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Role of endothelin receptors, calcium and nitric oxide in the potentiation by endothelin-1 of the sympathetic contraction of rabbit ear artery during cooling

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1 To examine further the potentiation by endothelin-1 on the vascular response to sympathetic stimulation, we studied the isometric response of isolated segments, 2 mm long, from the rabbit central ear artery to electrical field stimulation (1–8 Hz), under different conditions, at 37°C and during cooling (30°C).

2 Electrical stimulation produced frequency-dependent contraction, which was reduced (about 63% for 8 Hz) during cooling. At 30°C, but not at 37°C, endothelin-1 (1, 3 and 10 nM) potentiated the contraction to electrical stimulation in a dose-dependent way (from 43±7% to 190±25% for 8 Hz).

3 This potentiation by endothelin-1 was reduced by the antagonist for endothelin ET_A receptors BQ-123 (10 μM) but not by the antagonist for endothelin ET_B receptors BQ-788 (10 μM). The agonist for endothelin ET_B receptors IRL-1620 (0.1 μM) did not modify the contraction to electrical stimulation.

4 The blocker of L-type Ca^{2+} channels verapamil (10 μM l^{-1}) reduced (about 72% for 8 Hz) and the unspecific blocker of Ca^{2+}-channels $NiCl_2$ (1 mM) practically abolished (about 98%), the potentiating effects of endothelin-1 found at 30°C.

5 Inhibition of nitric oxide synthesis with N^G-nitro-L-arginine (L-NOARG, 0.1 mM) increased the contraction to electrical stimulation at 30°C more than at 37°C (for 8 Hz, this increment was 297±118% at 30°C, and 66±15% at 37°C). Endothelium removal increased the contraction to electrical stimulation at 30°C (about 91% for 8 Hz) but not at 37°C. Both L-NOARG and endothelium removal abolished the potentiating effects of endothelin-1 on the response to electrical stimulation found at 30°C.

6 These results in the rabbit ear artery suggest that during cooling, endothelin-1 potentiates the contraction to sympathetic stimulation, which could be mediated at least in part by increasing Ca^{2+} entry after activation of endothelin ET_A receptors. This potentiating effect of endothelin-1 may require the presence of an inhibitory tone due to endothelial nitric oxide.

Keywords: Cutaneous arteries; temperature; endothelin ET_A receptors; endothelium; Ca^{2+}-channels

Introduction

Endothelin-1 is a 21-amino acid peptide that is synthesized and released from endothelial cells. In addition to producing a powerful contraction of blood vessels (Yanagisawa *et al*., 1988), this peptide may modulate the sympathetic vascular response by increasing the constriction to sympathetic nerve stimulation in several vascular beds. This endothelin-1-mediated potentiation of sympathetic constriction has been observed in rat renal circulation (Reid, 1993), guinea-pig pulmonary artery (Wiklund *et al*., 1989) and rabbit saphenous (Mutafova-Yambolieva & Radomirov, 1994), jejunal (La & Rand, 1993) and central ear (Wong-Dusting *et al*., 1991) arteries. In rabbit jejunal (La & Rand, 1993) and in saphenous (Mutafova-Yambolieva & Radomirov, 1994) arteries, this potentiation is mainly due to facilitation of the response of postjunctional P2 receptors to adenosine 5′-triphosphate (ATP) released from sympathetic nerve endings. The noradrenergic component of the sympathetic response may also be facilitated by endothelin-1, as this peptide potentiates the vascular contraction to exogenous noradrenaline (Yang *et al*., 1990).

In a previous study from our laboratory (Padilla *et al*., 1997), we observed that in the rabbit ear artery endothelin-1 potentiates the contraction to sympathetic stimulation during cooling, but not during normotemperature and warming, thus this peptide partly reverted the inhibition that cooling produced on the sympathetic contraction. This cooling-induced decrease of the vascular contraction to sympathetic stimulation may be due to reduction of the α_1-adrenoceptor-mediated component without affecting the P2-receptor component, and endothelin-1 may potentiate this contraction during cooling by increasing the responsiveness of postjunctional α_2-adrenoceptors and P2-receptors (Garcia-Villalón *et al*., 1997.

