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Nitric oxide-dependent and -independent modulation of sympathetic vasoconstriction in the human saphenous vein

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Abstract

The possible modulation by the endothelium of the contractile responses to sympathetic nerve stimulation was examined in isolated superfused human saphenous vein. Contractile response curves for transmural nerve stimulation and noradrenaline were higher in endothelium-denuded than in intact human saphenous vein rings. In vessels with endothelium, transmural nerve stimulation- and noradrenaline-induced contractions were unaffected by the cyclooxygenase inhibitor, indomethacin (10 μM), but were potentiated by the nitric oxide (NO) synthase inhibitor, L-N^{ω}-nitro-L-arginine (L-NNA, 3 μM) even when combined with D-arginine (0.3 mM), but not with L-arginine (0.3 mM). As in the case of noradrenaline, contractile responses to 5-HT, but not to KCl, were enhanced by endothelium removal, L-NNA or L-NNA plus D-arginine, but were unaffected by L-NNA plus L-arginine. The guanylyl cyclase inhibitor, methylene blue (10 μM), potentiated both transmural nerve stimulation- and noradrenaline-induced contractions in endothelium intact rings, whereas it enhanced, although to a lesser degree, only the neurally evoked contractions in endothelium-denuded human saphenous vein. In the vessels without endothelium L-NNA failed to affect the vasoconstriction induced by both transmural nerve stimulation and noradrenaline. Our results suggest that at least two inhibitory factors are involved in modulating the sympathetic vasoconstriction in the human saphenous vein: (1) at a postjunctional level, NO, the release of which from endothelial cells is probably stimulated by the activation of specific receptors, and (2) at a prejunctional level, an unidentified vasodilator agent, which is unmasked by the removal of the endothelium layer and which is probably co-released along with noradrenaline, and which acts through the guanylyl cyclase pathway.

Keywords: Vasoconstrictor sympathetic nerve; Nitric oxide (NO); Saphenous vein, human; L-N^{ω}-Nitro-L-arginine (L-NNA); cGMP

1. Introduction

It is widely recognized that the vascular endothelium of several blood vessels has a strong capacity to produce vasodilator agents such as endothelium-derived relaxing factors (EDRFs), the most important of which has been identified as nitric oxide (NO), and prostacyclin (Furchgott, 1983; Ignarro et al., 1987a; Palmer et al., 1987). These relaxing factors exert an important modulatory action that contributes to the moment-to-moment maintenance of blood vessel tone (for reviews, see Moncada et al., 1991; Li et al., 1994). However, the release and the mechanisms by which EDRFs and prostacyclin modulate the vascular tone appear to be tissue- and species-dependent.

Different responses to vasodilator endogenous agents acting through the release of EDRFs have been reported in arteries and veins. In rabbits and lambs the relaxation induced by several of these agents was greater in veins than in arteries (McGrath et al., 1990; Gao et al., 1995); the opposite was true for monkeys and humans (Akar et al., 1994; Yang et al., 1989). In addition, in the same vascular bed and species, namely monkey mesenteric arteries and veins, the vasodilatation induced by certain endogenous agents was endothelium-dependent in the arteries, but not in the veins (Okamura et al., 1994).

The modulatory (inhibitory) role of EDRFs on the sympathetic vascular tone of isolated vessels has been functionally evidenced by indirect methods. Indeed, endothelium removal has been shown to enhance the contractions induced by sympathetic nerve stimulation (Tesfamariam et al., 1987; Hynes et al., 1988; Martinez et al., 1994). However, discrepant results regarding the mechanisms by which the endothelium can modulate the vascular

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tone have been obtained even in experiments carried out only on arterial vessels. The NO released from endothelial cells was identified as the main agent responsible for the inhibitory action of the endothelium on sympathetic contractions in guinea pig pulmonary and in human omental arteries, but with postjunctional or prejunctional mechanisms, respectively (Liu et al., 1991; Aldasoro et al., 1993). In addition to NO, prostacyclin and other endothelial factors appear to modulate the sympathetic vasoconstriction in rat aorta and tail artery (Jeremy et al., 1985; Thorin and Atkinson, 1994). The literature reports few data on the influence of the endothelium on the sympathetic tone of veins, but a modulation by EDRF has been suggested in canine pulmonary vein (Greenberg et al., 1989). Thus, whether such modulation and its mechanisms can be extrapolated to human veins remains to be determined.

The principal aim of our study was to verify whether the endothelium can modulate the sympathetic vasoconstriction of large human venous capacitance vessels, and to characterize the type(s) of EDRF and mechanisms involved in the phenomenon. The experiments were carried out in the human saphenous vein, which we have demonstrated to be a suitable model for studies on sympathetic neurotransmission (Fabi et al., 1993). We tested the effects of endothelium removal on the contractions induced by transmural nerve stimulation, exogenously applied noradrenaline, 5-hydroxytryptamine (5-HT), and potassium chloride (KCl). In addition, we investigated the effects of the cyclooxygenase inhibitor indomethacin, the NO-synthase inhibitor L-N^{ω}-nitro-L-arginine (L-NNA; Ishii et al., 1990; Moore et al., 1990), and the guanylyl cyclase blocker methylene blue (Martin et al., 1985; Gryglewski et al., 1992).

