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Regulation of nitric oxide-like activity by prostanoids in smooth muscle of the canine saphenous vein

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1 Organ bath experiments and measurements of prostanoids were performed to investigate the presence of nitric oxide synthase in venous smooth muscle and its interaction with cyclo-oxygenase.

2 In rings of canine saphenous vein without endothelium, the inhibitor of cyclo-oxygenase, indomethacin (10 μM), induced contraction. N^G-nitro-L-arginine (100 μM) (L-NOARG), an inhibitor of nitric oxide synthase did not affect the tone of rings of canine saphenous vein when administered alone. However, in the presence of indomethacin L-NOARG (100 μM) induced further contraction.

3 Similar results were obtained in response to N^G-monomethyl-L-arginine (L-NMMA)(300 μM or N^G-nitro-L-arginine methylester (L-NAME)(100 μM).

4 When rings of canine saphenous vein without endothelium were contracted with phenylephrine (1 μM) instead of indomethacin, neither L-NOARG or L-NMMA induced further contraction.

5 When rings of canine saphenous vein without endothelium were contracted with noradrenaline (0.3 μM) in the presence of indomethacin (10 μM) plus L-NOARG (100 μM), a relaxation to L-arginine was observed. Transient relaxations to superoxide dismutase (150 u ml^{-1}) were observed in all rings.

6 When rings of saphenous vein without endothelium were incubated with lipopolysaccharide (LPS) (100 μg ml^{-1}) or interleukin -1β (10 u ml^{-1}) the concentration-contraction curve to noradrenaline was not affected.

7 Rings without endothelium released prostaglandin E_2 and prostaglandin I_2, as measured by radiommunoassay. The basal production was abolished by indomethacin and not affected by L-NOARG.

8 These results suggest that when cyclo-oxygenase is inhibited, a nitric oxide synthase activity is revealed in rings of canine saphenous vein without endothelium.

Keywords: Nitric oxide; N^G-nitro-L-arginine; saphenous vein; indomethacin; lipopolysaccharide.

Introduction

Nitric oxide is produced from the terminal guanido nitrogen atom of L-arginine (Palmer *et al.*, 1988) by at least two isoforms of NO-synthase. The constitutive nitric oxide synthase, which is calmodulin and calcium-dependent was first isolated in the central nervous system (Bredt & Snyder., 1990) and thereafter described in endothelial cells (Busse & Mulsch, 1990a). In vascular smooth muscle cells, nitric oxide is produced by an enzyme that is induced by inflammatory agents such as cytokines or lipopolysaccharide (Busse & Mulsch., 1990b; Julou-Schaeffer *et al.*, 1990; Beasley *et al.*, 1991; Schini *et al.*, 1991). The activity of this inducible enzyme (NO synthase) is inhibited by analogues of L-arginine. The inducible smooth muscle isoform of the enzyme is not dependent on Ca^{2+} and is not blocked by calmodulin-inhibitors (Fleming *et al.*, 1990; Schini & Vanhoutte, 1992); its characteristics are similar to those of the inducible enzyme described in macrophages and fibroblasts (Fleming *et al.*, 1991). In contrast to arteries, the role of NO in venous relaxation is of less importance than certain metabolites of arachidonic acid (Altura *et al.*, 1972; DeMey & Vanhoutte, 1982). NO synthase can be induced in the smooth muscle of the rabbit jugular vein by endotoxaemia (Vallance *et al.*, 1992). Recent evidence suggests that nitric oxide can regulate the activity of cyclo-oxygenase enzymes (Salvemini *et al.*, 1993). In an attempt to characterize further the contribution of NO synthase to venous reactivity and of its interaction with cyclo-oxygenase, the present experiments were designed to examine the effects of inhibitors of NO synthase and cyclo-oxygenase on the tone of smooth muscle of canine isolated saphenous veins.

