ORIGINAL ARTICLE

Relaxation of Rat Main Pulmonary Artery to Electrical Stimulation: Role of Nitric Oxide

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Abstract

The present study was undertaken to determine participation of nitric oxide (NO) in noradrenergic, noncholinergic relaxation induced by electrical field stimulation (EFS) of perivascular nerves in isolated rat main pulmonary artery and its extralobar branches. Electrical field stimulation caused a frequency-dependent relaxation of main pulmonary artery of the rat precontracted with phenylephrine. N^G-nitro-L-arginine, inhibitor of nitric oxide synthase, inhibited the relaxation of pulmonary artery. Tetrodotoxin in concentration which was sufficient to block the contractile responses induced by EFS was unable to influence the relaxation response of phenylephrine- precontracted artery. These results suggest that EFS-induced relaxation in main pulmonary artery is mediated by NO mainly of nonneurogenic origin. 7-nitroindazole, neuronal NO synthase inhibitor, as well as capsaicin, a neurotoxin for sensory nerves, moderately attenuated the relaxant responses induced by EFS indicating that at least part of relaxation is mediated by neurally derived NO. The extension of EFS-induced relaxation in main pulmonary artery of spontaneously hypertensive rats was smaller than in normotensive control rats. The results suggest that EFS-induced release of NO contributes to maintaining the low resistance in lung circulation.

Introduction

One of the important regulators of the cerebral vascular tone and circulation is the network of perivascular nerves and their neurotransmitter discharges onto the postsynaptic smooth muscle (Gulbenkian *et al* 2001; Faraci & Heistad 1990). In large cerebral arteries isolated from most of mammals, including humans, electrical field stimulation (EFS) produces relaxations (Kimura *et al* 1997; Okamura *et al* 2002). The relaxant mechanism is found to be nonadrenergic and noncholinergic; based on several functional and histological studies with isolated cerebral arteries, nitric oxide (NO) is now considered to be a neurotransmitter of the vasodilator nerves and the nerves have been called nitroxidergic (nitrergic) nerves (Toda & Okamura 1991; 2003). Evidence for the neural origin of NO in mediating cerebral neurogenic vasodilation is supported by results from immunohistochemical, biochemical and pharmacological studies indicating that

cerebral perivascular nerves can recycle L-citrulline, the byproduct of NO synthesis, to L-arginine for synthetizing NO (Chen & Lee 1995).

The autonomic nervous system may also modify vascular tone of noncerebral arteries. Abundant evidence is now available that EFS-induced responses have been demonstrated in arteries of various organ systems including relaxation of pulmonary arteries (Ahlner *et al* 1991; Scott & McCormack 1999; Simonsen *et al* 1995). Relaxations are resistant to adrenergic and cholinergic blockade and they create inhibitory part of nonadrenergic, noncholinergic (NANC) system contributing to autonomic control of vascular tone.

Relaxations of pulmonary arteries to electrical field stimulation have been described in different species: bovine, guinea pig, cat, rat and human (Kubota *et al* 1988; Scott *et al* 1996; Török & Kyselá 2000; Gumusel *et al* 2001; Gonzáles-Luis *et al* 2007; Kristová *et al* 1986). There is still some controversy about the mechanisms underlying the relaxant response to NANC nerve stimulation in isolated pulmonary artery. Tetrodotoxin (TTX)-sensitive neurogenic relaxations were observed in guinea-pig pulmonary arteries (Liu *et al* 1992; Chen & Lee 1993; Scott & McCormack 1999), the others reported that pulmonary arteries were unaltered by TTX, therefore the major component of the arterial relaxant response seems to be nonneurogenic in nature (Hyman *et al* 1981; Buga & Ignarro 1992). The mediator of relaxation has not been determined yet; there are many putative neurotransmitters mediating NANC relaxations like sensory neuropeptide substance P, ATP, CGRP, or neuronal NO (Butler *et al* 1993; Maggi *et al* 1990).

Endogenous NO production appears to be important for maintaining a low pulmonary vascular resistance (Fineman *et al* 1991). It has been hypothesized that NO plays a crucial role in the relaxation of pulmonary arteries in response to electrical stimulation of perivascular nerves, since NO synthase inhibitors suppressed the relaxation, and L-arginine prevented and reversed the inhibitory effect of these inhibitors. The mechanism of the EFS-induced relaxation in pulmonary arteries is not up to date well clarified and therefore it desires increased attention.

