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Endothelial and neural factors functionally involved in the modulation of noradrenergic vasoconstriction in healthy pig internal mammary artery

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ABSTRACT

The role of endothelial and neural factors as modulators of neurogenic- and noradrenaline-induced vasoconstriction was examined in healthy pig internal mammary artery (IMA). Tetrodotoxin-, guanethidine-sensitive electrical field stimulation (EFS)-, and noradrenaline-elicited contractions were significantly diminished by prazosin ($n = 8$, $P < 0.001$) and less so by rauwolscine, indicating functional α_1- and α_2-adrenoceptor-mediated noradrenergic innervation of the IMA. Endothelium removal reduced neurogenic ($n = 8$, $P < 0.01$) but augmented noradrenaline responses ($n = 8$, $P < 0.01$), suggesting the release of two endothelium-dependent factors with opposite effects. In the presence of endothelium, neurogenic and exogenous noradrenaline vasoconstrictions were enhanced by L-NOArg ($n = 7$, $P < 0.05$ and $P < 0.01$ respectively) and ODQ ($n = 7$, both $P < 0.05$); in denuded arteries, nNOS inhibition with N^{ω}-propyl-L-arginine increased neurogenic contraction ($n = 7$, $P < 0.05$). Western blotting indicated the presence of neural and endothelial origin NO ($n = 6$, $P < 0.001$). Tetraethylammonium ($n = 9$, $P < 0.001$), iberiotoxin ($n = 7$, $P < 0.001$) and 4-aminopyridine ($n = 8$, $P < 0.01$) enhanced vasoconstrictions revealing a modulatory role of big conductance Ca^{2+}-activated K^+ (BK_{Ca}) and voltage-dependent K^+ (K_v) channels in noradrenergic responses. Bosentan pretreatment ($n = 8$, $P < 0.05$) suggested endothelin-1 as the inferred contractile neurogenic endothelial-dependent factor. Indomethacin-induced inhibition involved a muscular prostanoid ($n = 9$, $P < 0.05$), functionally and immunologically localized, and derived from cyclooxygenase (COX)-1 and COX-2, as revealed by Western blots ($n = 5$, $P = 0.1267$). Thus, noradrenergic IMA contractions are controlled by contractile prostanoid activation and endothelin-1 release, and offset by BK_{Ca} and K_v channels and neural and endothelial NO. These results help clarify the mechanisms of vasospasm in IMA, as the preferred vessel for coronary bypass.

1. Introduction

Among the available treatments for ischemic heart disease, coronary artery bypass grafting (CABG) is one of the most effective and long-lasting [1]. The unequivocal demonstration of improved graft patency and clinical outcomes using internal mammary artery (IMA) have made it the vessel of choice for CABG surgery. However, despite the continuous refinement of surgical techniques and the use of vasodilatory drugs, a 10% rate of closure or vasospasm has been reported during and after CABG surgery [2].

Under physiological conditions, different mechanisms, including humoral, local or neurally released factors, work together to achieve a delicate balance between vasodilators and vasoconstrictors thus tightly controlling vascular diameter. This balance could be upset in disease conditions or in the presence of cardiovascular risk factors and also in IMA case, during surgery. Spasm in human IMA may occur mainly in response to surgical manipulation *e.g.*, stretching or hypoxia, leading to the local release of contractile vasoactive factors. In addition, spasm may also be a consequence of the systemic response causing elevation of circulating levels of various potential spasmogens during and after CABG, mainly through sympathetic stimulation.

In an effort to avoid vasospasm, several studies have tried to improve our current understanding of the physiology of the IMA. Thus, it has been well established that IMA tone is influenced by

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vasoconstrictors such as noradrenaline, vasopressin, serotonin or endothelin [3–5], whose actions are counteracted by potent vasodilators like nitric oxide (NO), prostacyclin and endothelial-derived hyperpolarizing factor (EDHF) [6,7].

Stimulation of adrenergic nerve endings has been proposed as a factor that could trigger the spontaneous perioperative contraction of arterial grafts [3,8–11]. A better understanding of the functional role of IMA innervation could have interesting clinical implications since, during CABG surgery, the IMA may be harvested either as a pedicle together with concomitant veins, lymphatics, sympathetic plexus and internal thoracic fascia or may be skeletonized free of all surrounding tissue. Many studies, mostly in the field of cardiac surgery, have compared graft flow in skeletonized and pedicled IMA to determine which of these two techniques is the best option in terms of graft patency. The current state of the art mostly supports skeletonization but the benefits of this practice have not been assessed in detail [12]. The data published so far are controversial. One might expect that the loss of sympathetic nerve-mediated graft vasoconstriction could confer an advantage in skeletonized segments. However, Wendler et al. [13] reported that free flow even tends to be lower in skeletonized grafts. In recent reports, it has been shown that skeletonized and pedicled IMA grafts provide excellent patency rates, even during sympathetic stimulation [14], suggesting that some sympathetic innervation remains in the skeletonized IMA.

He et al. [3] were able to demonstrate *in vitro* the functional adrenergic innervation of the human IMA, but the mechanisms underlying such responses were not addressed. This finding was later confirmed by Gaudino et al. [15], who detected catecholaminergic nerve endings in the human IMA through immunohistochemistry. Other authors have identified co-transmitters such as neuropeptide Y (NPY) or ATP co-stored in IMA, suggesting a modulator role in human vascular sympathetic reflexes [8]. In addition, neural nitric oxide synthase (nNOS) expression and immunoreactivity have been detected mainly in the medial layer of the human IMA [10].

Most pharmacological and physiological information concerning noradrenergic responses of human IMA is focused on exogenous noradrenaline effects. However, underlying mechanisms to exogenous noradrenaline responses may differ from those to EFS, since some of these modulator mechanisms could be of neurogenic nature. As far as we are aware, no electrical field stimulation (EFS) experiments in any species have addressed the relation between neurogenic and endothelial factors in this arterial segment. Donoso et al. [8] indicated that cardiovascular risk factors, such as smoking, age, hypertension or diabetes, may alter the vascular reactivity of the IMA in different ways, and that the results obtained in their study could differ from the effects produced in healthy subjects. Nevertheless, data generated in *in vitro* studies often reflect function in diseased vessels only, and a reference to a normal non-diseased control is usually absent. Therefore, there is no existing data from cardiovascular risk factor-free model to determine how any of those deleterious factors might separately affect the behavior of this vessel. Since after their manipulation during CABG surgery, human IMA segments with functional nerve endings cannot feasibly be harvested, we obtained our IMA segments from the pig, the best non-primate model for human cardiovascular research [16].

In order to obtain further information on the origin of the vasospasm and establish whether or not a healthy endothelium could be able to offset a possible neurogenic contraction, in the present study we explore the functional interaction between endothelium and the sympathetic perivascular nerves. We assessed the specific roles of the endothelium integrity, NO-cGMP and cyclooxygenase (COX) pathways, potassium channels, endothelin-1 and reactive oxygen species (ROS) on EFS-induced contractions produced in pig IMA under basal tone.

2. Materials and methods

2.1. Experimental animal and tissue preparation

IMA segments were obtained from 37 cross-breed male pigs (weight 35–45 kg; 8 rings per animal) from the Experimental Surgery Department at Hospital Universitario Ramón y Cajal (Madrid, Spain), shortly after the animals were euthanized. Arteries were cleaned of adhered tissues and cut into 3 mm-long rings (external diameter = 3.2 ± 0.1 mm, internal diameter = 2.1 ± 0.1 mm; n = 37, without tension). The research protocol of this study was approved by the Ethical Committee for Animal Welfare of Hospital Universitario Ramón y Cajal. All animal experiments and procedures were carried out according to the European Union regulation (*86/609/EEC*) and Spanish Normative for the Care and Use of Laboratory Animals (*RD 1201/2005*).