Methods

Organ bath experiments

Experiments were performed on lateral saphenous veins taken from mongrel dogs of either sex (15-30 kg) anaesthetized with sodium pentobarbitone (30 mg kg^{-1}, i.v.). Immediately after excision, the tissues were placed in ice-cold modified Krebs-Ringer bicarbonate solution of the following composition (mM): NaCl 118.3, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, ethylenediamine tetraacetate 0.026 and glucose 11.1 (control solution). The blood vessels were cleaned of adherent connective tissue and cut into rings (4-5 mm in length). In each ring, the endothelium was removed by gently rubbing the intimal surface with the tip of a small forceps. Rings were suspended between two stirrups in organ baths (25 ml) filled with control solution (gassed with 95% O_2/5%CO_2, maintained at 37°C, pH 7.4). One of the stirrups was anchored inside the organ bath and the other connected to a force transducer (Gould CT 2) to record changes in isometric force. The rings were stretched to their optimal tension and the absence of endotheium was checked by the absence of re-

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laxation to thrombin (0.1 u ml^{-1}) during contraction to noradrenaline (0.1 μM) (DeMey & Vanhoutte, 1982). The rings were incubated either in control solution or in the presence of indomethacin (10 μM) and inhibitors of NO synthase, N^G-nitro-L-arginine (100 μM), or N^G-monomethyl-L-arginine (300 μM) were added 15 min later. In other experiments the rings were contracted with phenylephrine (1 μM) and inhibitors of NO synthase, N^G-nitro-L-arginine (100 μM), or N^G-monomethyl-L-arginine (300 μM) were added.

In some experiments, rings incubated with indomethacin (10 μM) with or without inhibitors of NO synthase, were contracted with noradrenaline (0.3 μM) and relaxations to L-arginine were obtained, followed by addition of superoxide dismutase (SOD; 150 u ml^{-1}).

In another set of experiments, rings of saphenous vein without endothelium were incubated for 6 h in cell culture medium plus lipopolysaccharide (LPS) 100 μg ml^{-1} of interleukin-1β (IL-1β) 10 u ml^{-1}. Following the incubation period, the rings were suspended between two stirrups in an organ bath, stretched to their optimal tension and concentration-response curves to noradrenaline were obtained in the presence of indomethacin.

Measurements of prostaglandin I_2 and E_2

Rings of saphenous vein, without endothelium, were placed in glass tubes containing 2 ml of control solution, oxygenated with 95% of O_2/5% CO_2, and incubated for 30 min at 37°C. The incubation buffer was than replaced with 2 ml fresh buffer in the presence or absence of indomethacin (10 μM). After an additional 30 min, the buffer was replaced once more with fresh solution with or without indomethacin. The preparations were then allowed to equilibrate for 30 min before 1 ml was collected for the determination of the basal generation of prostanoids. Prostaglandins were purified by solid phase extraction procedures (Powell, 1980; Kelly *et al.*, 1989). The samples were then freeze dried and reconstituted in assay buffer for the measurement of prostaglandins. Prostaglandin E_2 and 6-keto-prostaglandin-$F_{1\alpha}$ (the stable breakdown product of prostaglandin I_2) were measured with radioimmunoassay kits (Amersham Corporation). After the experiments, the tissues were blotted on absorbent paper and weighed. The content of prostaglandins is expressed as picograms per milligram (pg mg^{-1}) tissue.

Statistical analysis

Results are expressed as mean ± s.e. mean; n represents the number of separate experiments in veins from different dogs. Statistical comparisons were performed by means of a two way analysis of variance (ANOVA) or Student's t test for paired or unpaired observations. Differences were considered to be significant when P was less than 0.05.

Drugs

Indomethacin, N^G-nitro-L-arginine, N^G-monomethyl-L-arginine, N^G-nitro-L-arginine methylester, L-arginine, superoxide dismutase, (−)-noradrenaline, phenylephrine, lipopolysaccharide (LPS, from *Escherichia coli*, serotype 0127:B8) were obtained from Sigma Chemical (St. Louis, MO, U.S.A). Interleukin 1β (IL-1β) was purchased from Boehringer Mannheim Coporation (Indianapolis, IN, U.S.A.). Indomethacin was prepared in an equal amount of Na_2CO_3 (Sigma Chemical St. Louis, MO, U.S.A.).

Results

N^G-nitro-L-arginine (100μM) did not significantly affect the tone of quiescent rings (Figure 1). In contrast, indomethacin (10 μM) induced a small but significant contraction (1.6 ±0.6 g)(n=6) that was not observed with the vehicle (sodium carbonate solution). When N^G-nitro-L-arginine was added 15 min after indomethacin, the inhibitor of NO synthase produced a further contraction (1.3±0.4 g) (n=6). The contraction to N^G-nitro-L-arginine was not affected by superoxide dismutase (50 u ml^{-1})(n=4). When the rings were contracted with phenylephrine (1μM)(1.94 ±0.50 g) no change in tension was observed when N^G-nitro-L-arginine was added 15 min later (2.01 ±0.49 g)(n=6) (Figure 1).