The present study was undertaken to determine *a/* the extension of EFS-induced relaxation in main pulmonary artery from control normotensive Wistar rats and spontaneously hypertensive rats (SHR); b/ whether or not NO participates in NANC relaxation induced by EFS of perivascular nerves in isolated rat main pulmonary artery and its extralobar branches. We obtained evidence that the EFS-induced relaxation in rat main pulmonary artery is mediated by NO mainly of nonneurogenic origin.

Methods

Male normotensive Wistar rats and spontaneously hypertensive rats were used for experiments. All procedures used were in accordance with institutional guidelines and they were approved by the State Veterinary and Food Administration of the Slovak Republic.

At the beginning of the experiment, systolic blood pressure was measured by noninvasive tail-cuff plethysmography. Animals were killed by decapitation. Then pulmonary arteries (main pulmonary artery and its extralobar branches) were removed from rats, immersed in oxygenated Krebs solution and cleaned of adipose and connective tissues.

For measurement of contractile activity, arteries were cut into rings of 3.5–4.0 mm of length. In some preparations the endothelium was removed by gentle rubbing. The rings were fixed on stainless steel hooks and suspended in an organ bath containing modified Krebs solution which was continuously aerated with a mixture of 95 % O_2 + 5 % CO_2 and maintained at 37 °C. One side of the ring was connected by a thread to a force-displacement transducer (Sanborn FT 10) to measure changes in isometric tension which were recorded with a polygraph TZ 4200 (Labora).

The Krebs solution consisted of (in mmol/l): NaCl 118, KCl 5, $CaCl_2$ 2.5 $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, glucose 11, $CaNa_2$ EDTA 0.03, ascorbic acid 1.1. The resting tension of arterial rings was 10 mN. The preparations were allowed to equilibrate for 90 min before experimental observation. During this period, the Krebs solution was changed at 15 min intervals.

Electrical field stimulation (EFS) was performed with the aid of two parallel platinum electrodes placed 5–6 mm apart, on either side of the rings, and connected to and electronic stimulator ST-3 (Hungary) as described previously (Török *et al* 1993). The following parameters were used: square-wave pulses 0.5 ms duration, 0.5 – 32 Hz, supramaximal voltage (approx. 32 V), duration of stimulation 20 s. Parameters of EFS were determined to avoid the generation of free radicals and direct stimulation of vascular smooth muscle. When the effects of pharmacological agents were examined, the vessels were exposed to the agent for at least 20 min before electrical stimulation. Relaxation of arterial rings was measured as the decrease in tension below the elevated tension elicited by submaximal precontraction with phenylephrine (1 μmol/l).

Chemicals used (N^G-nitro-L-arginine, tetrodotoxin, phenylephrine, noradrenaline, acetylcholine, propranolol, atropine sulphate, indomethacin, guanethidine, 7-nitroindazole and capsaicin) were from Sigma.

Results are expressed as a mean ± S.E.M. Differences between means were evaluated by one-way analysis of variance (ANOVA) or Student's test for unpaired observations.

Results

Under basal conditions, short-lasting electrical field stimulation of the main pulmonary artery resulted in reversible and reproducible contractions, as shown in Fig. 1. These responses were blocked by tetrodotoxin (1 μmol/l; Fig. 1) and/or by guanethidine (10 μmol/l) suggesting that they were mediated by noradrenaline released as a result of nerve activity.

During active tone produced by phenylephrine (1 μmol/l), EFS (4 Hz) caused relaxation of the main pulmonary artery which was not abolished by TTX (Fig. 1). The response was dependent on the frequency of electrical stimulation with the most pronounced decrease of tone up to 4 Hz (Fig. 2). Magnitude of EFS-induced relaxations in extralobar branches of main pulmonary artery was smaller than in main pulmonary artery. Acute *in vitro* deendothelization of artery had no significant effect on these responses.

In phenylephrine-precontracted pulmonary arteries from SHR with developed hypertension (mean systolic blood pressure: 176±3 mmHg in SHR *vs.* 107±2 mmHg in normotensive Wistar rats, $p<0.001$), EFS-induced relaxant responses were smaller than in those from control normotensive rats (Fig. 2).