The objective of the present study was to analyse further the mechanisms underlying the potentiating effects of endothelin-1 on the vascular contraction to sympathetic stimulation, by studying the subtype of endothelin receptors, ET_A and ET_B, involved, as well as the role of nitric oxide and Ca^{2+}-channels in these effects of endothelin-1. The experiments were performed in isolated segments from rabbit ear artery, which has been used as a model of cutaneous blood vessels (Patton & Wallace, 1978; Roberts & Zygmunt, 1984; Harker & Vanhoutte, 1988). In this artery, the effects of endothelin-1 on electrical field stimulation were recorded isometrically at 37°C and during cooling to 30°C; 30°C was selected as it was the temperature in the 34–24°C range where the maximal potentiating effects of endothelin-1 on electrical field stimulation in rabbit ear artery were found (Padilla *et al*., 1997).

Methods

Twenty Male New Zealand White rabbits, weighing 2–2.5 kg, were killed by intravenous injection of sodium pentobarbitone, 100 mg kg^{-1}. Central ear arteries were dissected free and cut into cylindrical segments 2 mm in length. Each segment was prepared for isometric tension recording in a 6 ml organ bath containing modified Krebs-Henseleit solution of the following

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composition (mM): NaCl 115, KCl 4.6, KH_2PO_4 1.2, $MgSO_4$ 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 25 and glucose 11.1. The solution was equilibrated with 95% oxygen and 5% carbon dioxide to give a pH of 7.3–7.4, which was measured with a pH-meter micropH 2001 (Crison Instruments). Briefly, the method consists of passing two fine, stainless steel pins, 150 μm in diameter, through the lumen of the vascular segment. One pin is fixed to the organ bath wall, while the other is connected to a strain gauge for isometric tension recording, thus permitting the application of passive tension in a plane perpendicular to the long axis of the vascular cylinder. The recording system included a Universal Transducing Cell UC3 (Statham Instruments, Inc.), a Statham Microscale Accessory UL5 (Statham Instruments, Inc.) and a Beckman Type RS Recorder (model R-411, Beckman Instruments, Inc.). A previously determined resting passive tension of 0.5 g was applied to the vascular segments and they were then allowed to equilibrate for 60-90 min before any drug was added. The temperature of the bath was adjusted from the beginning of the experiment at 37°C and 30°C (cooling), and the arteries remained at the chosen temperature throughout the duration of the experiment.

Electrical field stimulation (1, 2, 4 and 8 Hz, 0.2 ms pulse duration, at a supramaximal voltage of 70 V, for 5 s) was applied to the arteries via two platinum electrodes placed either side of the artery and connected to a CS-14 stimulator (Cibertec). An interval of at least 5 min was imposed between stimulation periods to allow recovery of the response and the stimulation trains were repeated until the responses were reproducible during at least 40 min under control conditions. Then, the effects of endothelin-1 (1, 3 and 10 nM) on the arterial response to electrical stimulation were studied by cumulative addition to the organ bath. Electrical field stimulation (1–8 Hz) was applied twice after each concentration of the peptide, each arterial segment was treated with the three concentrations of endothelin-1. The effect of this peptide on the response to electrical stimulation was studied in the arterial segments at 37°C and 30°C, and each arterial segment was tested at only one of these temperatures.

In some cases, the highest concentration of endothelin-1 produced contraction of the arterial segments, but usually this contraction diminished after electrical stimulation. If the contractile tone induced by endothelin-1 did not return to less than 25% of the maximal contraction achieved with electrical stimulation, the data of these particular segments were discarded.

To analyse the role of endothelin ET_A and ET_B receptors, the effects of endothelin-1 on the arterial response to electrical stimulation were recorded at 37°C and 30°C in the presence of the endothelin ET_A receptor antagonist BQ-123 (10 μM) and in the presence of the endothelin ET_B receptor antagonist BQ-788 (10 μM). Also, the effect of the endothelin ET_B receptor agonist IRL-1620 (0.1 μM) on the response to electrical stimulation was studied at both temperatures.