In rings of canine saphenous vein without endothelium, in the presence of indomethacin (10 μM), N^G-nitro-L-arginine methylester (100 μM) or N^G-monomethyl-L-arginine (300 μM) induced contractions similar to those evoked by N^G-nitro-L-arginine (1.15 ±0.35 and 1.24 ±0.45 g respectively) (n=3).

During the contractions to noradrenaline (0.3 μM), in the presence of indomethacin, L-arginine evoked small relaxations in the rings treated with the NO-synthase inhibitors but not in control rings (Figure 2). At the end of the experiment, superoxide dismutase (150 u ml^{-1}) evoked transient relaxations in all preparations (n=4)(Table 1). Incubation for 6 h with LPS (100 μg ml^{-1}) or IL-1β (10 u ml^{-1}) did not affect the concentration-response curve to noradrenaline (Figure 3)(n=4).

Rings without endothelium released prostaglandin E_2 and prostaglandin I_2 (Table 2). This basal production was abolished by indomethacin and not affected by N^G-nitro-L-arginine (n=3).

Discussion

The present experiments suggest that cells other than endothelial cells (presumably smooth muscle cells) of the canine saphenous vein can express a nitric oxide-synthase activity, when the cyclo-oxygenase pathway is inhibited by in-

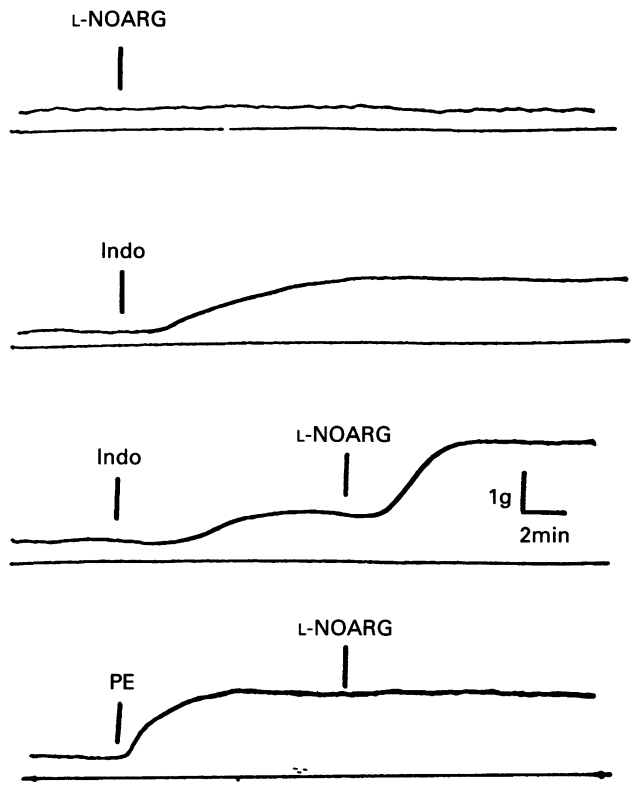

Figure 1 Isometric tension recordings showing the effects of N^G-nitro-L-arginine (L-NOARG; 100 M), indomethacin (Indo; 10 μM), alone or in combination, on the basal tone of rings of canine saphenous vein without endothelium and the effects of L-NOARG (100 μM) after phenylephrine (PE; 1 μM). Representative traces from 6 separate experiments.

domethacin.