In phenylephrine-precontracted both endothelium-intact and denuded arterial preparations treated with guanethidine (10 μmol/l), propranolol (1 μmol/l), atropine (10 μmol/l) and indomethacin (1 μmol/l), and pre-contracted with phenylephrine (1 μmol/l), EFS evoked frequency-dependent relaxations. The EFS-induced relaxations were not influenced by TTX in concentrations sufficient to abolish the contraction induced by nerve stimulation before elevation of vascular tone with phenylephrine (Fig. 3). Administration of N^G-nitro-L-arginine (L-NNA; 0.1 mmol/l), a general NO synthase inhibitor, produced further increase of tension over the PE-induced contraction. Relaxation induced by EFS (4 Hz) was completely abolished with L-NNA. The inhibition by L-NNA could be reversed, at least in part, by the addition of L-arginine (0.1 mmol/l).

Tamoxifen, a strong inhibitor of neuronal nitric oxide synthase, in concentration 10 μmol/l did not influence the magnitude of EFS-induced relaxation of main pulmonary artery. 7-nitroindazole (10 μmol/l), an inhibitor specific for the neuronal isoform of NO synthase, produced a small transient contraction of the phenylephrine-pretreated pulmonary artery. Then artery gradually relaxed. For this reason the inhibition of EFS-induced relaxation (by 45 %) could be overestimated.

In the next step we compared the magnitude of reduction in relaxant response of Wistar rat main pulmonary artery induced by stimulation at 4 Hz after pharmacological drugs that interfere with the actions and synthesis of NO. Tetrodotoxin and/or tamoxifen did not significantly change EFS-induced relaxation. Capsaicin and 7-nitroindazole moderately reduced

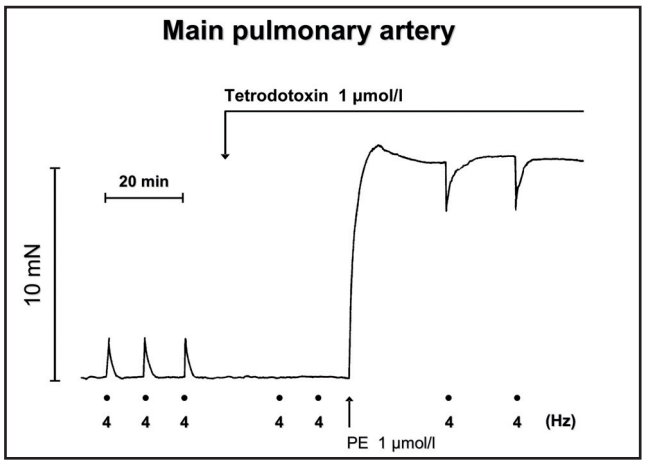

FIGURE 1. Effect of tetrodotoxin on the electrical field stimulation (4 Hz)-induced responses in endothelium intact rat main pulmonary artery before and during contraction caused by phenylephrine (PE).

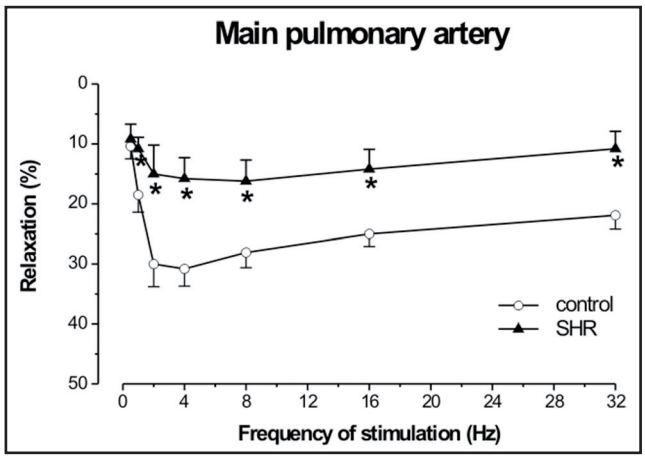

FIGURE 2. Frequency-dependent relaxations produced by electrical field stimulation (0.5–32 Hz) in normotensive (control) and spontaneously hypertensive rats (SHR). Arterial rings were precontracted with phenylephrine (1 μmol/l). Relaxant responses are expressed as a percentage of PE-induced contraction. Each point represents the mean ± S.E.M. of 6–9 arteries (one per animal). * $p<0.05$.