2. Materials and methods

2.1. Preparation of tissue

Vessels were prepared as described elsewhere (Fabi et al., 1993). Briefly, human saphenous vein segments were taken from patients undergoing surgery for aorta-coronary bypass grafting. Veins from patients who were not receiving adrenoceptor agonists or antagonists, nor drugs that influence the storage or release of noradrenaline, but who were taking Ca^{2+} channel antagonists, angiotensin-converting enzyme inhibitors, aspirin-like drugs and nitrovasodilators, were selected for the studies. The terminal segment of the vessel, 2–3 cm in length, before it penetrates the fascia lata (less desirable for bypass purposes) was selected. Immediately after excision the tissue was placed in an oxygenated Krebs solution at 4°C and transported to the laboratory within 10 min. Most vessels were used on the day of surgery, and all tissues were used within 18 h. The vessels were cleaned of the adherent connective tissue and cut into 4- to 5-mm wide rings. The vein segments were mounted in an organ chamber on L-shaped stainless steel rods, to record smooth muscle force. The preparations were superfused with Krebs oxygenated solution at 37°C by a constant perfusion pump (Gilson Minipuls II, Villiers Le Bel, France) at a flow rate of 5 ml/min under a resting tension of 2 g and allowed to equilibrate 90–120 min. The composition of the Krebs solution was (mM): $NaHCO_3$ 25, NaCl 118, KCl 4.7, $CaCl_2 \cdot 2H_2O$ 2.5, KH_2PO_4 1.2, $MgSO_4 \cdot 7H_2O$ 1.17 and glucose 5.6; the solution was aerated with a mixture of 95% O_2 and 5% CO_2. The tension of the circular muscle layer was recorded with a Grass FT 0.3 T isometric force transducer (Grass Instrument Company, Quincy, MA) coupled to a polygraph (Grass model 7).

2.2. Transmural nerve stimulation of the vessel rings

Transmural nerve stimulation was delivered for 1 min through platinum wire electrodes placed on both sides of the vessel. The rectangular pulses applied by an electronic stimulator at 0.5–16 Hz (Grass model S 11) were 0.3 ms in duration and had supramaximal voltage (14 V measured across the electrodes).

Contractile response curves for transmural nerve stimulation and for exogenously administered noradrenaline were obtained. To verify whether the contractions evoked by transmural nerve stimulation were mediated by endogenous noradrenaline released from adrenergic nerves, the vessel rings were superfused with Krebs containing 2 μM tetrodotoxin or with 10 μM guanethidine for 30 min. The contractions induced by transmural nerve stimulation, but not by exogenously administered noradrenaline, were inhibited by the two antagonists, confirming that the transmural nerve stimulation-induced contractions were neurogenic and mediated by endogenous noradrenaline released from adrenergic nerves. Following the addition of phentolamine (1 μM) to the perfusing medium, the contractile responses produced by both transmural nerve stimulation and noradrenaline were substantially blocked, indicating the involvement of α-adrenoceptors (data not shown).

2.3. Experimental protocol

After the preparations were allowed to equilibrate and a stable tension was obtained, they were stimulated with 1-min transmural nerve stimulation at 16 Hz and with a 1-min infusion of Krebs containing noradrenaline (5 μM), which caused near maximal contraction in pilot experiments, and were then allowed to return to base-line tension. Submaximal tone was then elicited by exposing rings to 1 μM noradrenaline. The tone was maintained for the period of time during which relaxant responses to 1-min infusion of Krebs containing 3–10 μM acetylcholine were tested. In the endothelium denudation study, paired vessel rings from the same patients were prepared. In one venous

ring the endothelium was removed mechanically by inserting a roughened stainless steel wire into the lumen and gently rolling the ring on wet filter paper. Endothelium denudation or integrity was confirmed in each experiment by the loss or the presence of vasorelaxant responses to exogenous acetylcholine, respectively.

A control series of contractile responses to transmural nerve stimulation and noradrenaline was then performed. Stimulations lasting 1 min were applied at 0.5, 1, 2, 4 and 8 Hz, with a period of at least 10 min between each stimulation. Exogenous noradrenaline was administered by superfusing the vessel segment for 1 min with Krebs solution containing 0.1, 0.3, 1, 3 μM noradrenaline. After the control series of transmural nerve stimulation and noradrenaline responses was completed, antagonists were added to the superfusing medium and allowed to bathe the blood vessels for 30 min. A second series of transmural nerve stimulation and noradrenaline addition was repeated in the presence of antagonists. In vessels with intact endothelium, control experiments demonstrated that contractile responses to transmural nerve stimulation and to exogenous noradrenaline (Fig. 1A,B) were reproducible in two experimental periods 30 min apart ($F = 2.17$ and $F = 3.83$, respectively; $P > 0.05$ for both transmural nerve stimulation and noradrenaline). Conversely, in endothelium-denuded rings, the second series of transmural nerve stimulation and noradrenaline addition (Fig. 1C,D) gave higher responses than the control series ($F = 66.8$ and $F = 15.6$, respectively), while a third series carried out in 2 experiments (data not shown) overlapped the second one. In consideration of this, to study the effect of endothelium removal, only the second series of transmural nerve stimulation- and noradrenaline-induced contractions in rings with and without endothelium were compared.

To normalize the data, the contractile responses of each preparation were expressed as the percentage of the maximum force generated in response to 16 Hz and to 5 μM noradrenaline in the control series (unless otherwise stated). As in the case of noradrenaline, contractile response curves for KCl and for 5-HT were obtained in separate experimental groups by superfusing the vein rings for 1 min with a Krebs solution containing 20, 40, 60 and 80 mM KCl and 0.1, 0.3, 1 and 3 μM 5-HT. The mean frequency- and concentration-response curves were obtained in rings from different patients. Each ring was exposed to only one antagonist, but various antagonists were tested at the same time, using separate venous rings from the same patient.