2.2. Immunohistochemistry studies

IMA segments were fixed by immersing in 4% paraformaldehyde prepared in 0.1 M sodium phosphate buffer (PB), pH 7.4 at 4 °C for 3–4 h, and then placed in a cryoprotective phosphate buffer solution containing 30% sucrose for 24 h at 4 °C. Specimens were frozen in CO_2 and stored at −80 °C until cross-sections of 10 μm were obtained using a cryostat microtome.

For immunohistochemistry using avidin–biotin–peroxidase complex procedures [17], the tissue sections were immersed in a mixture of 1% H_2O_2 and 90% methanol in distilled water and then washed in PB (3 × 10 min). Specimens were preincubated for 3 h in 10% normal goat serum in PB containing 0.3% Triton X-100 to detect dopamine-beta-hydroxylase and neuronal nitric oxide synthase, and in 5% bovine serum albumin and 0.25% triton X-100 to detect choline-acetyltransferase, cyclooxygenase-1 and cyclooxygenase-2. Next, the sections were treated with rabbit anti-dopamine-beta-hydroxylase antibody (Chemicon International Inc., Phillipsburg, NJ, USA) diluted 1:1500 in PB rabbit anti-neuronal nitric oxide synthase (Chemicon International Inc., Phillipsburg, NJ, USA) diluted 1:1000 in PB, goat anti-choline-acetyltransferase antibody (Chemicon International Inc., Phillipsburg, NJ, USA) diluted 1:150 and rabbit anti-cyclooxygenase-1 and cyclooxygenase-2 (Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA) diluted 1:100. Sections were then incubated for 2 h at room temperature with biotinylated anti-rabbit serum raised in goat (Chemicon International Inc., Phillipsburg, NJ, USA) diluted 1:400 to detect dopamine-beta-hydroxylase, neuronal nitric oxide synthase, cyclooxygenase-1 and cyclooxygenase-2, and with anti-goat serum raised in donkey (Chemicon International Inc., Phillipsburg, NJ, USA) diluted 1:400 to detect choline-acetyltransferase. Once treated with the avidin–biotin complex (ABC, Vector) diluted 1:100 for 90 min at room temperature, the resultant immunocomplex was visualized using 0.05% 3,3′-diaminobenzidine and 0.001% H_2O_2 in PB.

2.3. Western blot analysis of eNOS, iNOS, nNOS, COX-1 and COX-2

IMA segments were homogenized (MagNA Lyser, Roche, IN, USA) in radioimmunoprecipitation assay (RIPA) buffer containing protease inhibitor cocktail tablets at the concentration supplied by the manufacturer (Complete Mini, Roche, IN, USA). Protein contents were determined using the BCA (bicinchoninic acid) Protein Assay Kit (Thermo Scientific Pierce, Madison, WI, USA) and equal amounts of whole vessel homogenates were electrophoresed on 7.5% SDS-polyacrylamide and transferred to polyvinylidene difluoride membranes. After blocking, the membranes were incubated overnight (4 °C) with primary antibodies against endothelial nitric oxide synthase (eNOS) (Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA), inducible nitric oxide synthase (iNOS)

(BD Transduction Laboratories, Madrid, Spain), nNOS (BD Transduction Laboratories, Madrid, Spain), COX-1 (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) and COX-2 (Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA). As positive controls were used cell lysate from mouse macrophages stimulated with 10 ng/mL interferon gamma (IFNγ) and 1 μg/mL lipopolysaccharide (LPS), for iNOS (BD Transduction Laboratories, Madrid, Spain); rat cerebrum lysate for nNOS (BD Transduction Laboratories, Madrid, Spain); cell lysates from human umbilical vein endothelial cells (HUVEC) for eNOS and COX-1; and cell lysate from HUVEC treated with 1 μg/mL LPS for COX-2. As internal control of the amount of protein, β-actin (Sigma–Aldrich, St. Louis, MO, USA) was used. After incubation with secondary antirabbit or anti-mouse antibodies as appropriate, antibody binding was assessed by enhanced chemiluminescence (Thermo Scientific Pierce ECL Western Blotting Substrate, Madison, WI, USA). Blots were digitalized using a Gelprinter Plus (TDI, Madrid, Spain) and the densities of spots assessed using Image Gauge 4.0 software (Fujifilm Science Lab, Madrid, Spain).

2.4. Vascular reactivity studies

IMA segments were transferred to 5 mL organ baths containing physiological saline solution (PSS) at 37 °C and aerated with a mixture of 95% O_2 and 5% CO_2 to maintain the final pH at 7.4. The rings were mounted between two parallel L-shaped stainless steel wires. One wire was fixed to a displacement unit allowing fine adjustment of tension while the other was attached to a force transducer (Grass FT03C, Grass Instruments Division Astro-Med, Inc., West Warwick, RI, USA). Care was taken not to damage the luminal surface of the preparation during mounting. Nevertheless, in some experiments the endothelium was mechanically removed by gently rubbing the intimal surface of the rings with a stainless steel wire before starting experimental protocol. The isometric tension of the vessel wall was displayed and recorded using a data acquisition system, PowerLab hardware and Chart v5.3 software (DMT, Aarhus, Denmark).

After an equilibration period of 30 min, each ring was stretched in a stepwise manner to its optimal length-tension ratio (≈22 mN). The contractile ability of the preparations was tested by exposing the arterial rings to a 123.7 mM potassium-enriched solution (K-PSS, 15.19 ± 0.99 mN, n = 37). Segments with the K-PSS response less than 10 mN were discarded from further investigation.

The integrity of the vascular endothelium was confirmed by immediate relaxation (>70%) induced by acetylcholine (10^{-6} to 3×10^{-6} M) in vessels precontracted with phenylephrine (10^{-6} M). The vessel rings were considered to lack a functional endothelium when their response to acetylcholine was under 10%.

EFS was achieved using a stimulator (Cibertec CS20, Madrid, Spain) connected to two platinum electrodes placed on each side of the vessel parallel to its longitudinal axis. Two consecutive reproducible frequency–response curves (0.5–32 Hz, 400 mA, 15 s trains and 1 ms duration) were obtained in preparations under resting tension. In every experiment, the first frequency–response curve served as control and the time between the first and second frequency–response curve was 60 min. Frequency–response curves to EFS were development in the presence of cocaine (3×10^{-6} M), a catecholamine uptake inhibitor and propranolol (3×10^{-6} M), a β-adrenoceptor blocker, and cumulative concentration-dependent curves to noradrenaline were performed in the presence of propranolol, with the exception of the set of experiments in which we specifically tested β-adrenoceptor. Previous experiments have shown that repeated exposure to noradrenaline induces tachyphylaxis. Thus, we used consecutive segments from the same pig in parallel experiments, with one acting as the control for the others.

To assess the nature of EFS- and noradrenaline-induced contractile responses, and the mechanisms underlying those responses, different antagonists, inhibitors and blockers were added to the organ bath 30–60 min before conducting the frequency- and concentration–response curves, respectively. EFS induced contractions and concentration–response curves to noradrenaline were obtained from separate preparations.

2.5. Data analysis

Contractile responses to EFS and noradrenaline observed under conditions of basal tone were expressed as a percentage of the contractile response to K-PSS.

For each concentration–response curve, the agonist concentration needed for a half-maximal response (EC_{50}) was estimated by computerized nonlinear regression analysis (GraphPad Software). The sensitivity of the drugs is expressed in terms of pD_2, which is defined as the negative logarithm of the EC_{50} for the agonist used ($pD_2 = -\log EC_{50}$). E_{max} refers to the maximum response achieved.