To analyse the role of Ca^{2+} channels, the effects of endothelin-1 on the response to electrical stimulation were also recorded at 37°C and 30°C in the presence of $NiCl_2$ (1 mM) and verapamil (10 μM), unspecific (Narahashi *et al*., 1987) and L-type-specific (McDonald *et al*., 1994) Ca^{2+}-channel blockers, respectively.

To analyse the role of nitric oxide and of vascular endothelium, the effects of endothelin-1 on the response to electrical stimulation at 37°C and 30°C were studied in arteries without endothelium or in arteries with endothelium pretreated with the inhibitor of nitric oxide synthesis N^G-nitro-L-arginine (L-NOARG, 0.1 mM). Endothelium removal was accomplished by gently rubbing the vascular lumen with a steel rod, and tested by the abolition of the relaxing response to acetylcholine (10 μM) after precontraction with endothelin-1 (0.1 μM).

After reproducible responses to electrical stimulation (1–8 Hz) were obtained over 40 min, one of the antagonists was added to the organ bath, and then, 1 to 8 Hz stimulation applied twice in the presence of the corresponding antagonists. After these series of electrical stimulations, endothelin-1 (1–10 nM) was added cumulatively to the organ bath, which contained the antagonist previously applied, and the response to electrical stimulation was recorded in the arteries in the presence of each concentration of endothelin-1. As a control one vascular segment, which was treated with endothelin-1 but not with any of the antagonists was studied at each temperature.

Contraction amplitudes are expressed as mean ± s.e.mean and the results were evaluated by two-way analysis of variance applied to each group of data, followed by paired Student's *t* test corrected by the Bonferroni method for multiple comparisons. A probability value of less than 0.05 was considered significant when a single pair of means were compared, or 0.05 divided by the number of comparisons when multiple means were compared with a control.

Drugs used were: N^G-nitro-L-arginine (L-NOARG; nickel chloride hexahydrate ($NiCl_2$); verapamil hydrochloride, all from Sigma; endothelin-1 (human, porcine) from Peninsula Laboratories Europe, Ltd; cyclo (D-α-aspartyl-L-prolyl-D-valyl-L-leucyl-D-tryptophyl, peptide free base (BQ-123); N-(N-(N- (2,6- dimethyl -1- piperidinyl) carbonyl) -4- methyl - L-leucyl) -1- (methoxycarbonyl) -D- tryptophyl) D-norleucine monosodium (BQ-788) and endothelin-1 (8.21), N-Suc-(Glu^9, $Ala^{11,15}$), peptide free base (IRL1620) from Research Biochemicals International. All drugs were dissolved in distilled water and further diluted in isotonic NaCl.