2.4. Drugs

The following drugs were used: (−)-noradrenaline bitartrate, acetylcholine chloride, 5-hydroxytryptamine maleate, potassium chloride, guanethidine sulphate, tetrodotoxin, phentolamine hydrochloride, indomethacin, N^{ω}-nitro-L-arginine, L-arginine, D-arginine, methylene blue (obtained from Sigma Chemical Co, St Louis, MO, USA).

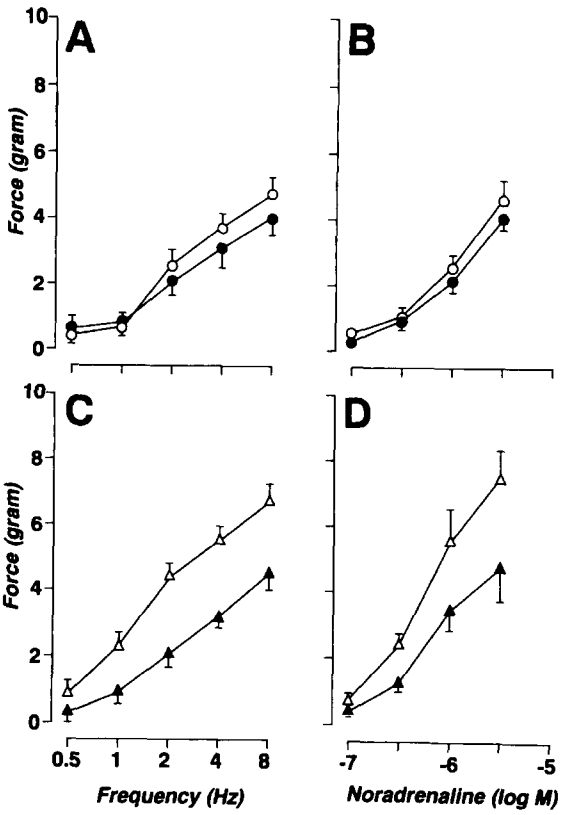

Fig. 1. Comparison of the first series of vasoconstrictor responses to 1-min transmural nerve stimulation and 1-min noradrenaline infusion with the second series of vasoconstrictor responses to the same stimuli obtained after a 30 min interval in superfused human saphenous vein rings. Contractions are expressed in g. Values are the means ± S.E.M. (A,B) Frequency- and concentration-response curves for superfused human saphenous vein rings with intact endothelium ($n = 8$). The first frequency- and concentration-response curves for transmural nerve stimulation and noradrenaline (●) did not differ from the second series (○). (C,D) The contractile effects of the first series of transmural nerve stimulation and noradrenaline (▲) in human saphenous vein rings deprived of endothelium ($n = 8$) are significantly different from those of the second series (△). $P < 0.025$ and $P < 0.01$ vs. control period for transmural nerve stimulation and noradrenaline, respectively.

Noradrenaline was dissolved in 0.9% saline containing 0.1% ascorbic acid and kept at +4°C. Indomethacin was dissolved in a small amount of absolute ethanol and sodium bicarbonate (150 mM) solution and then in Krebs solution readjusted to pH 7.4 with HCl before use. All the other drugs were dissolved in distilled water and freshly prepared upon use.

2.5. Statistical analysis of results

Values are presented as means ± S.E.M. and n indicates the number of experiments in each group. Frequency-response curves for transmural nerve stimulation and concentration-response curves for noradrenaline (with and without endothelium) were compared by analysis of variance with repeated measures according to Winer (1971). The comparison of the contractile responses in the

absence and presence of antagonists on the same experimental vessel was made by analysis of variance and covariance with repeated measures (BMDP Statistical Software, Inc., Los Angeles, CA, USA). A P value < 0.05 was considered to be significant.

3. Results

3.1. Vasoconstrictor responses to transmural nerve stimulation and noradrenaline infusion in superfused human saphenous vein: influence of endothelium removal

Transmural nerve stimulation of the superfused venous rings with intact endothelium (Fig. 1A) produced a frequency-dependent vasoconstriction which reached its maximum at 16 Hz (5.087 ± 0.590 g, $n = 8$). In the same preparations, 1-min infusion of noradrenaline (Fig. 1B) produced a concentration-dependent vasoconstriction that mimicked the response to transmural nerve stimulation (maximum contraction at 5 μM 5.312 ± 0.583 g; $n = 8$).

As shown in Fig. 1C,D, frequency- and concentration-dependent vasoconstrictions in response to transmural nerve stimulation and noradrenaline were also obtained in human saphenous vein rings denuded of endothelium. By comparison of the second series of contractions, according to the protocol described in Materials and methods, the responses induced by transmural nerve stimulation and noradrenaline in endothelium-denuded human saphenous vein resulted in significantly higher values than those obtained in the venous rings with intact endothelium ($F = 6.69$ and $F = 10.02$, respectively).