Results are expressed as the mean ± standard error of the mean (S.E.M.) of number of animals used (n). Statistical comparisons were performed using the one way analysis of variance (ANOVA) for multiple comparisons and Student's t-test for paired or unpaired data as appropriate. A P value of less than 5% ($P < 0.05$) was taken to denote a significant difference.

2.6. Drugs and solutions

The following drugs were used: acetylcholine, 4-aminopyridine (10^{-4} M), apamin (5×10^{-7} M), 4′-hydroxy-3′-methoxyacetophenone (apocynin, 10^{-4} M), atropine (10^{-6} M), cocaine (3×10^{-6} M) glibenclamide (10^{-6} M), guanethidine (10^{-5} M), iberiotoxin (10^{-7} M), indomethacin (3×10^{-6} M), N^{ω}-nitro-L-arginine (L-NOArg, 10^{-4} M), noradrenaline, 1H-1,2,4oxadiazolo4,3-aquinoxalin-1-one (ODQ, 3×10^{-6} M), phentolamine (10^{-5} M), phenylephrine, prazosin (10^{-8} M), propranolol (3×10^{-6} M), superoxide dismutase (SOD, 150 U/mL), 1-(2-chlorophenyl)diphenylmethyl-1H-pyrazole (TRAM-34, 3×10^{-6} M), tetraethylammonium (10^{-3} M) and tetrodotoxin (10^{-6} M) (all from Sigma–Aldrich, St. Louis, MO, USA). Rauwolscine (2×10^{-7} M) and N^{ω}-propil-L-arginine (10^{-4} M) was obtained from Tocris (Bristol, United Kingdom) and bosentan (10^{-5} M) was a gift from F. Hoffman-La Roche Laboratories (Basel, Switzerland).

All drugs were dissolved in distilled water except indomethacin, which was prepared in ethanol (96%), and glibenclamide, ODQ and TRAM-34, which required dimethylsulphoxide. Preliminary experiments revealed no effects of the solvent used on the preparations and these were added in volumes not exceeding 0.1% of the tissue bath volume. Stock solutions of drugs were freshly prepared daily. Concentrations of drugs were expressed as the final concentration in the organ bath.

The composition of PSS (mM) was: NaCl 119, KCl 4.7, $CaCl_2$ 1.5, $MgSO_4$ 1.2, $NaHCO_3$ 25, glucose 11, KH_2PO_4 1.2 and ethylenediaminetetraacetic acid (EDTA) 0.027. To prepare K-PSS, the NaCl in PSS was replaced with KCl on an equimolar basis.

3. Results

3.1. Immunohistochemistry studies of IMA innervation

Noradrenergic nerve fibres were immunohistochemically labeled using an antibody against dopamine-beta-hydroxylase in sections prepared from the pig IMA segments. Immunostained fibres were mainly observed in the adventitial layer (Fig. 1A and B). Immunohistochemical assays using an antibody against choline-acetyltransferase revealed no cholinergic innervation of the pig

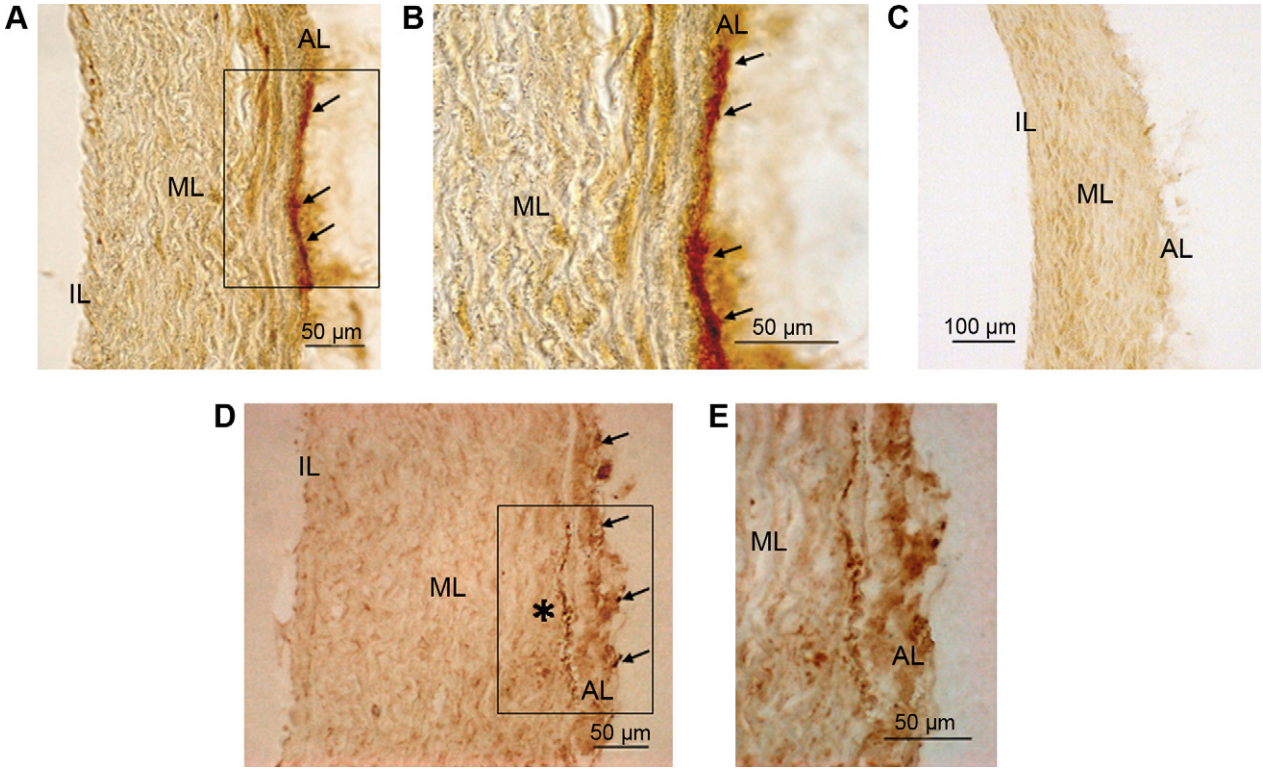

Fig. 1. Representative original pictures of cross-sections of pig IMA showing: (A) and (B) immunoreactivity to dopamine-beta-hydroxylase, (C) lack of immunoreactivity to choline-acetyltransferase and, (D) and (E) immunoreactivity to nNOS in the adventitia (arrows) and a moderate number of nerve trunks at the boundary between the muscle and adventitia layer (asterisk). Immunohistochemical studies were performed by avidin–biotin–peroxidase complex counterstaining. IL: intimal layer, ML: medial layer, AL: adventitial layer.

IMA wall (Fig. 1C). When we immunohistochemically stained the pig IMA with a polyclonal antibody against the neural isoform of NO, we observed a moderate number of nerve trunks in the adventitia and at the boundary between the muscle and adventitia layer (Fig. 1D and E).

3.2. Vasoconstrictor responses to EFS and exogenous noradrenaline. Effect of adrenergic and muscarinic receptor antagonists on noradrenergic contractile responses

Frequency-dependent IMA contractions were produced by stimulating nerve endings by applying EFS to baseline tension. The neurogenic and adrenergic nature of these responses (E_{max} at 32 Hz was 71.6 ± 5.7%; n = 17) was confirmed by the significant reduction induced by tetrodotoxin (10^{-6} M; 12.5% ± 2.8%; n = 8), a neural Na^+ channel blocker (Fig. 2A), and by guanethidine (10^{-5} M; 16.5 ± 4.8%; n = 9), an adrenergic neurotransmission blocker, respectively.

Noradrenaline (10^{-10} to 3×10^{-5} M) caused concentration-dependent increases on basal tension (pD_2 = 6.93 ± 0.08 and E_{max} = 147.3 ± 4.9%; n = 41) (Fig. 2B).