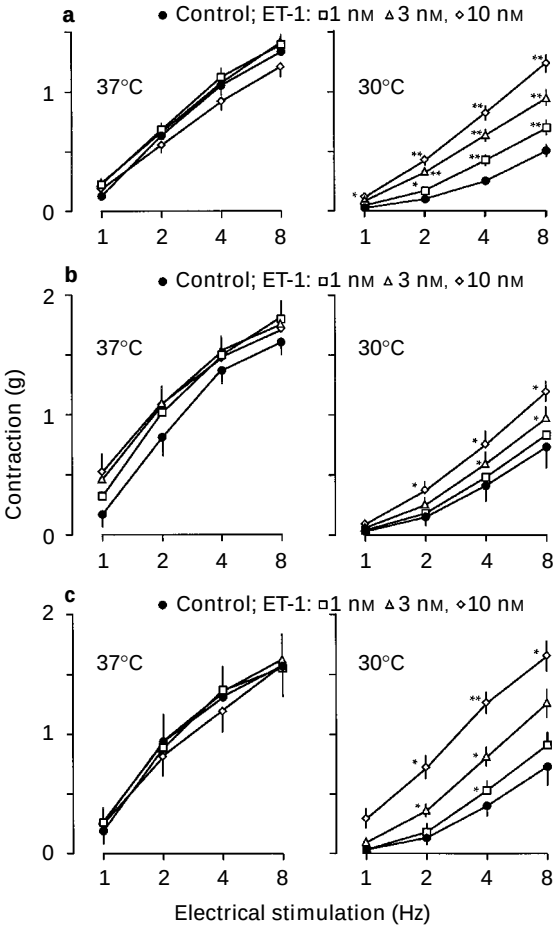

Figure 1 Contraction of rabbit central ear arteries to electrical field stimulation (1–8 Hz, 0.2 ms pulse duration, 70 V, 5 s) in control conditions and in the presence of endothelin-1 (ET-1, 1–10 nM), at 37°C and 30°C. This was performed: (a) in the absence of antagonists; (b) in the presence of the antagonist of endothelin ET_A receptors BQ-123 (10 μM) and (c) in the presence of the antagonist of endothelin ET_B receptors BQ-788 (10 μM). Points are means and vertical lines show s.e.means. Data significantly different from that found in the absence of endothelin-1: $^*P<0.01$, $^{**}P<0.001$. Data were averaged from 20 animals in (a) and from 6 animals in (b) and (c).

Results

Response to electrical stimulation

Electrical stimulation (1–8 Hz) produced frequency-dependent contraction of the vascular segments at 37°C and 30°C, and at 30°C the response at every frequency of stimulation applied was significantly lower ($P<0.001$) than at 37°C (0.5 ± 0.07 vs 1.35 ± 0.13 g for 8 Hz) (Figure 1a).

Effects of endothelin-1 on the response to electrical stimulation

Control At 37°C, addition of endothelin-1 (1–10 nM) did not modify significantly the contraction to electrical stimulation, but at 30°C it produced a concentration-dependent increase in this response (Figure 1a).

Endothelin ET_A and ET_B receptor subtypes involved Pretreatment with either the ET_A receptor antagonist BQ-123 (10 μM) or the ET_B receptor antagonist BQ-788 by themselves did not modify the arterial response to electrical stimulation. At 37°C, addition of endothelin-1 after pretreatment with BQ-123 did not modify the response to electrical stimulation (Figure 1b). At 30°C, in the presence of BQ-123, endothelin-1 still increased the response to electrical stimulation, but this increment was lower ($P<0.001$) than the control potentiation (Figures 1a and b). At 37°C, in the presence of BQ-788 (10 μM), endothelin-1 did not modify the response to electrical stimulation, and at 30°C it potentiated the contraction to electrical stimulation in a similar degree than in the absence of this antagonist (Figure 1a and c).

The ET_B receptor agonist IRL-1620 (0.1 μM) did not modify the contraction of ear arteries to electrical stimulation at either 37°C or 30°C (not shown).

Role of Ca^{2+} The blocker of Ca^{2+} channels $NiCl_2$ (1 mM) reduced ($P<0.001$) the response to electrical stimulation at 37°C ($48\pm9\%$ for 8 Hz) and at 30°C ($26\pm7\%$ for 8 Hz) (Figure 2a), whereas the blocker of L-type Ca^{2+} channels verapamil (1 μM) did not modify this response at either temperature (Figure 2b).

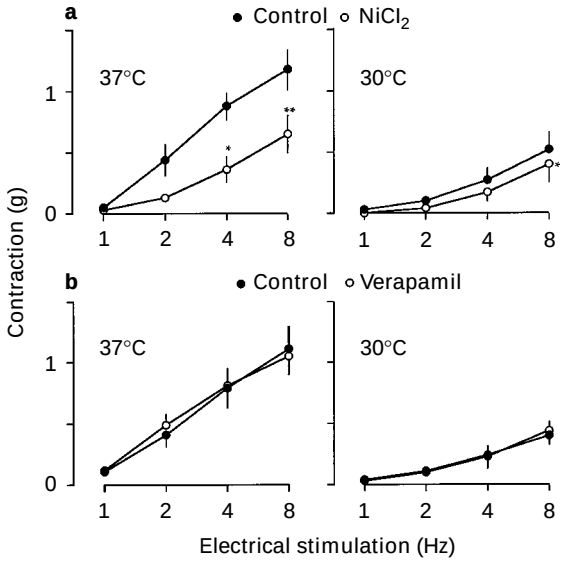

Figure 2 Contraction of rabbit central ear arteries to electrical field stimulation (1–8 Hz, 0.2 ms pulse duration, 70 V, 5 s) in arteries in control conditions and in the presence of: (a) $NiCl_2$ (1 mM) and (b) verapamil (10 μM), at 37°C and 30°C. Points are mean and vertical lines show s.e.mean. Data significantly different from control: $*P<0.05$, $**P<0.01$. In each case, data were averaged from 6–7 animals.