3.2. Effects of indomethacin on vasoconstrictor responses to transmural nerve stimulation and noradrenaline

As shown in Fig. 2, after the first series of transmural nerve stimulation- and noradrenaline-induced contractions were elicited in venous rings with endothelium, the addition of the cyclooxygenase inhibitor indomethacin (10 μM) to the perfusing medium did not significantly affect the frequency- and concentration-dependent contractile responses of the venous rings to either transmural nerve stimulation or exogenous noradrenaline ($F = 0.07$ and $F = 2.19$, respectively; $P > 0.05$ for both responses).

3.3. Effects of L-NNA, L-arginine and D-arginine on vasoconstrictor responses to transmural nerve stimulation and noradrenaline

As shown by the original tracing of a superfused venous ring with intact endothelium (Fig. 3), the presence of the NO synthase inhibitor L-NNA (3 μM) in the superfusing medium increased the contractions produced by both transmural nerve stimulation and noradrenaline, with respect to the control response for the same vessel. Accordingly, the

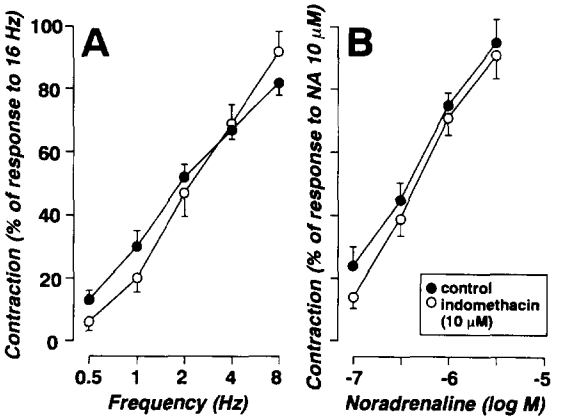

Fig. 2. Frequency- and concentration-response curves for transmural nerve stimulation (A) and noradrenaline (B) in the absence (●) and presence (○) of 10 μM indomethacin in human saphenous vein rings with intact endothelium. Indomethacin had no significant effect on either transmural nerve stimulation- or noradrenaline-induced contractile responses. Contractions are expressed as the percentage of the maximal contraction in response to transmural nerve stimulation (16 Hz) and noradrenaline (NA, 10 μM) at the beginning of the experiments. Value are the means ± S.E.M. of 8 experiments.

neurally mediated contractions at all tested frequencies were significantly enhanced ($F = 65.11$) by the presence of L-NNA (Fig. 4A). As in the case of transmural nerve stimulation-induced contractions, a significant difference ($F = 13.21$) between the control contractile responses and those elicited in the presence of the antagonist was also found for the concentration-response curves for exogenous noradrenaline (Fig. 4B). The combined presence of 3 μM L-NNA and 0.3 mM L-arginine in the medium (Fig. 5A,B) did not cause any potentiating effect of the contractions produced by transmural nerve stimulation and noradrenaline ($F = 0.26$ and $F = 4.80$, respectively; $P > 0.05$ for both transmural nerve stimulation and noradrenaline-induced contractions), whereas the presence of 0.3 mM

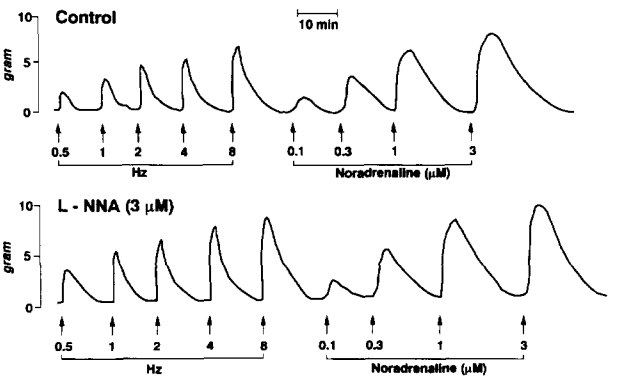

Fig. 3. Typical recording showing the vasoconstrictor responses to 1-min transmural nerve stimulation (0.5–8 Hz) and 1-min infusion of noradrenaline (0.1–3 μM) in a superfused human saphenous vein ring with intact endothelium. After a control series of transmural nerve stimulation- and noradrenaline-induced responses was obtained, 3 μM L-NNA was added to the superfusing Krebs solution 30 min before the start of the second series of contractile responses.

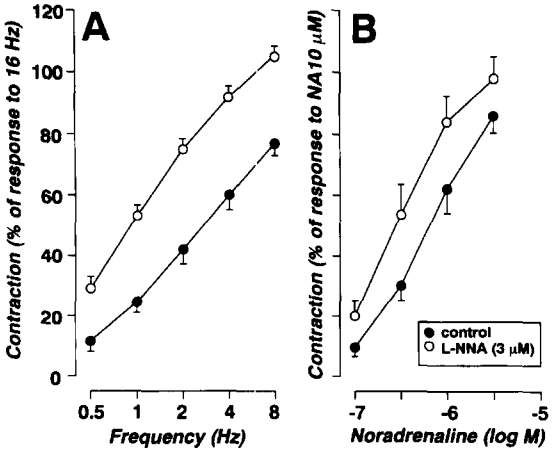

Fig. 4. Frequency- and concentration-response curves for transmural nerve stimulation (A) and noradrenaline (B) in human saphenous vein rings with intact endothelium. The contractile curves were obtained in the absence (●) and presence (○) of 3 μM L-NNA. Values are the means ± S.E.M. of 7 experiments. $P < 0.001$ for transmural nerve stimulation and $P < 0.01$ for noradrenaline vs. control.