Both EFS- and exogenous noradrenaline-induced contractions were significantly inhibited by the nonspecific α-adrenoceptor and specific α_1-adrenoceptor antagonists, phentolamine (10^{-5} M) and

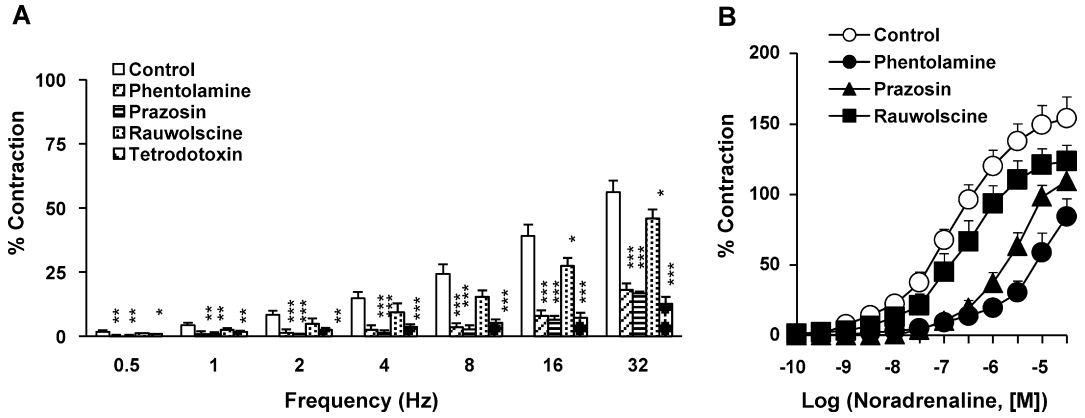

Fig. 2. (A) Electrical field stimulation-evoked contractions in pig IMA segments in the absence (control) and presence of phentolamine (10^{-5} M), a nonspecific α-adrenoceptor antagonist, of prazosin (10^{-8} M), a specific α_1-adrenoceptor antagonist, of rauwolscine (2×10^{-7} M), a specific α_2-adrenoceptor antagonist and, of tetrodotoxin (10^{-6} M), a neural Na^+ channel blocker. (B) Exogenous noradrenaline-evoked contractions in the absence (control) and presence of phentolamine (10^{-5} M), of prazosin (10^{-8} M) and of rauwolscine (2×10^{-7} M). Values are means ± S.E.M. of the data recorded in 8 animals. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$, significance compared to control rings.

Table 1
Effects of adrenoceptor antagonists on exogenous noradrenaline response in pig internal mammary artery.

	n	pD_2	E_{max} (%)
Control	8	6.87 ± 0.15	157.3 ± 15.1
Phentolamine 10^{-5} M	8	$4.70 \pm 0.20^{***}$	$96.6 \pm 10.0^{***}$
Prazosin 10^{-8} M	8	$5.72 \pm 0.13^{***}$	$116.8 \pm 8.1^{***}$
Rauwolscine 2×10^{-7} M	8	$6.45 \pm 0.15^{**}$	$124.3 \pm 4.3^{**}$
Control	7	6.35 ± 0.08	117.0 ± 2.3
Propranolol 3×10^{-6} M	7	$6.64 \pm 0.02^{*}$	123.2 ± 6.4

Data represent means ± S.E.M. n, number of animals. $pD_2 = -\log EC_{50}$, where EC_{50} is the concentration of agonist producing 50% of the E_{max}. E_{max} is the maximal contraction expressed as a percentage of the K-PSS-induced contraction obtained for noradrenaline.
* Significant differences between control *versus* treatment groups: $P < 0.05$.
** Significant differences between control *versus* treatment groups: $P < 0.01$.
*** Significant differences between control *versus* treatment groups: $P < 0.001$.

prazosin (10^{-8} M) respectively, and in smaller measure, by the specific α_2-adrenoceptor antagonist, rauwolscine (2×10^{-7} M) (Fig. 2A and B; Table 1).

The addition of propranolol (3×10^{-6} M), a β-adrenoceptor antagonist, did not alter the EFS-contraction curves. However, propranolol induced a significant increase in noradrenaline-induced responses only in endothelium-intact segments (Table 1).

Incubation with the muscarinic receptor antagonist atropine (10^{-6} M) failed to modify neurogenic contractions (data not shown).

3.3. Role of the endothelium and nitric oxide in vasoconstrictor responses to EFS and exogenous noradrenaline

Mechanical removal of the endothelium significantly diminished contractions in IMA segments produced in response to EFS (Fig. 3A). In contrast, the sensitivity and E_{max} to noradrenaline were significantly enhanced in endothelium-denuded IMA rings (Fig. 3B; Table 2).

In the presence of endothelium, neurogenic and exogenous noradrenaline vasoconstrictions were significantly enhanced when L-NOArg (10^{-4} M), a NOS inhibitor, and ODQ (3×10^{-6} M), a soluble guanylate cyclase inhibitor, were added to the organ bath (Fig. 4A and B; Table 2). NOS inhibition in denuded arteries only increased neurogenic contraction (Fig. 4C and D). Under the same conditions, no significant changes in the frequency- and noradrenaline–response curves were observed after adding ODQ (Fig. 4C and D). L-NOArg and ODQ produced an increase in basal tone during pre-incubation (24.8 ± 2.4 and 32.0 ± 7.1% of K-PSS, respectively; $n = 7$).

Table 2
Effects of endothelium removal, NO synthase, soluble guanylate cyclase and cyclooxygenase inhibition, antioxidant systems, and endothelin receptor blockade on exogenous noradrenaline response in pig internal mammary artery.

	n	pD_2	E_{max} (%)
Control	8	6.66 ± 0.15	135.3 ± 7.0
Without endothelium	8	$7.36 \pm 0.07^{**}$	$176.0 \pm 8.8^{***}$
Control	7	6.27 ± 0.17	136.0 ± 6.7
L-NOArg (10^{-4} M)	7	$7.14 \pm 0.10^{**}$	$166.1 \pm 7.3^{**}$
ODQ (3×10^{-6} M)	7	$6.74 \pm 0.10^{*}$	$157.8 \pm 10.2^{**}$
Control	9	6.73 ± 0.16	166.2 ± 10.6
Indomethacin (3×10^{-6} M)	9	6.66 ± 0.13	174.4 ± 12.3
Control	7	6.69 ± 0.10	163.6 ± 15.2
SOD (150 U/mL)	7	6.55 ± 0.15	156.5 ± 15.8
Control	8	6.14 ± 0.10	140.6 ± 4.6
Apocynin (10^{-4} M)	8	6.27 ± 0.10	135.7 ± 11.4
Control	8	6.53 ± 0.22	152.9 ± 11.6
Bosentan (10^{-5} M)	8	6.60 ± 0.20	157.8 ± 8.8

Data represent means ± S.E.M. n, number of animals. $pD_2 = -\log EC_{50}$, where EC_{50} is the concentration of agonist producing 50% of the E_{max}. E_{max} is the maximal contraction expressed as a percentage of the K-PSS-induced contraction obtained for noradrenaline.
* Significant differences between control *versus* treatment groups: $P < 0.05$.
** Significant differences between control *versus* treatment groups: $P < 0.01$.
*** Significant differences between control *versus* treatment groups: $P < 0.001$.

In endothelium-denuded rings, the specific inhibition of nNOS by incubation with N^{ω}-propyl-L-arginine (10^{-4} M) increased neurogenic responses (Fig. 4E).

Our Western blot analysis revealed eNOS and nNOS protein expression in the pig IMA as shown in Fig. 4F ($P < 0.001$, $n = 6$). In basal conditions, iNOS was undetectable (Fig. 4F).