In quiescent canine coronary artery rings with endothelium or in the coronary circulation of the perfused guinea-pig heart, inhibition of the constitutive nitric oxide synthase by nitric oxide synthase inhibitors, or inhibition of guanylate cyclase by methylene blue, are associated with an increase in tone (Kelm & Schrader, 1990). This is due to inhibition of the basal release of nitric oxide and abolition of its inhibitory effect on basal tone. In the present study, in rings without endothelium, the inhibitors of nitric oxide synthase did not affect the basal tone of the saphenous vein. This is consistent with the presence of only minimal amount of inducible nitric oxide synthase in venous smooth muscle under control conditions. However, when indomethacin was present, the inhibitors of nitric oxide synthase caused contraction. Methylene blue, an inhibitor of soluble guanylate cyclase, causes contraction in dog renal arteries by non specific inhibition of the synthesis of prostaglandin I_2 (Okamura *et al.*, 1990). Following its inhibition by N^G-nitro-L-arginine methyl ester, nitric oxide synthase can produce superoxide anions which induce contraction (Pou *et al.*, 1992). However, the production of superoxide anions do

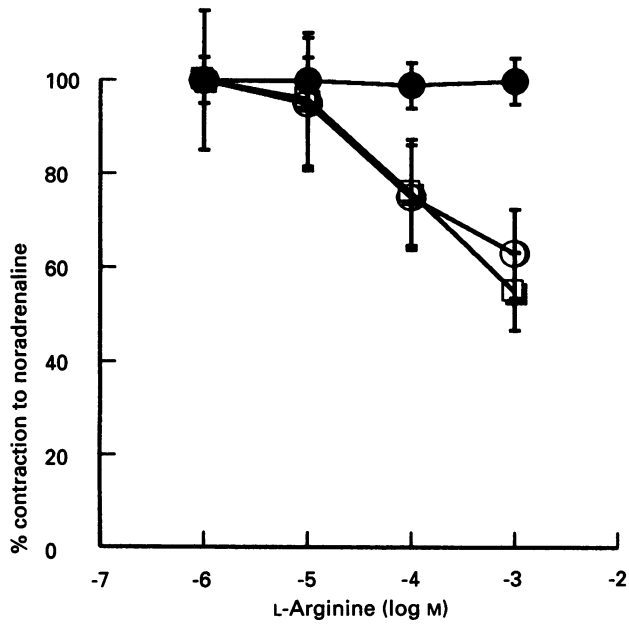

Figure 2 Relaxations to L-arginine (1 μM – 1 mM) in rings of canine saphenous vein without endothelium contracted with noradrenaline (0.3 μM). Experiments were performed in control solution (●) in the presence of indomethacin (10 μM) or in the presence of indomethacin (10 μM) and N^G-monomethyl-L-arginine (300 μM) (○) or N^G-nitro-L-arginine (100 μM)(□). Results are expressed as a percentage of the contraction to noradrenaline (0.1 μM)($n=4$).

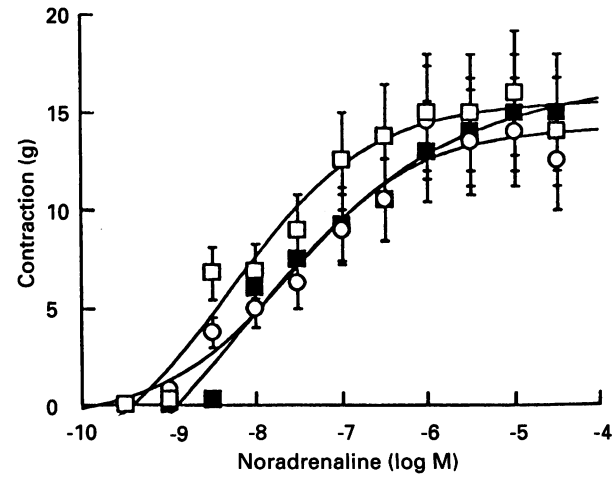

Figure 3 Concentration-response curve to noradrenaline in rings of canine saphenous vein without endothelium pre-incubated for 6 h, in control solution (■), in the presence of LPS (100 μg ml^{-1})(□) or in the presence of interleukin-1β (10 u ml^{-1})(○)($n=4$).

Table 1 In rings of canine saphenous vein without endothelium contracted to noradrenaline (0.1 μM), in the presence of indomethacin (10 μM) and the NO-synthase inhibitors (L-NOARG or L-NMMA), concentration-response curve to L-arginine were obtained and superoxide dismutase (SOD 150 u ml^{-1}) was applied at the end of the experiment: The transient relaxation to SOD is expressed as a percentage of the maximal relaxation to sodium nitroprusside (SNP 100 μM)

	Control	*L-NOARG* (100 μM)	*L-NMMA* (300 μM)
SOD (150 u ml^{-1}) (% relaxation to SNP)	35.3 ± 15.2	45.5 ± 10.1	52.2 ± 11.3

Values are ± s.e.mean. $n=4$.