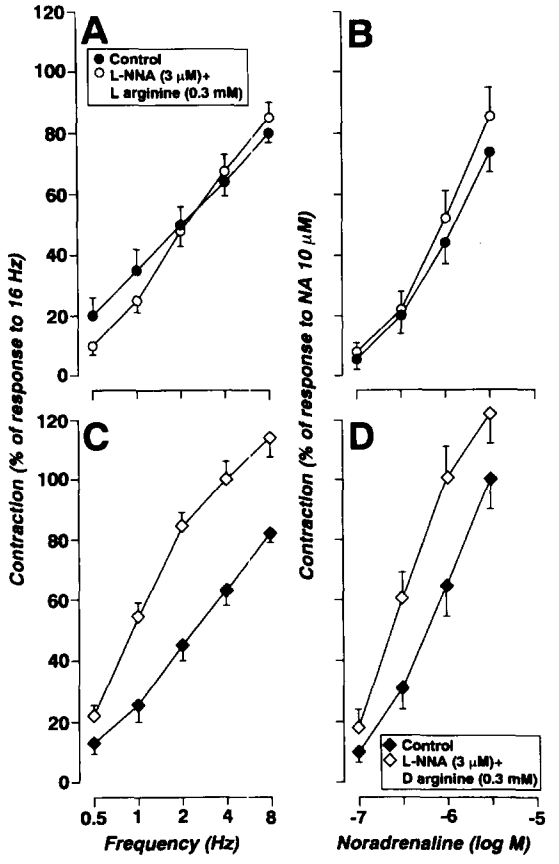

Fig. 5. Effects of the combined presence of 3 μM L-NNA with 0.3 mM L-arginine (A,B; $n = 7$) or with 0.3 mM D-arginine (C,D; $n = 7$) on the frequency- and concentration-contractile curves for transmural nerve stimulation (A,C) and noradrenaline (B,D) in superfused human saphenous vein rings with intact endothelium. The contractions evoked by transmural nerve stimulation and noradrenaline in the presence of L-arginine + L-NNA (○) did not differ from the control series (●), but the transmural nerve stimulation and noradrenaline contractile responses obtained when D-arginine (◇) was added to L-NNA were significantly different from those of the control series (◆) – $P < 0.01$ for both transmural nerve stimulation and noradrenaline vs. control.

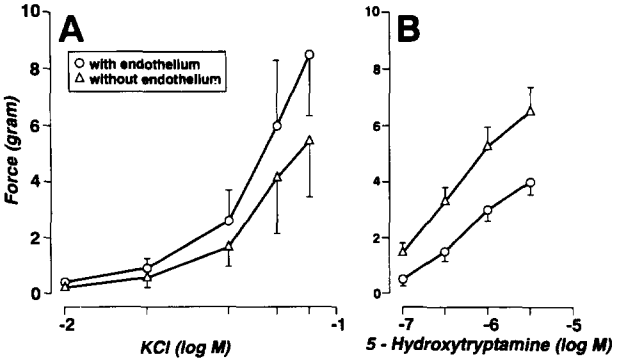

Fig. 6. Effects of endothelium removal on the concentration-response curves for (A) potassium chloride (KCl) and (B) 5-hydroxytryptamine (5-HT). The contractions induced by KCl in human saphenous vein rings without endothelium (△; $n = 6$) did not differ from those of the rings with endothelium (○; $n = 6$), whereas the contractile responses to all tested concentrations of 5-HT in endothelium-denuded rings (△; $n = 7$) were significantly different ($P < 0.005$) from those of the control group (○; $n = 7$). Contractions are expressed in g. Means ± S.E.M. are shown.

D-arginine (Fig. 5C,D) was not able to counteract the potentiating effect of L-NNA on contractions elicited by transmural nerve stimulation and noradrenaline ($F = 12.25$ and $F = 17.49$, respectively).

3.4. Effects of endothelium removal on the vasoconstriction induced by KCl and 5-HT

One-min infusion of KCl (10, 20, 40, 60, 80 mM) caused concentration-dependent contractile responses in vein rings with endothelium which were not significantly different ($F = 0.69$; $P > 0.05$) from those obtained in vessels without endothelium (Fig. 6A). Conversely, the concentration-dependent responses to 5-HT (0.1, 0.3, 1 and 3 μM) were significantly higher ($F = 12.66$) in endothelium-denuded human saphenous vein rings than in human saphenous vein rings with intact endothelium (Fig. 6B).

3.5. Effects of L-NNA, L-arginine and D-arginine on vasoconstrictor responses to 5-HT

As shown in Fig. 7A, the presence of 3 μM L-NNA significantly enhanced ($F = 51.63$) the contractions induced by 5-HT in human saphenous vein rings with intact endothelium. However, the contractile responses to 5-HT were not significantly ($F = 0.07$; $P > 0.05$) modified by the combined presence of 3 μM L-NNA and 0.3 mM L-arginine (Fig. 7B), whereas the potentiating effect ($F = 11.22$) of the NO synthase inhibitor was still observed when 0.3 mM D-arginine was combined with L-NNA (Fig. 7C).