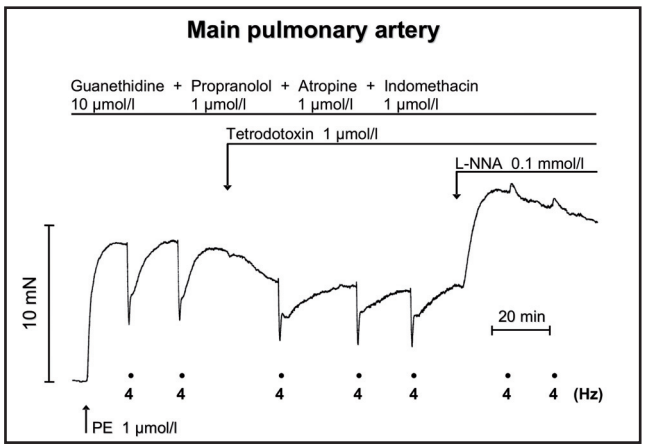

FIGURE 3. Effect of tetrodotoxin and N^G-nitro-L-arginine (L-NNA) on electrical field stimulation (4 Hz)-induced relaxations in endothelium intact rat main pulmonary artery precontracted with phenylephrine (PE) in the presence of guanethidine, propranolol, atropine and indomethacin.

relaxation, but N^G-nitro-L-arginine was the most potent inhibitor of relaxation of all drugs tested, because it nearly completely abolished EFS-induced relaxations (Table 1).

Discussion

The present experiments indicate that an electrically induced relaxation in main pulmonary artery may not be only due to activation of perivascular nerves since it is totally unaffected by TTX in concentrations up to 1 µmol/l. The use of TTX blockade of the response induced by EFS is a strong criterion to define neurogenic mechanisms. Tetrodotoxin blocks the spread of action potentials by inhibiting the voltage-dependent Na^+ channels. In nerves that depend on the action potential for the release of transmitter, TTX should block transmitter release and, thus, block its function. In our experiments TTX in concentration 1 µmol/l which was sufficient to block the contractile responses induced by EFS (4 Hz) was unable to influence the relaxation response. These findings are consistent with those of Buga & Ignarro (1992) observed on isolated bovine pulmonary artery, and in other vessels where the major component of the arterial relaxant response is nonneurogenic in nature (Feletou & Vanhoutte 1987; Hyman *et al* 1981; Kalsner & Quillan 1989; Saito *et al* 1999).

The vasorelaxant function of NO synthase-containing perivascular nerves has been clearly demonstrated in isolated cerebral arteries from many different species (Chen & Lee 1993; Gonzalez & Estrada 1991; Toda & Okamura 1990; 1992; Iadecola *et al* 1994) but this function has not been clearly demonstrated in isolated noncerebral arteries including pulmonary ones.

TABLE 1. Effect of tetrodotoxin, capsaicin, 7-nitroindazole, tamoxifen and NG-nitro-L-arginine on the magnitude of relaxation induced by 4 Hz in rat main pulmonary artery.

Treatment	Relaxation (%)
Control	20.3 ± 1.7
Tetrodotoxin (1 mmol/l)	17.6 ± 1.8
Control	21.5 ± 2.4
Capsaicin (0.5 µmol/l)	16.0 ± 1.3 *
Control	19.3 ± 2.7
7-nitroindazole (10 µmol/l)	12.1 ± 1.4 *
Control	18.2 ± 2.1
Tamoxifen (10 µmol/l)	19.2 ± 2.5
Control	24.3 ± 2.9
N^G-nitro-L-Arginine (0.1 mmol/l)	0.9 ± 0.5 ***

Relaxation is expressed as a percentage of the contraction elicited by phenylephrine (1 µmol/l). Values represent the mean ± SEM of 6-8 rats. * $p<0.05$; *** $p<0.001$.

Our findings in pulmonary arteries regarding TTX sensitivity are not in agreement with those of several authors who utilize the same methodology and similar parameters of electrical field stimulation. Tetrodotoxin-sensitive neurogenic relaxations mediated mainly by NO were observed in guinea-pig pulmonary arteries (Liu *et al* 1992; Chen & Lee 1993; Scott & McCormack 1999). These were supported by histological findings which demonstrated perivascular neurons containing NADPH diaphorase or NOS immunoreactivity (Haberberger *et al* 1997).

The present as well as previous our observations (Török & Kyselá 2000) clearly indicate that EFS stimulates the formation and/or release of NO in rat pulmonary artery: TTX resistant relaxations induced by EFS are not influenced with anticholinergic (atropine) and neuronblocking drugs (guanethidine) and a beta-adrenergic antagonist (propranolol) – therefore they are nonadrenergic, noncholinergic. They are not mediated by prostaglandins since they were not affected by indomethacin. In our experiments the EFS-induced relaxation in the pulmonary artery was abolished by L-NNA, indicating that EFS causes relaxation by mechanism involving the L-arginine-NO-cGMP pathway. The fact that L-arginine reversed in part the inhibitory effect of L-NNA on EFS-induced relaxation confirms this statement.