Table 2 Effect of indomethacin (10 μM), N^G-nitro-L-arginine (NOARG, 100 μM) on the basal production of prostaglandin I_2 (PGI_2) and prostaglandin E_2 (PGE_2) in rings of canine saphenous vein

Prostanoid production (pg mg^{-1} tissue)	*Control*	*Indomethacin* (10 μM)	*L-NOARG* (100 μM)
PGI_2	19.6 ± 1.8	2.3 ± 1*	19.2 ± 1.7
PGE_2	14.4 ± 0.7	<0.5†	13.3 ± 0.9

Data are expressed as pg mg^{-1} tissue ± s.e.mean ($n=4$).
*Denotes differences from the basal production in non-treated vein (control) ($P<0.05$).
†Indicates values below the limit of sensitivity of the assay (0.5 pg mg^{-1} tissue).

not account for the changes in tension induced by N^G-nitro-L-arginine, since the contraction was not affected by superoxide dismutase which degrades these radicals (Rubanyi & Vanhoutte, 1986). A non specific effect of nitric oxide synthase inhibitors can be ruled out in the present study since similar observations were made with different arginine analogues. Furthermore, the basal production of prostanoids by rings of canine saphenous vein was not affected by these inhibitors. Earlier observations demonstrate a contractile effect of nitric oxide synthase inhibitors that is enhanced when tone is applied to the tissue (Rees *et al.*, 1990). However, in the present study, nitric oxide synthase inhibitors did not induce contraction when the tone was increased beforehand with an agent like phenylephrine, which does not stimulate cyclo-oxygenase in rings of canine saphenous vein.

In the presence of NOS inhibitors relaxation to L-arginine was observed when rings of canine saphenous vein were contracted with noradrenaline. Similar observations have been made in the rat aorta without endothelium in control conditions or in endotoxaemic rats (Schini & Vanhoutte, 1991; Schott *et al.*, 1992). In the present study, the amplitudes of the relaxation evoked by L-arginine are equivalent to the amplitude of the contraction induced by the inhibitors of nitric oxide synthase. This suggests that the relaxations observed are mainly due to the reversal of the effect of the inhibitors. However, the relaxation observed in response to superoxide dismutase which protect nitric oxide breakdown by superoxide anions suggests that, in the presence of indomethacin, nitric oxide is produced in small amounts. The isoform of the enzyme, i.e. constitutive or inducible, involved in the synthesis of the relaxing factor in the present study is not clear. Following treatment with IL-1β or lipopolysaccharide for 6 h in arterial vessels the contraction to noradrenaline is reduced. This effect may be due to the induction of nitric oxide synthase in smooth muscle cells and the subsequent production of nitric oxide which inhibits the tone of the blood vessel (Fleming *et al.*, 1990). Under the same experimental conditions, the concentration-response curve to the catecholamine was not affected in the canine saphenous vein. The possiblity that induction of nitric oxide synthase is present under 'control conditions' cannot be excluded. However, the time course of the responses and the results with LPS in the present study suggest that the small amount of nitric oxide produced by the rings without endothelium in the canine saphenous vein might not be due to the induction of nitric oxide synthase.

The induction of nitric oxide synthase in a murine macrophage cell line is inhibited by eicosanoids (Marotta *et al.*, 1992). In the present study, evidence of NO synthase activity was recorded only in the presence of indomethacin. Although the direct effect of prostanoids on crude preparations of nitric oxide synthase extracted from the tissue was not examined in this study, the present results suggest that in canine saphenous vein endogenous prostanoids inhibit nitric oxide synthase. The mechanism underlying this regulation is not known. A possible explanation may be activation of the cyclic AMP pathway by prostacyclin or prostaglandin E_2. In venous tissue, myogenic tone is regulated both by NO and prostanoids (Berczi *et al.*, 1992). In the canine saphenous vein, the basal production of these prostanoids by smooth muscle cells could be sufficient to initiate the activation of cyclic AMP-dependent protein kinase which in turn may catalyse the down regulation of nitric oxide synthase. The kinetics of the response that are observed in the present study, suggest that prostanoids may modulate the activity rather than regulate the induction of nitric oxide synthase in smooth muscle of canine saphenous vein.

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