3.6. Effects of methylene blue on vasoconstrictor responses to transmural nerve stimulation and noradrenaline

In human saphenous vein with intact endothelium, the addition of the guanylyl cyclase inhibitor methylene blue

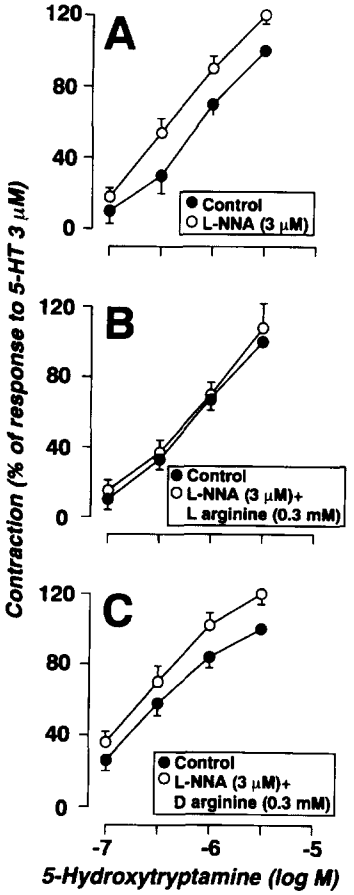

Fig. 7. (A) Concentration-response curves for 5-HT ($n = 7$) in the absence (●) and presence (○) of 3 μM L-NNA ($P < 0.01$ vs. own controls) in human saphenous vein rings with intact endothelium. (B) Contractile responses to 5-HT in the absence (●) and presence (○) of 3 μM L-NNA combined with 0.3 mM L-arginine ($n = 6$) were not different from those of controls. (C) Contractions evoked by 5-HT in the presence (○) of 3 μM L-NNA + 0.3 mM D-arginine ($n = 6$) were significantly different ($P < 0.05$) from those of controls (●). Contractions are expressed as the percentage of the maximal contraction to 3 μM 5-HT of the control series. Means ± S.E.M. are reported.

(10 μM; Fig. 8) to the superfusing medium caused a significant shift to the left of both the frequency- and concentration-response curves for transmural nerve stimulation and noradrenaline with respect to the control series of contractile responses ($F = 14.86$ and $F = 7.09$, respectively).

3.7. Effects of L-NNA and methylene blue on vasoconstrictor responses to transmural nerve stimulation and noradrenaline in human saphenous vein without endothelium

In endothelium-denuded vein rings (Fig. 9A,B), the presence of L-NNA in the perfusing medium did not potentiate the contractile responses to either transmural nerve stimulation or noradrenaline in comparison to those of the control experimental group ($F = 0.76$ and $F = 3.17$,

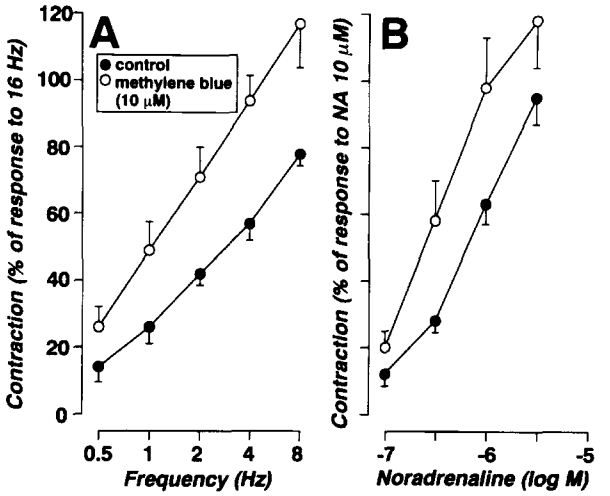

Fig. 8. Effect of 10 μM methylene blue (○) on the vasoconstrictor responses to transmural nerve stimulation (A) and noradrenaline (B) in superfused human saphenous vein rings with endothelium. $P < 0.01$ and $P < 0.05$ for transmural nerve stimulation and noradrenaline, respectively, in comparison to control responses (●). Values are the means ± S.E.M. of 7 experiments.

respectively; $P > 0.05$). Conversely, when methylene blue was added to the Krebs solution, the contractions evoked by transmural nerve stimulation were significantly higher ($F = 4.90$) than those of the control preparations. However, the contractions produced by noradrenaline in the

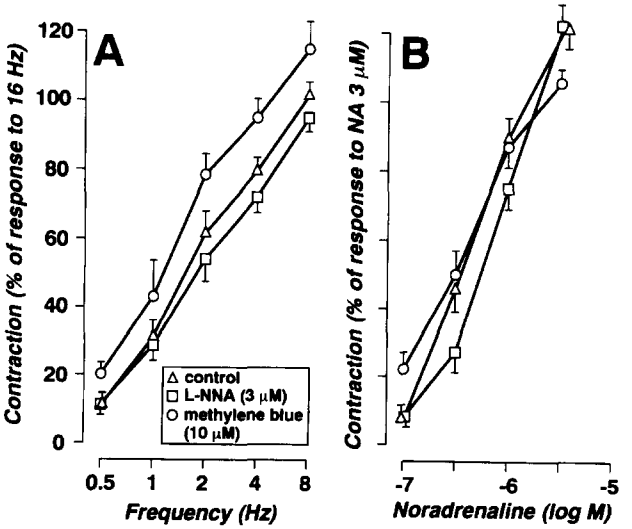

Fig. 9. Frequency- and concentration-response curves for transmural nerve stimulation (A) and noradrenaline (B) in human saphenous vein rings without endothelium. The contractions evoked by transmural nerve stimulation and noradrenaline in the rings superfused with Krebs containing 3 μM L-NNA (□; $n = 10$) did not differ from those of the control group (△; $n = 10$). Conversely, in the presence of 10 μM methylene blue (○; $n = 10$), the transmural nerve stimulation-induced contractile responses were significantly different from those of controls ($P < 0.05$), while the contractions produced by noradrenaline overlapped the control responses. Means ± S.E.M. are shown.

presence of methylene blue were similar to those found in control rings ($F = 0.05$; $P > 0.05$).