3.4. Role of Ca^{2+}-activated K^+ (K_{Ca}), voltage-dependent K^+ (K_v), and ATP-sensitive K^+ (K_{ATP}) channels in noradrenergic contractile responses

The non-selective K_{Ca} channel blocker, tetraethylammonium (10^{-3} M), significantly enhanced both neurogenic- and exogenous noradrenaline-elicited contractions in endothelium intact IMA rings (Fig. 5A and B; Table 3). In contrast, in endothelium-denuded IMA segments, tetraethylammonium only caused an increase in EFS-induced contractions.

Different subtypes of K_{Ca} channels have been explored using specific blockers such as the blockers of big conductance K_{Ca} channels (BK_{Ca}) iberiotoxin (10^{-7} M), intermediate conductance K_{Ca} channels (IK_{Ca}) TRAM-34 (3×10^{-6} M), or small conductance K_{Ca} channels (SK_{Ca}) apamin (3×10^{-7} M) and apamin plus TRAM-34. However, only iberiotoxin affected the noradrenergic responses. This BK_{Ca} blocker, mimicking the TEA effect, evoked a

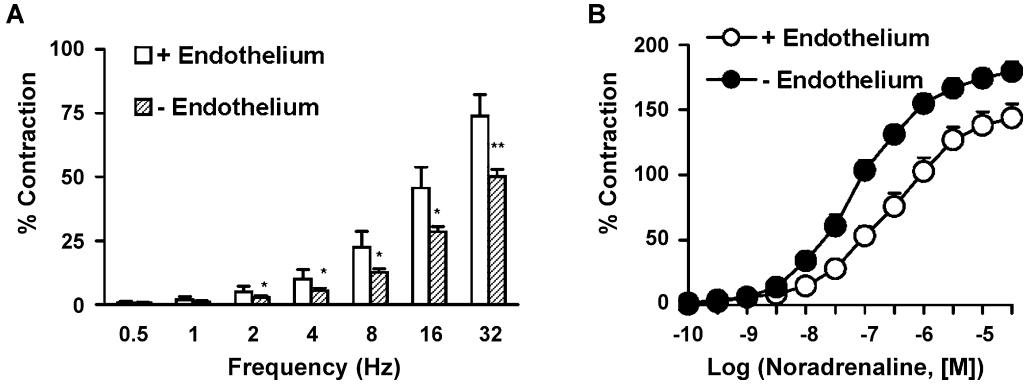

Fig. 3. Role of the endothelium in contractile responses to: (A) electrical field stimulation and (B) exogenous noradrenaline in pig IMA segments. Values are means ± S.E.M. of the data recorded in 8 animals. $^{*}P < 0.05$; $^{**}P < 0.01$, significance compared to endothelium-intact rings.

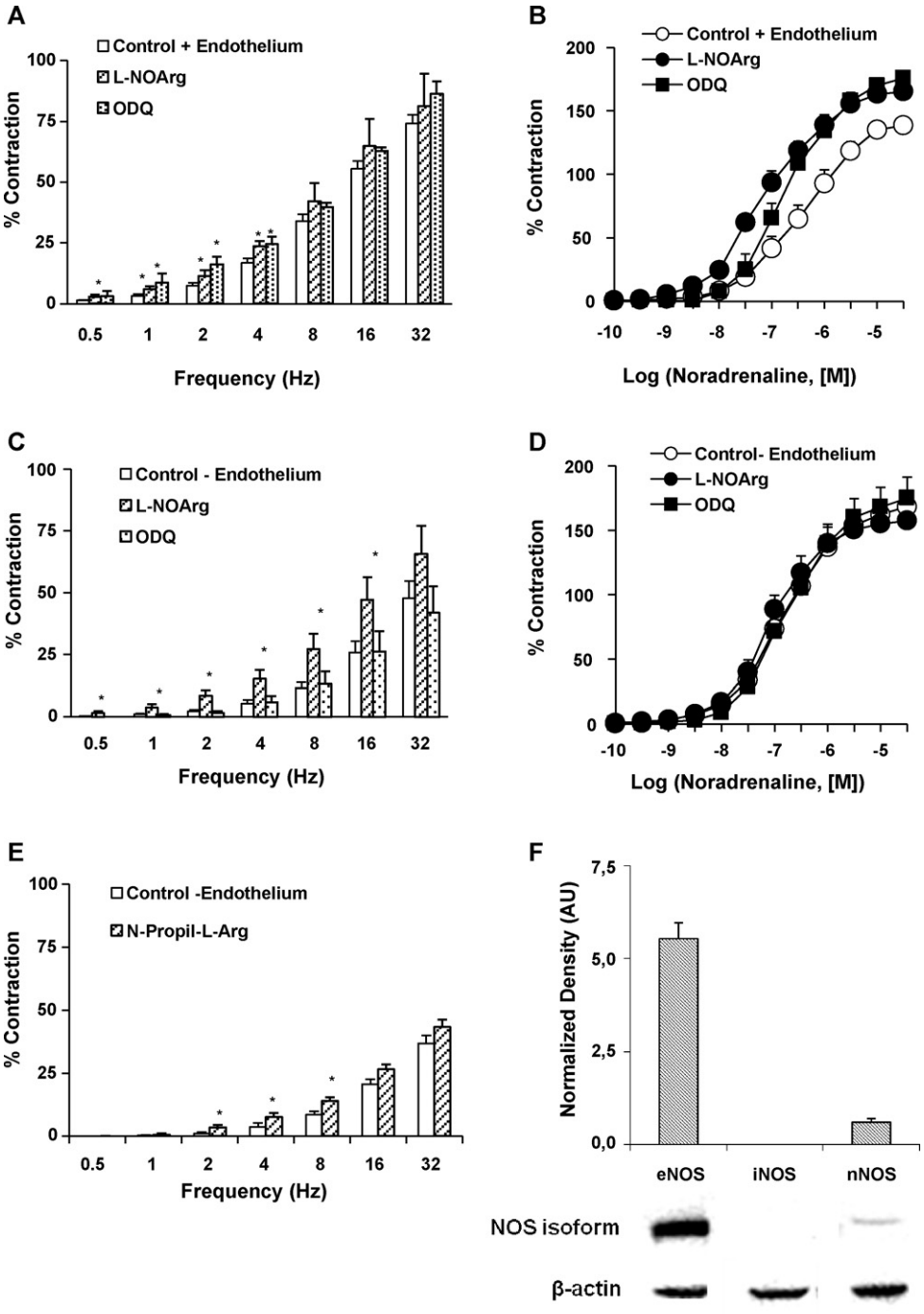

Fig. 4. Effect of L-NOArg (10^{-4} M), a nitric oxide synthase inhibitor, and ODQ (3×10^{-6} M), a soluble guanilate cyclase inhibitor on (A) electrical field stimulation- and (B) exogenous noradrenaline-evoked contractions in endothelium-intact pig IMA segments. Δ Basal tone induced by L-NOArg = 24.8 ± 2.4% and by ODQ = 32.0 ± 7.1% of K-PSS. Effect of L-NOArg and ODQ on (C) electrical field stimulation- and (D) exogenous noradrenaline-evoked contractions in endothelium-denuded pig IMA segments. (E) Effect of N^{ω}-propil-L-arginine (10^{-4} M), a specific neural nitric oxide synthase inhibitor on electrical field stimulation-evoked contractions in endothelium-denuded IMA segments. Values are means ± S.E.M. of the data recorded in 7 animals. $^{*}P < 0.05$, significance compared to control rings. (F) eNOS, iNOS and nNOS protein expression in pig internal mammary artery tissue. A representative immunoblotting image and relative levels assessed by densitometry of different NOS isoforms are presented. eNOS *versus* nNOS $^{***}P < 0.001$. Values are means ± S.E.M. of the data obtained from 6 animals.

significant increase in the contractions elicited by EFS independently of the presence or absence of endothelium, and in contractions produced in response to exogenous noradrenaline but only in endothelium intact rings (Fig. 5A and B; Table 3).

