K^+$ (625 ± 28 mg); $n$ = number of paired experiments.

capsaicin, 10 $\mu$M methylene blue, 1 $\mu$M clenbuterol or pretreatment with cycloheximide, did not modify the contractions caused by NA.

In basal conditions, the addition of 10 $\mu$M clenbuterol did not alter basal tone. However, when a concentration-response curve was performed in segments precontracted with 1 $\mu$M NA (837 ± 42 mg; $n=7$) a small relaxation was observed at 10 $\mu$M, the value of which was 90 ± 11 mg ($n=7$). This relaxation was not modified by pre-incubation with L-NAME or L-NMMA, but it was abolished by 1 $\mu$M propranolol (results not shown).

## Discussion

The present results show that the contractile responses induced by EFS in rat mesenteric artery segments, appear to be due to NA release from noradrenergic nerve terminals and subsequent interaction with $\alpha$-adrenoceptors as shown by Li & Duckles (1991). This assumption is supported by the fact that tetrodotoxin and phentolamine, which block nerve impulse propagation and $\alpha$-adrenoceptors, respectively, markedly reduced such responses.

Frequency-dependent vasoconstriction was significantly reduced by the $\beta_2$-agonist clenbuterol, and this effect was antagonized by the selective $\beta$-adrenoceptor antagonist propranolol. Similar results have been observed by Kawasaki *et al.* (1982) in the same artery with high concentrations of the $\beta$-agonist isoprenaline. Our results suggest that the effect of clenbuterol is mediated by $\beta_2$-adrenoceptors and may be related to a small prejunctional effect, or to a more dominant effect on postjunctional $\beta$-adrenoceptor-mediated relaxation that reduces the vasoconstrictor response induced by the released NA.

Although different prejunctional receptors have been demonstrated on adrenergic endings than can facilitate (Encabo *et al.*, 1996) or inhibit NA release (Starke, 1987), this modulator effect has not been observed in all tissues (Langer, 1981; Misu & Kubo, 1986; Sadeghi & Eikenburg, 1993).

The possible mechanisms involved in the negative effect of clenbuterol on noradrenergic neurotransmission were investigated by assessing its capacity to inhibit the tritium overflow induced by EFS. Clenbuterol did not significantly alter this overflow, so the prejunctional $\beta$-adrenoceptor would only have a very limited influence on adrenergic neurotransmission in rat mesenteric artery, as shown earlier by Sadeghi & Eikenburg (1993). These results agree with most findings, which indicate the existence of prejunctional facilitatory $\beta$-adrenoceptors in the rat mesentery only in the spontaneously hypertensive rat (Kawasaki *et al.*, 1982; Kubo *et al.*, 1984).

Activation of postjunctional $\beta$-adrenoceptors induces a variable vasodilator response that may attenuate the vasoconstrictor response to NA in several vascular beds (Vanhoutte *et al.*, 1981). We observed that clenbuterol produced a very modest relaxation in arteries precontracted with NA and that this relaxation was abolished by propranolol. This small effect indicates that the relaxation might not be associated with the marked reduction induced by clenbuterol of the response to EFS.

The existence of an interaction between the $\alpha$- and $\beta$-adrenoceptors which mediate vasoconstriction and vasodilatation is controversial. Thus, an increase (McGrath, 1982) or a decrease (Wilffert *et al.*, 1983) of $\alpha$-adrenoceptor-mediated contraction in the presence of $\beta$-agonists have both been described. In our experimental conditions, pre-incubation with clenbuterol did not modify the contraction caused by exogenous NA, as previously found with isoprenaline in this artery (Cohen & Wiley, 1977), indicating that the clenbuterol modulation of EFS-induced contraction is not exerted the postjunctional receptor level.

It has been proposed that the sensory nerves, present in this artery, play a part in vascular tone regulation, and that CGRP is the essential neurotransmitter in these capsaicin-sensitive nerves (Kawasaki *et al.*, 1988). The participation of sensory innervation in the reduced EFS response caused by clenbuterol was assessed by the use of capsaicin which selectively depletes neurotransmitters from sensory nerves (Duckles & Buch, 1982; Li & Duckles, 1992). Acute *in vitro* denervation of rat mesenteric arteries with capsaicin had no significant effect on the vasoconstrictor response to either EFS or exogenous NA (Ralevic *et al.*, 1995). The reduction induced by clenbuterol in the EFS-induced contraction in these denervated arteries was similar to the reduction observed in the control situation, indicating that sensory nerves are not involved in the vasodepressor effect of clenbuterol.

The nitrergic innervation that has recently been demonstrated in several blood vessels, including the rat mesenteric artery (Yu *et al.*, 1993), may participate in the modulation of the vasomotor response (Li & Duckles, 1992; Yu *et al.*, 1993). We have investigated the possible existence of a non-endothelial NO synthase that would regulate the response to EFS and exogenous NA, by use of the NO synthase inhibitors L-NMMA and L-NAME and the substrate of this enzyme L-arginine (Palmer *et al.*, 1988; Moore *et al.*, 1990). Although the contractions elicited by EFS were markedly reduced by L-arginine, they were increased by the NO synthase inhibitors. However, the vasoconstrictor response caused by exogenous NA was unaltered. Our results indicate participation by non-endothelial NO in the vasomotor response induced by EFS. L-NAME, L-NMMA and L-arginine did not alter basal tone, suggesting that the absence of resting NO release was not due to a lack of substrate.

L-NAME and L-NMMA diminished the inhibitory effect of clenbuterol and the inhibition was re-established by the subsequent addition of L-arginine. This finding indicates the participation of NO in the effect of clenbuterol.

Vascular smooth muscle has been shown to synthesize and release NO by activating inducible NO synthase and NO has been found to have the ability to modulate the response caused

by sympathetic nerve stimulation (González *et al*., 1992). Arteries were treated with cycloheximide, a protein synthesis inhibitor to assess the participation of this inducible NO synthase on the inhibitory response of clenbuterol. Since the results were similar to the control situation the NO synthase involved in the effect of clenbuterol is not inducible and may be the neuronal constitutive isoform.

NO and other nitrovasodilators can stimulate the soluble guanylate cyclase in smooth muscle, thus increasing the levels of intracellular guanosine 3′ : 5′-cyclic monophosphate (cyclic GMP) that mediate the vasodilator response; this enzyme is inhibited by methylene blue (Gruetter *et al*., 1981; Martin *et al*., 1985). Methylene blue also reduced the inhibitory effect of clenbuterol on EFS thereby re-confirming the participation of NO, which would increase cyclic GMP levels.

In contrast, our results showed that NO released from nitrergic nerves did not alter NA release from the sympathetic nerves in the rat mesenteric artery, because clenbuterol did not modify tritium overflow. This rules out a paracrine mechanism of NO in the modulation of adrenergic transmission (Levi *et al*., 1995).

In conclusion, our results suggest that clenbuterol reduces EFS-induced contraction in endothelium-denuded mesenteric arteries of the rat by inducing the release of NO from nitrergic nerves; this release seems to be mediated by activation of the $\beta_2$-adrenoceptors, which are probably present in nitrergic nerves.

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