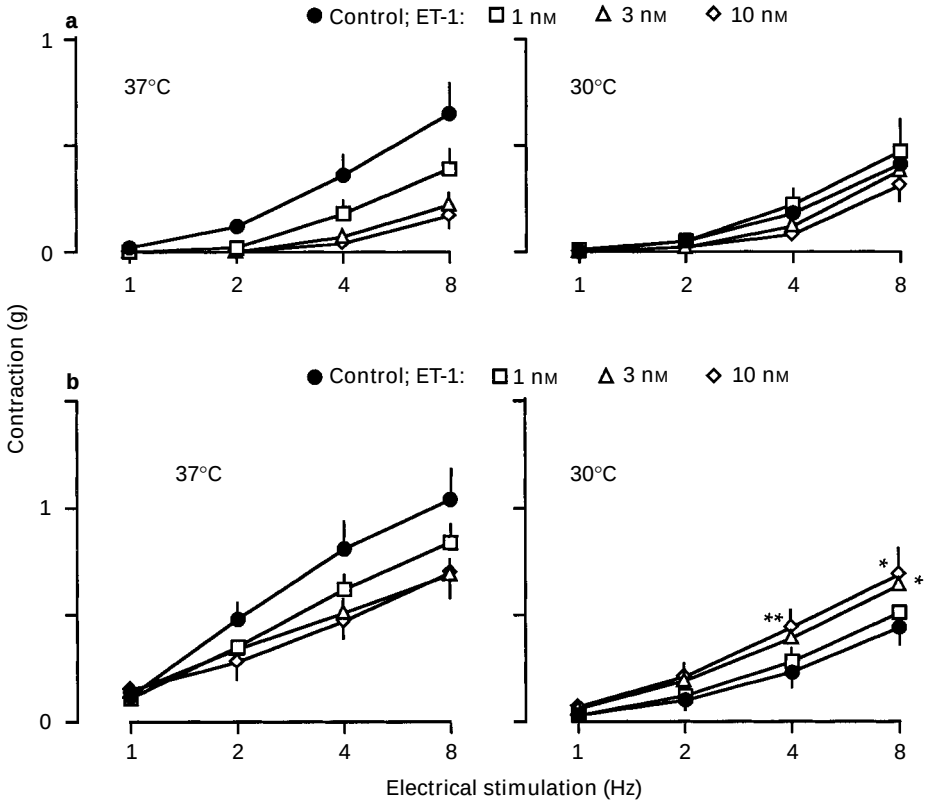

Figure 3 Contraction of rabbit central ear arteries to electrical field stimulation (1–8 Hz, 0.2 ms pulse duration, 70 V, 5 s) in control conditions and in the presence of endothelin-1 (ET-1; 1–10 nM), at 37°C and 30°C. This was performed: (a) in the presence of $NiCl_2$ (1 mM) and (b) in the presence of verapamil (10 μM). Points are mean and vertical lines show s.e.mean. Data significantly different from that in the absence of endothelin-1: $*P<0.01$, $**P<0.001$. In each case, data were averaged from 6–7 animals.

At 37°C, in the presence of $NiCl_2$ (Figure 3a) or verapamil (Figure 3b), endothelin-1 reduced the response to electrical stimulation. At 30°C, $NiCl_2$ abolished ($P<0.001$) (Figure 3a) and verapamil reduced ($P<0.001$) (Figure 3b) the potentiating effect of endothelin-1 on the contraction to electrical stimulation.