The K_v channel blocker, 4-aminopyridine (10^{-4} M), also enhanced the EFS-elicited contractions produced in pig IMA segments with or without an intact endothelium (Fig. 5C). Conversely, despite the presence of the endothelium, this treatment did not alter the sensitivity to noradrenaline (Fig. 5D; Table 3).

In addition, we observed an increase in basal tension during pre-incubation with tetraethylammonium (26.3 ± 3.4% of K-PSS; $n = 9$), iberiotoxin (14.31 ± 14.3% of K-PSS; $n = 7$), and 4-aminopyridine (33.8 ± 4.3% of K-PSS; $n = 8$). These responses were independent of their excitatory effects on electrically evoked contractions in pig IMA.

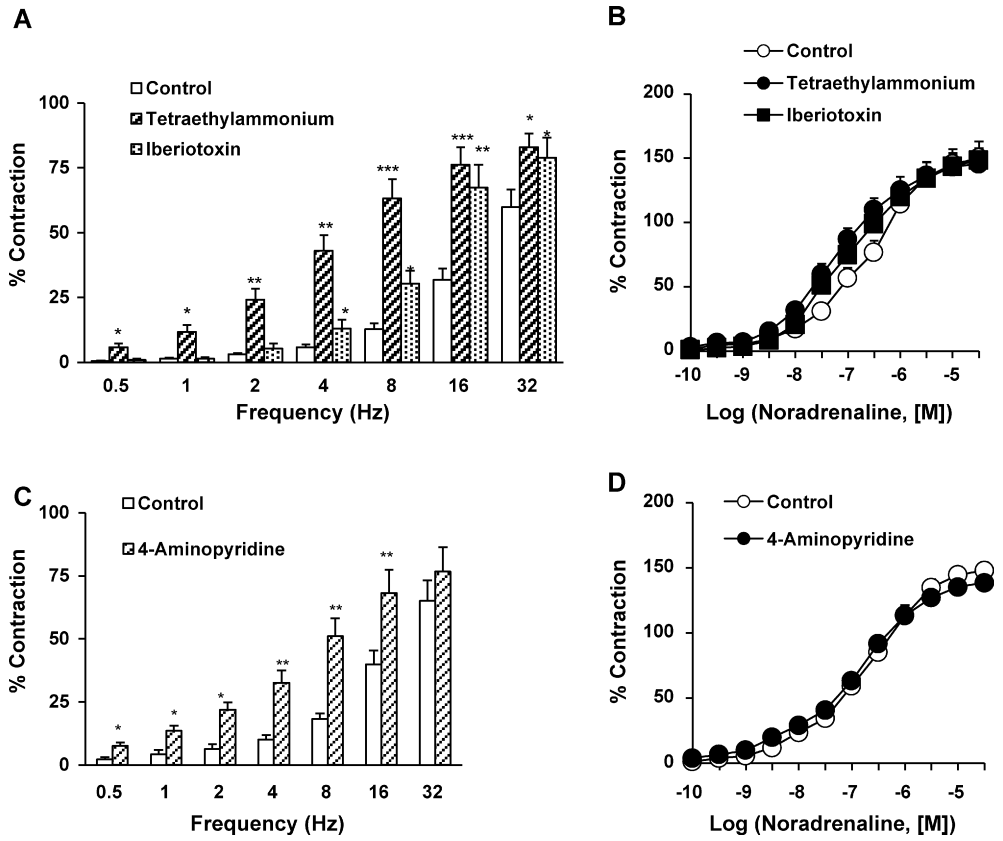

Fig. 5. (A and C) Electrical field stimulation- and (B and D) exogenous noradrenaline-evoked contractions in endothelium-intact pig IMA segments, in the absence (control) and presence of tetraethylammonium (10^{-3} M), a Ca^{2+}-activated potassium channel blocker, of iberiotoxin (10^{-7} M), a specific blocker of the big conductance K_{Ca} channels and of 4-aminopyridine (10^{-4} M), a voltage-dependent potassium channel blocker. Δ Basal tone induced by tetraethylammonium = 26.3 ± 3.4% of K-PSS, by iberiotoxin = 14.31 ± 14.3%, and by 4-aminopyridine = 33.8 ± 4.3%. Values are means ± S.E.M. of the data recorded in 7–9 animals. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$, significance compared to control rings.

Finally, the lack of effect of glibenclamide (10^{-6} M), a K_{ATP} channel blocker, on the EFS- (data not shown) or noradrenaline-elicited responses (Table 3), was unaffected by the presence of the endothelium.

3.5. Contribution of prostanoids, endothelin receptor activation and ROS to noradrenergic contractile responses

Immunohistochemical staining of the pig IMA with polyclonal antibodies against COX-1 and COX-2 revealed that these constitutive isoforms of COX are widely and uniformly distributed in the muscle layer, being absent in both the endothelial and adventitia layer (Fig. 6A and B). Western blotting revealed a similar COX-1 and COX-2 protein expression in the pig IMA as shown in Fig. 6C ($n = 5$, $P = 0.1267$).

Preincubation with indomethacin (3×10^{-6} M), a COX inhibitor, significantly reduced frequency-dependent contractions, even in the absence of endothelium. In contrast, no changes in exogenous noradrenaline-induced contractions were produced in response to the same treatment (Fig. 6D and E; Table 2).

Endothelin-A (ET_A) and endothelin-B (ET_B) receptor blockade by bosentan (10^{-5} M) reduced the contractile responses to EFS detected in endothelium-intact rings but not in denuded rings (Fig. 7A and B). This treatment did not affect vasoconstriction in response to noradrenaline in the presence or absence of endothelium (Table 2).

Neurogenic contractions and those produced by exogenous noradrenaline were unaffected by the addition of the antioxidizing enzyme, superoxide dismutase (SOD, 150 U/mL) to the organ bath (Table 2), regardless of the presence or absence of endothelium. Also, irrespective of endothelium removal, both EFS- and noradrenaline-contractile curves were unaltered by the NADP(H)-oxidase inhibitor apocynin (10^{-4} M) (Table 2).

Table 3
Effects of potassium channel blockers on exogenous noradrenaline response in pig internal mammary artery.

	n	pD_2	E_{max} (%)
Control	9	6.68 ± 0.12	151.8 ± 7.7
Tetraethylammonium (10^{-3} M)	9	7.25 ± 0.08**	147.6 ± 11.1
Iberiotoxin (10^{-7} M)	7	7.01 ± 0.09*	150.3 ± 15.0
Apamin (5×10^{-7} M)	7	6.76 ± 0.20	165.7 ± 11.4
TRAM 34 (3×10^{-6} M)	7	6.53 ± 0.15	158.4 ± 5.7
Apamin + TRAM 34	7	6.62 ± 0.15	167.7 ± 16.4
Control	8	6.80 ± 0.16	149.8 ± 6.0
4-Aminopyridine (10^{-4} M)	8	6.83 ± 0.10	140.8 ± 6.8
Control	9	6.69 ± 0.10	158.1 ± 10.4
Glibenclamide (10^{-6} M)	9	6.75 ± 0.12	161.2 ± 18.8

Data represent means ± S.E.M. n, number of animals. $pD_2 = -\log EC_{50}$, where EC_{50} is the concentration of agonist producing 50% of the E_{max}. E_{max} is the maximal contraction expressed as a percentage of the K-PSS-induced contraction obtained for noradrenaline.

* Significant differences between control *versus* treatment groups: $P < 0.05$.