4. Discussion

The aim of the present study was (i) to verify whether factors released by vascular endothelial cells modulate the adrenergic vasoconstriction in human saphenous veins and (ii) to identify the type of EDRF involved in this modulatory effect and to characterize the mechanisms by which such an effect may be exerted.

The results obtained show that the vasoconstrictor responses to transmural nerve stimulation were higher in endothelium-denuded than in intact endothelium human saphenous vein rings, suggesting that endothelium-derived factors have an inhibitory role on the vasoconstrictor effects of sympathetic nerve stimulation. To test whether arachidonic acid metabolites are involved in the contraction induced by transmural nerve stimulation, we studied the effects of the cyclooxygenase inhibitor indomethacin on unrubbed vein rings. Contractile responses to transmural nerve stimulation were unaffected by the presence of indomethacin, thus suggesting that in human saphenous vein, unlike in rat isolated aorta (Jeremy et al., 1985; Jeremy and Dandona, 1989), adrenergic contraction does not stimulate the synthesis of vasodilator prostanoids. Conversely, inhibition of NO synthesis by L-NNA produced an enhancement of the contractions induced by the neurally evoked adrenergic responses. Such enhancement was reversed by L-arginine, but not by D-arginine, in vessels with an intact endothelium. This indicates that NO synthesized in the endothelial cells from its precursor L-arginine can attenuate the vasoconstriction induced by sympathetic nerve stimulation.

All the above described effects on the contractions induced by endogenously released noradrenaline were also obtained for the contractions evoked in the same vessel rings by the stimulation of α-adrenoceptors through the exogenous administration of noradrenaline. In contrast to our results, in a study with human saphenous veins, Yang et al. (1991) reported that the presence of 10^{-4} M N^G-monomethyl-L-arginine (L-NMMA) did not affect the concentration-response curve for noradrenaline in human saphenous vein with an intact endothelium, although they demonstrated that the endothelium of these veins released NO. The likely explanation for these discrepant results is the more powerful inhibiting effect of L-NNA on vascular NO biosynthesis than that of L-NMMA (Hobbs and Gibson, 1990; Ishii et al., 1990; Moore et al., 1990) and/or to different experimental conditions (e.g., dose-response curves for single 1-min infusions in superfused vein rings vs. cumulative dose-response curves on vessels placed in an organ bath).

In any case, in our experiments the enhancement of the response to exogenous noradrenaline was observed in three different experimental groups, that is in endothelium-denuded vs. intact endothelium vessels and in the presence of L-NNA or L-NNA + D- arginine vs. control series. Hence, our data clearly demonstrate that factors produced by the endothelium, NO in particular, play an inhibitory role in the contractions evoked by noradrenaline in human saphenous veins. Although these veins have been shown to have a lower capacity for producing NO than human mammary arteries (Lüscher et al., 1988, Yang et al., 1991), this small quantity of NO released from the venous endothelium can modulate the contractions produced by both sympathetic stimulation and exogenous noradrenaline, as it does in human deferential arteries (Martinez et al., 1994). Thus, our data confirm the important physiological role of EDRF/NO in the control of the human venous tone (Collier and Vallance, 1989).

As mentioned in the Introduction, the mechanisms of the modulatory effect of EDRF/NO on sympathetic contractions appear to be controversial. In guinea-pig pulmonary artery, inhibition of the continuous basal release of NO from endothelial cells has been suggested as an explanation for the enhancing effect of L-NMMA on the contraction induced by adrenergic nerve stimulation (Liu et al., 1991), whereas the enhancing effect of endothelium removal on contractions elicited by transmural nerve stimulation in rat caudal artery has been attributed to endothelial factors released either at basal levels or by the stimulation of α-adrenoceptors (Hynes et al., 1988). Moreover, stimulation of NO production may be associated with an increase in vascular smooth muscle tone, as suggested by the results of experiments performed in rats in vivo (Vargas et al., 1990).

Our data regarding human saphenous veins provide further insight into the mechanisms by which NO may modulate adrenergic contractions. Firstly, since L-NNA, at the concentration used in the present work, had no effect on the basal resting tone, an inhibition of the basal release of NO from endothelial cells cannot account for the enhancement of the vasoconstrictor responses to sympathetic nerve stimulation or to noradrenaline. Secondly, in our experiments, the removal of the endothelium did not affect the contraction of the human saphenous vein rings evoked by KCl, as in the case of the vasoconstriction produced by endothelin-1 in the same veins (Lüscher et al., 1990). This may indicate that the contractions produced by KCl, which are mainly due to the influx of extracellular calcium into the smooth muscle cells, and by endothelin, which are also dependent on the release of calcium from intracellular stores (Tanoi et al., 1992), are not a sufficient stimulus to increase the production of NO. Thus, specific modulation by the endothelium of contractions elicited by transmural nerve stimulation and noradrenaline may be suggested.