Nitric oxide synthesis inhibition and endothelium removal Pretreatment of the arteries with L-NOARG (0.1 mM) increased the contraction to electrical stimulation at 30°C and 37°C, and this increase was higher ($P<0.001$) in percentage but not in absolute values, at 30°C ($297\pm118\%$ for 8 Hz) than at 37°C ($66\pm15\%$ for 8 Hz) (Figure 4a).

Endothelium removal increased the response to electrical stimulation at 30°C (about 90% for 8 Hz), but not at 37°C, as compared with that recorded in intact arteries at the corresponding temperatures (Figure 4b).

Application of endothelin-1 after L-NOARG failed to modify further that response at 30°C or 37°C as compared with that found in the presence of L-NOARG alone (Figure 4c).

In arteries with endothelium removed, the presence of endothelin-1 did not modify the contraction to electrical stimulation at 37°C or 30°C (Figure 4d).

Discussion

We have found in this study that cooling decreases the contraction of rabbit central ear artery to electrical field stimulation and that endothelin-1 potentiates this contraction during cooling, but not at 37°C, thus confirming previous studies from our laboratory (Padilla *et al*., 1997; García-Villalón *et al*., unpublished data). In one of these studies (García-Villalón *et al*., 1997) the data suggest that cooling depresses the response mediated via α_1-adrenoceptors, perhaps by a prejunctional mechanism, and that the potentiation by endothelin-1 during cooling may be due to this peptide increasing the responsiveness of postjunctional α_2-adrenoceptors and P2-receptors.

In the present study we have analysed further possible mechanisms of the interaction of endothelin-1 with the vascular sympathetic response. The present results suggest that the potentiating effects of endothelin-1 on the arterial contraction to electrical stimulation during cooling may be mediated by the ET_A receptor subtype, as this potentiating effect was reduced by the antagonist for these receptors BQ-123. The observation that BQ-123 only partially blocked the endothelin-1 potentiating effects could suggest that the receptors involved are of the ET_{A2} subtype, as they are relatively resistant to this antagonist (Sudjarwo *et al*., 1994). However, to clarify this issue, more specific antagonists should be used. We also found that the antagonist for ET_B receptors BQ-788 did not modify the endothelin-1 potentiating effects and that the agonist for ET_B receptors IRL 1620 did not modify the arterial response to electrical stimulation. These findings suggest that ET_B receptors are probably not involved and as BQ-788 is antagonist for both ET_{B1} and ET_{B2} subtypes of ET_B receptor (Karaki *et al*., 1994), the data indicate that neither of these two receptor subtypes are involved in the endothelin-1-induced effects in the vascular contraction to sympathetic stimulation.

Moreover, we have analysed the role of Ca^{2+} in the interaction between endothelin-1 and sympathetic contraction by using Ca^{2+}-channel antagonists. The potentiation produced by endothelin-1 during cooling was abolished by $NiCl_2$, which at the concentration used (1 mM) may be a relatively unspecific Ca^{2+}-channel blocker (Narahashi *et al*., 1987), therefore this potentiating effect of endothelin-1 may be related to activation of membrane Ca^{2+}-channels. Verapamil reduced this potentiating effect of endothelin-1, but a significant response was still present after treatment with this blocker. The concentration of verapamil used (10 μM) should be enough to block L-type Ca^{2+}-channels, as lower or similar concentrations (3–10 μM) abolished the contraction to the L-type Ca^{2+}-channel agonist Bay K 8644 (Hagiwara *et al*., 1993; Asano *et al*., 1995). Therefore, it is hypothesized that the potentiating effects of endothelin-1 are mediated in part by L-type Ca^{2+}-channels and in part by non-L-type Ca^{2+}-channels. These non-L, verapamil resistant, $NiCl_2$-sensitive channels might be of the T-type, as this type of channel, together with the L-type has been found in rabbit ear artery smooth muscle cells (Benham *et al*., 1987). Findings in porcine coronary artery suggest that endothelin-1 activates Ni^{2+}-sensitive, non-L-type Ca^{2+}-channels (Blackburn & Highsmith, 1990). Nevertheless, in rabbit mesenteric artery (Yoshida *et al*., 1994), rat aorta (Shimamoto *et al*., 1992) and human coronary artery (Stork & Cocks, 1994)