** Significant differences between control *versus* treatment groups: $P < 0.01$.

4. Discussion

This is the first study comprehensively characterizing IMA neurogenic and endothelial regulation in healthy pigs. The present findings indicate that α_1- and α_2-adrenoceptor-mediated nerve contractions in pig IMA rings are modulated by NO from two

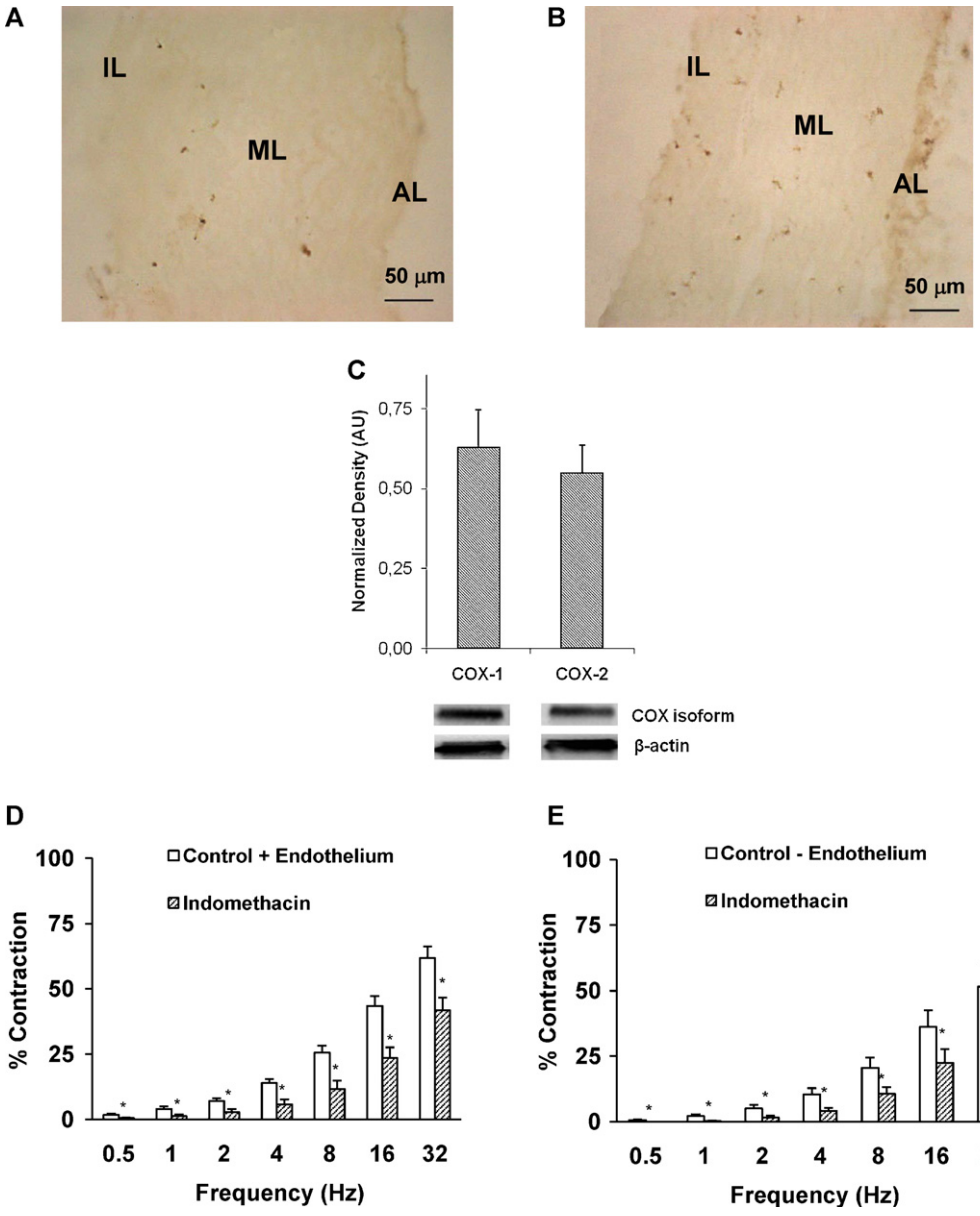

Fig. 6. Representative original pictures of cross-sections of pig IMA showing immunoreactivity to COX-1 and to COX-2 (A and B). (C) COX-1 and COX-2 protein expression in pig internal mammary artery. A representative immunoblotting image and relative levels assessed by densitometry of COX-1 and COX-2 isoforms are presented. COX-1 *versus* COX-2 $P = 0.1267$. Values are means ± S.E.M. of the data obtained from 5 animals. Electrical field stimulation-evoked contractions in endothelium-intact (D) and endothelium-denuded (E) pig IMA segments, in the absence (control) and presence of indomethacin (3×10^{-6} M), a cyclooxygenase inhibitor. Values are means ± S.E.M. of the data recorded in 9 animals. $^{*}P < 0.05$, significance compared to control rings.

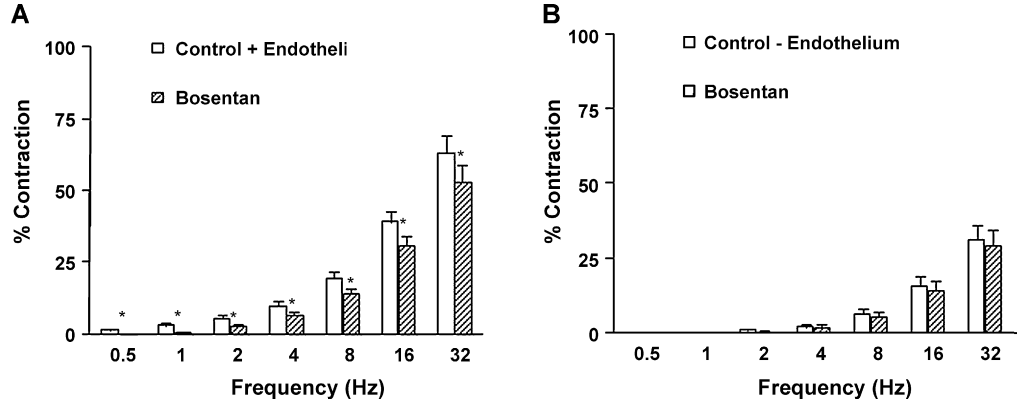

Fig. 7. Electrical field stimulation-evoked contractions in endothelium-intact (A) and endothelium-denuded (B) pig IMA segments, in the absence (control) and presence of bosentan (10^{-5} M), an endothelin receptor blocker. Values are means ± S.E.M. of the data recorded in 8 animals. $^{*}P < 0.05$, significance compared to control rings.

different sources, neural and endothelial, and also by BK_{Ca} and K_v channels, contractile prostanoids and endothelin.

Noradrenergic but not cholinergic nerve fibres were visualized by immunohistochemistry in the adventitia of the pig IMA wall. This observation is in agreement with those described by Gaudino et al. [15], who identified the sympathetic innervation of the human IMA. Our functional results suggest that perivascular nerve stimulation was able to elicit neurogenic α_1- and α_2-adrenoceptor-mediated contractions. Contractile α_2-adrenoceptors appear to play a minor role in the vasoconstriction produced in response to EFS and exogenous noradrenaline, since these responses were inhibited by rauwolscine to a lesser extent than by prazosin. In contrast, propranolol increased the sensitivity of the IMA rings to exogenous noradrenaline but did not modify contractions elicited by EFS, thus indicating that relaxing β-adrenoceptors are masked by predominant neural contractile responses or that neurally released noradrenaline finds it difficult to reach the endothelium. These results are consistent with those reported for human IMA [3,18,19] indicating that α_1-, α_2- and β-adrenoceptor subtypes, which contribute to neurogenic response, are conserved between species in specific vascular beds.