Involvement of other mediators which interfere with the synthesis and action of NO in EFS-induced relaxation cannot be excluded. Pretreatment of pulmonary artery with capsaicin, a neurotoxin that shows selectivity for sensory nerves (Zheng *et al* 1997), moderately inhibited the NANC relaxation, suggesting that the EFS-induced relaxation is at least partly mediated by mediator released from sensory nerves. Similar effect of capsaicin on EFS-induced relaxation has been also observed in isolated guinea-pig pulmonary (Maggi *et al* 1990; Liu *et al* 1992; Butler *et al* 1993) and mesenteric arteries (Zheng *et al* 1997) suggesting involvement of CGRP in sensory nerves.

7-nitroindazole, an inhibitor specific for the neuronal isoform of NO synthase (Xavier *et al* 2004), produced significant inhibition of EFS-induced relaxation. Since in the presence of 7-NI in incubating medium the arterial tone gradually declined and did not reach steady state, the magnitude of EFS-induced inhibition of relaxant response could be overestimated. Moreover, the fact that 7-nitroindazole attenuated the response induced by EFS does necessarily mean that NO is released from perivascular nerve endings, because neuronal NO synthase is equally expressed in vascular smooth muscle (Boulanger *et al* 1998) and vascular endothelium (Huang *et al* 2002). Tamoxifen, a strong inhibitor of neuronal nitric oxide synthase (Renodon *et al* 1997), did not influence the magnitude of EFS-induced relaxation of pulmonary artery.

Free radical generation in oxygenated salt solution in responses to EFS does not appear to be involved

in the mechanism by which EFS causes relaxation of pulmonary artery in the present study. The electrical stimulation parameters employed, such as square wave pulses of short duration (0.5 ms) and short stimulation trains of 20 s, are not conducive to the generation of free radicals in aqueous solution (Feletou & Vanhoutte 1987). The possibility of free radicals found itself to cause relaxation upon EFS at higher frequency of stimulation (greater production of reactive oxygen species) was excluded because magnitude of relaxation was not changed or reduced in comparing to relaxation observed at low frequency of stimulation (Fig. 2).

Unlike noradrenaline, NO itself cannot be stored in nerve endings. It is generally accepted that NO is synthesized on demand when the nerve endings are pharmacologically or electrically excited. Other sources than neurons (endothelium, smooth muscle cells) for the NO involved in nonadrenergic noncholinergic inhibitory transmission have also been proposed (Geary *et al* 1997; Buchwalow *et al* 2002). During electrically induced relaxation of artery, the current generated by EFS causes the endothelium to release the both NO and EDHF; higher frequencies primarily cause NO release from endothelium. This statement was supported by experiment in which effluent taken from the bath of freshly isolated and electrically stimulated bovine aortic endothelial cells caused relaxation in endothelium-denuded ring arterial segments contracted with phenylephrine (Geary *et al* 1997). In our experiments, persistence of EFS-induced relaxation of pulmonary artery after deendothelization also suggests that the source of the transmitter must not be necessarily endothelial cells. Buchwalow *et al* (2002) showed that, in contrast to the currently accepted view, smooth muscle cells in blood vessels express all three NO synthase isoforms. These findings suggest an alternative mechanism by which local NO synthase expression may modulate vascular functions in endothelium-independent manner.

Vascular generation of superoxide anions are increased in hypertensive animals (Wu *et al* 2001). Nitric oxide in vessel wall is rapidly inactivated in the presence of superoxide anions (Gryglewski *et al* 1986), and it is reflected in attenuated vascular relaxant responses. We have shown that in pulmonary artery from SHR EFS-induced relaxations were reduced at each frequency. This could indicate a decrease of NO release from NO-generating vascular components (endothelium, sympathetic neurons, smooth muscle) and /or increased inactivation of NO by reactive oxygen species.

We conclude that electrical field stimulation causes relaxation of precontracted rat pulmonary artery which is mediated by NO mainly of nonneurogenic origin. In normotensive animals EFS-induced release of NO contributes to maintaining the low resistance in lung circulation. In SHR, the reduced EFS-induced relaxation can contribute to maintenance of elevated blood pressure.

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