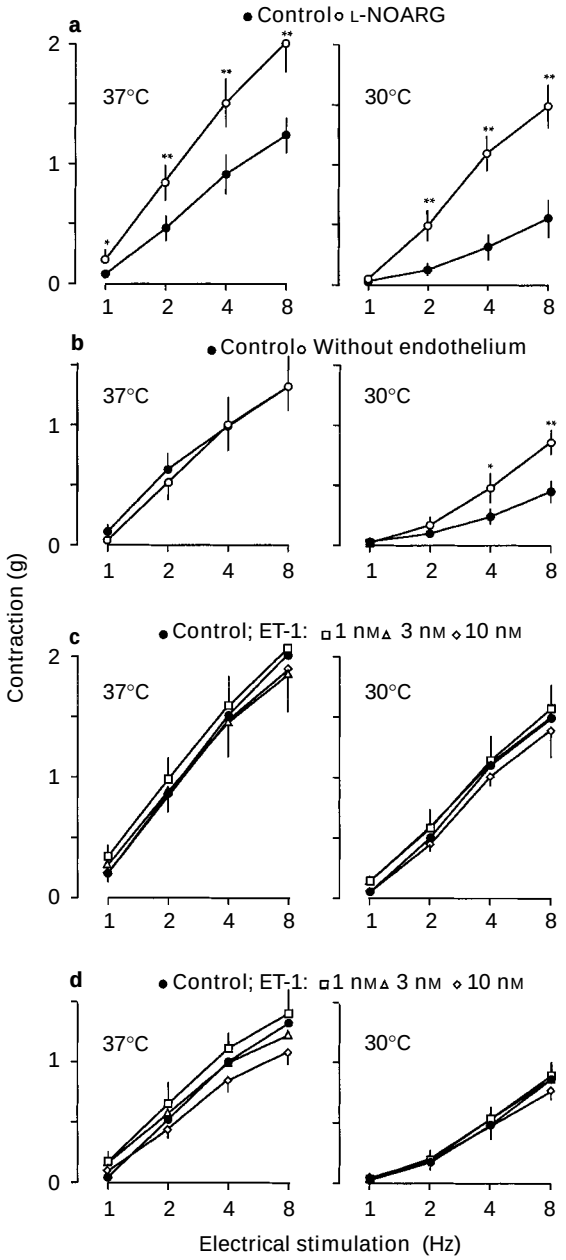

Figure 4 Contraction of rabbit central ear arteries to electrical field stimulation (1–8 Hz, 0.2 ms pulse duration, 70 V, 5 s): (a) in arteries under control conditions and in the presence of L-NOARG (0.1 mM), (b) in arteries under control conditions and without endothelium; (c) in arteries pretreated with L-NOARG (0.1 mM) and (d) in arteries without endothelium, in the absence (control) and in the presence of endothelin-1 (1–10 nM); at 37°C and 30°C. Points are mean and vertical lines show s.e.mean. Data significantly different from control: $*P<0.05$, $**P<0.01$. In each case, data were averaged from 6–7 animals.

the direct contraction by endothelin-1 seems to be mediated mainly by a dyhydropiridine-sensitive (probably L-type) Ca^{2+}-channel. La & Rand (1993) found that the direct contractile effect of endothelin-1, but not its potentiating effect on the purinergic contraction of the rabbit ear and jejunal arteries is dependent on L-type channels, suggesting that these two effects of endothelin-1 may be mediated by different mechanisms.