Given that the endothelium modulates vascular adrenergic responses [20–23], we assessed the contribution of endothelium to EFS- and exogenous noradrenaline responses. In our study, mechanical endothelium removal significantly diminished neurogenic contractions but augmented exogenous noradrenaline responses. Thus, the net effect of the endothelium on the neurogenic responses of the pig IMA points to the release of an endothelium-derived contractile factor that offsets the vasodilatory actions of the endothelium-derived factor involved in the response to exogenous noradrenaline.

Higher endothelial NO production has been observed in IMA than other CABG grafts, and this has been linked to its good long-term patency [24]. Our results are similar to those of Pesic et al. [23], who showed that contractions elicited by phenylephrine in human IMA are modulated by endothelial NO. Further, increases in basal tone during incubation with L-NOArg in endothelium-intact rings also suggest the involvement of endothelial NO in regulating IMA vascular tone. NO can also be released from perivascular nerves [25]. We observed that IMA contractions to EFS were significantly enhanced by L-NOArg in both endothelium-intact and denuded segments, pointing to a role of neurogenic NO, whose synthesis enzyme has been detected immunohistochemically in the adventitial layer of the pig IMA. This was confirmed by the enhancement induced by the specific inhibitor of the nNOs, N^{ω}-propyl-L-arginine, in EFS contractions on endothelium-denuded rings. In addition, significant nNOS and eNOS protein expression was detected by Western blotting, supporting the data obtained in the vascular reactivity assays. In line with our results, Webb et al. [10] confirmed the presence of constitutive NOS isoforms, nNOS and eNOS, in the human IMA wall though they also detected iNOS protein expression. We were unable to detect an iNOS band in our blots derived from pig IMA specimens. This discrepancy could be attributed to the cardiovascular risk factors present in the patients included in Webb's study. Furthermore, it has been reported that NO from both neural and/or endothelial sources can modulate adrenergic responses in several blood vessels [9,20,26], but this has not yet been functionally proven in the IMA. Some of these authors claim that NO might inhibit noradrenaline release acting at the presynaptic level. We observed different mechanisms underlying the NO effect, depending on whether this effect was endothelial or neurogenic. The increases in EFS- and exogenous noradrenaline-elicited contractions produced by ODQ only in IMA rings with an intact endothelium, suggest that endothelial NO produced its relaxant effect mainly by increasing smooth muscle cGMP levels. However, neural NO seems to rely on other mechanisms since no effect of ODQ was detected in endothelial-denuded rings. It appears that expression and function of nNOS in perivascular nerves provide a mechanism for rapid vasodilation to oppose sympathetic vasoconstriction [27].

EDHF, acting through potassium channel opening, could counteract EFS- and agonist-induced increases in pig IMA tone, as proposed for several vascular beds [4,21,28]. In human IMA, PCR and Western blot analyses have revealed the gene and protein expression of BK_{Ca} channels [29]. Consistently, in smooth muscle cells the patch clamp technique has indicated that K^+ currents are significantly decreased by tetraethylammonium and iberiotoxin, while 4-aminopyridine only has a weak effect. Alterations in K_v channel expression and function have been noted in disease conditions. This could be the case of patients undergoing CABG surgery in the above study.

The results obtained here suggest that BK_{Ca} and K_v but not K_{ATP} channels, also contribute to offsetting noradrenergic contractions. In addition, increases in tension produced during tetraethylammonium, iberiotoxin and 4-aminopyridine pretreatment also indicate that both BK_{Ca} and K_v channels modulate the basal tone of this artery. Given that tetraethylammonium and iberiotoxin enhanced the responses to exogenous noradrenaline, we may conclude that endothelium-dependent postjunctional BK_{Ca} channels are involved in these responses, as has been proposed by Mauricio et al. [28] in the saphenous vein. Additionally, according to the hypothesis of Tagaya et al. [30], the effect of these channel blockers only on neurogenic responses but not in those elicited by exogenous noradrenaline in endothelium-denuded IMA rings points to the possibility that prejunctional BK_{Ca} channels also exist. Moreover, since 4-aminopyridine also only increased neurogenic responses, we could suggest that K_V channels in the pig IMA are located presynaptically, as previously reported in rat, pig and human radial artery [20,21,31]. We cannot rule out an additional unspecific effect of tetraethylammonium on prejunctional K_v channels that was perhaps responsible for the greater effect of tetraethylammonium observed compared to iberiotoxin.

Noradrenaline could enhance the release of arachidonic acid derivatives from the vascular wall [32]. Gupte et al. [33] detected COX-1, COX-2 and TXA_2 synthases in the endothelial and vascular smooth muscle cells of the human IMA. Here, COX-1 and COX-2 synthases are also expressed and located at the medial layer of the pig IMA. Also, we have performed Western blotting analysis for COX-1 and COX-2. In addition, indomethacin diminished the EFS-elicited contractions in an endothelium independent manner, suggesting a release of contractile prostanoids and ruling out the possibility that the neurogenic contractile factor dependent on the endothelium, observed here, is an arachidonate derivative. In contrast, contractile responses to exogenous noradrenaline were unaffected by the same treatment, precluding a postjunctional effect of this catecholamine through the COX pathway, and indicating that another neurotransmitter released by terminal nerves is related to this pathway. In effect, endothelin, whose release is increased during CABG and whose synthesis expedites post-hypoxic contraction of human IMA [33], is considered an autonomic nervous system neurotransmitter [34].

We next induced ET_A and ET_B receptor blockade using bosentan, which diminished EFS-induced contractions only in endothelium-intact rings. This could explain the differences observed in endothelial-intact and denuded pig IMA rings, attributed to an endothelium-dependent contractile factor. In contrast, bosentan did not affect contractions produced by exogenous noradrenaline. We suggest that other non-adrenergic neurotransmitters, could be responsible for endothelial endothelin-1 synthesis, causing pig

IMA contractions by acting on postjunctional receptors. Additionally, our data also may point to a presynaptic role for endothelin enhancing noradrenaline release, as reported by Mutafova-Yambolieva and Westfall [35] in the rat tail artery. In any case, an interaction between activation of ET-receptors and the COX-pathway is possible, as suggested in human IMA [33].

Other endothelium-dependent vasoconstrictors such as ROS could explain the differences observed in endothelial-intact and denuded pig IMA rings. Notably, these vasoactive mediators are produced in abundance during CABG and have been incriminated in the genesis of vasospasm [36]. Although NO normally acts as a cardiovascular protective factor, under oxidative stress situations, its bioavailability can be reduced by superoxide anions, which may be produced by different enzymes such as NAD(P)H oxidases and counteracted by anti-oxidant systems including SOD [37,38]. Our experiments revealed that neither apocynin nor SOD affect EFS- and noradrenaline-induced contractions. Under disease or stress oxidative conditions, ROS production is considerable, as observed in IMA specimens obtained from active smokers [39]. Thus, it is not surprising that we did not detect significant ROS production in our cardiovascular risk factors-free experimental model.

In conclusion, the data reported here provide new insights into the neural regulation of the healthy IMA. Potent vasoconstrictions induced by perivascular nerve stimulation trigger a complex mechanism of modulation. These α_1- and α_2-adrenoceptor-mediated neurogenic contractions involve activation of contractile prostanoids and endothelin-1 release and are counteracted by BK_{Ca} and K_v channels and neural and endothelial NO. Thus, therapeutic tools targeted at increasing NO release and opening potassium channels, as well as those inhibiting the synthesis of prostanoids and blocking endothelin receptors, could be beneficial as postoperative therapy for preventing the genesis of vasospasm. Moreover, results emerging from the reference model we propose may have implications for the surgical use or not of skeletonized IMA rings. In future work, the relaxant innervation of the IMA and the role of neurotransmitters other than noradrenaline will need to be addressed.

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