By contrast with the results we obtained with KCl, the contractions evoked by 5-HT, as well as those evoked by noradrenaline, were greater in vein rings without endothelium than in rings with an intact endothelium. Since 5-HT-induced contractions were potentiated by L-NNA even

in the presence of D-arginine, but not in the presence of L-arginine, our data allow us to identify NO as the major factor released from endothelial cells and to suggest that it is involved in the modulation of the contractions elicited by 5-HT, as it is in the case of sympathetic stimulation and noradrenaline.

Hence, these data can be reasonably interpreted only if the activation of α-adrenergic and 5-HT receptors located on the endothelial cells of human saphenous veins leads to an increased release of NO. Even though we cannot provide any evidence for the existence of such receptors, this possibility cannot be ruled out. Stimulation of 5-HT receptors located on endothelial cells has also been suggested by Urabe et al. (1991) as being responsible for the endothelium removal-induced potentiation of the contractions elicited by 5-HT in rat mesenteric and femoral arteries. Moreover, the stimulated release of EDRF by exogenous agonists via endothelial α_2-adrenoceptors has been suggested in dog coronary artery (Cocks and Angus, 1983) and in rat aorta (Egleme et al., 1984). It is of interest to note that adrenoceptors of the α_2 type have been shown to be preponderant in human saphenous vein (Smith et al., 1992). Furthermore, given that L-NNA enhances the contractile responses to both transmural nerve stimulation and noradrenaline to the same extent (mean potentiating effects: $94 \pm 12\%$, $n = 35$ and $99 \pm 35.3\%$, $n = 28$, for transmural nerve stimulation and noradrenaline, respectively), we suggest that NO produced by endothelial cells inhibits sympathetic contractile responses, probably by acting at a postjunctional level.

Because NO causes vasorelaxation by activating soluble guanylyl cyclase, resulting in increased intracellular cGMP (Ignarro, 1989), we also studied the effects of methylene blue. This synthetic phenothiazine dye has been shown to inhibit vasorelaxations induced by NO, hydrogen peroxide (Burke and Wolin, 1987) or organic peroxide (Thomas and Ramwell, 1986), and to suppress the activation of soluble guanylyl cyclase by nitrovasodilators (Ignarro et al., 1987b). Also in the presence of methylene blue, the vasoconstrictor responses induced by transmural nerve stimulation and noradrenaline in human saphenous vein with endothelium were enhanced. This fact further confirms the involvement of a vasodilator factor like NO, that acts at a postsynaptic level on vascular smooth muscle cells through the guanylyl cyclase pathway.

We reasoned that if these hypotheses were correct, the inhibition of either L-arginine or guanylyl cyclase pathways would fail to enhance the contractions elicited by transmural nerve stimulation and noradrenaline in human saphenous vein denuded of endothelium. Indeed we found that the contractions induced by both transmural nerve stimulation and exogenous noradrenaline were not potentiated in the presence of L-NNA, confirming the endothelial source of NO. Moreover, in view of the fact that the contractions induced by transmural nerve stimulation were unaffected by L-NNA, these data may also provide evidence against the hypothesis of modulation by NO released by electrical stimulation of NANC nerves, as suggested for various arteries (Liu et al., 1992; Rand, 1992). However, by contrast with the results obtained with L-NNA, in endothelium-denuded human saphenous vein rings an enhancement of the contractions evoked by transmural nerve stimulation was obtained in the presence of methylene blue, even though this increase was smaller than that observed in human saphenous vein with endothelium. Indeed, in intact rings 10 μM methylene blue enhanced the mean neural adrenergic contractions at 1 and 2 Hz by 81 and 72%, respectively, whereas in human saphenous vein rings without endothelium the contractions evoked by transmural nerve stimulation at 1 and 2 Hz in the presence of the guanylyl cyclase inhibitor were augmented by only 25 and 15%, respectively, in comparison to those of control preparations. Moreover, the fact that this potentiation was observed only for the vasoconstrictor responses to transmural nerve stimulation, and not for those induced by exogenous noradrenaline, suggests that neurally evoked adrenergic contractions are modulated also by an endothelium-independent component different from NO. The release of an unidentified relaxing factor that acts through cGMP and which is activated by sympathetic stimulation may represent a stimulating hypothesis.

In conclusion, these results, taken together with those for human saphenous vein with an intact endothelium, suggest that methylene blue enhances sympathetic contractions mainly through the inhibition of endothelial-derived NO, although the inhibition of the vasorelaxing effect of an unknown neurally released agent, different from NO but acting through the guanylyl cyclase pathway, may also be contributory. Several physiological compounds, such as atrial natriuretic factor and some other atrial peptides, have been found to increase cGMP and relax vascular smooth muscle (Lincoln, 1989). However, apart from its guanylyl cyclase inhibiting property, methylene blue has been claimed to increase the release of noradrenaline from adrenergic nerve endings (Soares-Da-Silva and Caramona, 1988). Thus an increased release of noradrenaline by methylene blue during sympathetic nerve stimulation cannot be excluded.

In summary, in the human saphenous vein, sympathetic vasoconstriction is modulated by at least two pathways: one endothelium-dependent, the other endothelium-independent. The former is mediated by the release of NO from endothelial cells, probably through the activation of α_2-adrenoceptors located on these cells. The nitric oxide-independent pathway probably involves the release of an unknown comediator of noradrenaline, acting predominantly through cGMP. Thus, these results indicate that: (1) in addition to its important role in controlling arterial vascular tone, NO may also regulate venous capacitance and (2) cGMP is a key modulatory component in the vasoconstriction induced by the stimulation of sympathetic nerves.

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