Nitric oxide is another endothelial factor which probably plays an important role in the regulation of cardiovascular function, by producing relaxation or limiting contraction of blood vessels to several types of vasoactive stimuli (Umans & Levi, 1995). In the present study we have found that inhibition of nitric oxide synthesis with L-NOARG increased the contraction of rabbit ear arteries to electrical stimulation, proportionally more during cooling than at 37°C, suggesting that nitric oxide inhibits the contraction to sympathetic stimulation, particularly during cooling. This nitric oxide may be released, at least in part, from the endothelium as we found that during cooling endothelium removal also increased the vascular response to sympathetic stimulation. This agrees with previous results from our laboratory where it is suggested that cooling may increase the release of nitric oxide in the rabbit ear artery (Fernández *et al*., 1994b), resulting in reduced contraction to endothelin-1, noradrenergic stimulation, histamine or 5-hydroxytryptamine (Monge *et al*., 1991; García-Villalón *et al*., 1992; Fernández *et al*., 1994a; 1995) and increased relaxation in response to muscarinic stimulation (Monge *et al*., 1993; García-Villalón *et al*., 1995). Therefore, an increased nitric oxide release may be involved in the reduced vascular response to sympathetic stimulation during cooling. In addition to this, prejunctional α_2-adrenoceptors and reduction of neurotransmitter release at perivascular nerve endings may also be involved in this reduction of the vascular response (García-Villalón *et al*., 1997).

After nitric oxide inhibition with L-NOARG or endothelium removal, endothelin-1 failed to increase further the contraction to electrical stimulation, as compared with that obtained in the absence of this peptide, at 37°C and during cooling. These observations suggest that the potentiating effect of endothelin-1 observed during cooling may require of the presence of an inhibitory vascular tone produced by nitric oxide. Studies by others have shown that endothelin-1 (0.1–1 nM) potentiates the effects of electrical stimulation in rabbit ear (La *et al*., 1991) and jejunal (La & Rand, 1993) arteries at 37°C, and that this potentiation is endothelium-independent (La *et al*., 1991). On the other hand, Yang *et al*. (1990) have shown that endothelin-1 (0.3–1 nM) amplifies the response of human mammary arteries to exogenous noradrenaline at 37°C, and that this potentiation is nitric oxide independent. As we did not find a potentiating effect by endothelin-1 at 37°C, the discrepancy between these studies (Yang *et al*., 1990; La *et al*., 1991; La & Rand, 1993) and ours may reside in differences in experimental preparations and approach used. One of these differences may be that in these studies lower endothelin-1 concentrations than in ours were used. Also, in human mammary artery (Yang *et al*., 1990) and rabbit jejunal arteries (La & Rand, 1993) distribution of endothelin receptor subtypes may differ from the rabbit ear artery. Discrepancies with the observations of La *et al*. (1991), who also used rabbit ear arteries, may be because they used arteries perfused through the arterial lumen and superfused through the adventitial surface, and stimulated with 1 ms square wave pulses. Also, mechanisms involved in the interaction between endothelin-1 and sympathetic response may differ at 37°C and 30°C.

In conclusion, the present results in the rabbit ear artery (cutaneous artery) suggest that endothelin-1 potentiates the response to sympathetic stimulation during cooling through both T-type and L-type Ca^{2+} channels after activation of ET_A receptors. This potentiating effect of endothelin-1 may require the presence of an inhibitory vascular tone by endothelial nitric oxide. A potentiating effect of endothelin-1 on arterial contraction during cooling may counteract the inhibitory effects of nitric oxide, which may be augmented at low temperature. Therefore, endothelin-1 and nitric oxide may have an opposite effect on the sympathetic vascular response, and the opposite effects of these two substances may be in balance in cutaneous vasculature during cooling. By modulating this balance, the endothelium through endothelin-1 and nitric oxide may regulate vascular reactivity and participate in the regulation of cutaneous blood flow during changes of temperature.

If the present results can also be obtained in man, they might be of relevance for understanding the pathophysiology of some alterations of the cutaneous circulation, e.g., Raynaud's phenomenon. Our results suggest that endothelin-1 might facilitate vasospasm by increasing the cutaneous vasoconstriction to sympathetic noradrenergic stimulation during cooling. The potential importance of this hypothesis is supported by the observation that exposure of the body surface to cold in patients with Raynaud's phenomenon induces an intense cutaneous vasoconstriction, and that plasma levels of endothelin-1 are increased during cooling in these patients (Kanno *et al*., 1991). The present results suggest that specific antagonists of endothelin ET_A receptors might be potentially useful in the treatment of this disturbance.

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