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Mechanical stretch reveals different components of endothelial-mediated vascular tone in rat aortic strips

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1 Since the role of mechanical stretches in vascular tone regulation is poorly understood, we studied how stretch can influence endothelial tone.

2 Isometric contractions of isolated rat aortic helical strips were recorded. The resting tension was set at 0.7 g, 1.2 g or 2.5 g. Endothelium-preserved strips were precontracted with either phenylephrine or prostaglandin F_{2α} (PGF_{2α}).

3 In control conditions, acetylcholine (ACh) dose-dependently relaxed phenylephrine-precontracted strips independently of resting tension.

4 At 0.7 g resting tension, nitric oxide synthase (NOS) inhibitors did not reduce ACh-induced relaxation, while either a guanylyl cyclase inhibitor or a NO trapping agent prevented it. At 1.2 g and 2.5 g resting tensions, NOS inhibitors shifted the ACh dose-response curve to the right.

5 After preincubation with indomethacin (5 μM) or ibuprofen (10 and 100 μM), at 0.7 g and 1.2 g resting tensions, ACh induced an endothelium-dependent, dose-dependent contraction. ACh (10⁻⁶ M) increased the contraction up to two times greater the phenylephrine-induced one. Lipoxygenase inhibitors prevented it. At high stretch, the ACh vasorelaxant effect was marginally influenced by cyclooxygenase (COX) inhibition. Similar results were obtained when aortic strips were precontracted with PGF_{2α}.

6 Our data indicate that when resting tension is low, ACh mobilizes a stored NO pool that, synergistically with COX-derived metabolites, can relax precontracted strips. COX inhibition up-regulates the lipoxygenase metabolic pathway, accounting for the ACh contractile effect. At an intermediate resting tension, NO production is present, but COX inhibition reveals a lipoxygenase-dependent, ACh-induced contraction. At high resting tension, NO synthesis predominates and COX metabolites influence ACh-induced relaxation marginally.

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Keywords: rat aortic strips; resting tension; stored nitric oxide; lipoxygenase metabolite-induced contraction; nitric oxide synthase inhibitors; guanylyl cyclase inhibitor; nitric oxide trapping agent

Abbreviations: ACh, acetylcholine; COX, cyclooxygenase; cyclic GMP, guanosine 3':5'-cyclic monophosphate; ecNOS, endothelial constitutive NO synthase; EC₅₀s, median effective concentrations; EDHF, endothelial-derived hyperpolarizing factor; EDRFs, endothelial-derived relaxant factors; ETI, 5,8,11-triynoic acid; 6-keto PGF_{1α}, 6-keto prostaglandin F_{1α}; L-NAME, ω-nitro-L-arginine methylester; L-NMMA, ω-monomethyl-L-arginine; L-NIO, L-N⁵-iminoethylornithine; LY83583, 6-anilino-5,8-quinolinequinone; MK-886, 3-(1-(4-chlorobenzyl)-3t-butyl-thio-5-isopropylindol-2-yl)-2,2-dimethylpropanoic acid; PGF_{2α}, prostaglandin F_{2α}; PGI₂, prostacyclin; PTIO, 2-phenyl-4,4,5-tetramethyl-imidazoline-1-oxyl-3-oxide

Introduction

At least three different endothelial-derived relaxant factors have been thus far identified: prostacyclin (PGI₂), nitric oxide (NO), and the endothelial-derived hyperpolarizing factor (EDHF). PGI₂ is the main metabolite of cyclooxygenase (COX) in endothelial cells and its role as an anti-aggregating agent is well defined (Moncada & Vane, 1979). It seems, however, to affect vascular relaxation less, since in the presence of indomethacin, endothelial cell activation can still relax precontracted vascular preparations (Furchgott & Zawadzki, 1980). NO is produced by endothelial constitutive NO synthase (ecNOS) from L-arginine (Palmer *et al.*, 1988). Throughout guanylyl cyclase activation, guanosine 3':5'-cyclic monophosphate (cyclic GMP) increase and cyclic GMP-dependent protein kinase stimulation, NO can induce smooth

muscle cell relaxation (Cornwell *et al.*, 1991). EDHF has been identified as a cytochrome P450 metabolite of arachidonic acid (Fisslthaler *et al.*, 1999), which, by activation of potassium channels, can induce hyperpolarization of vascular smooth muscle cell membrane and artery relaxation (Campbell & Harder, 1999).

Depending on the experimental and pathophysiological conditions, all these compounds can contribute to endothelial-dependent vasorelaxation.

Moreover, endothelial cells also produce contractile substances. Contracting factors include lipid-derived eicosanoids, such as arachidonic acid and lipoxygenase metabolites (Ezra *et al.*, 1983; Rimele & Vanhoutte, 1983; Berkowitz *et al.*, 1984; Marsault *et al.*, 1997; Filipeanu *et al.*, 1998). Leukotrienes are also produced by endothelial cells in culture (Kähler *et al.*, 1993; Hollenberg *et al.*, 1994). Therefore, from common precursor arachidonic acid, endothelial cells may produce either vasorelaxant factors or vasoconstricting

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substances according to the prevalence of the different metabolic pathway.

Among the physiological regulators of endothelial-derived vasoactive substance production, shear stress and cell stretch may be of primary relevance. However, the influence of cell stretch on endothelial-derived vasoactive substance production and release is still partially unclear.

The effect of stretch has been investigated by Dainty *et al.* (1990) by changing either the resting length or the resting tension of rat aortic preparations *in vitro*. The authors show that the initial length of preparation (and hence stretch degrees) modifies the contractile response to phenylephrine. The decrease in tone induced by 1 μ M acetylcholine (ACh), is indeed dependent on the initial stretch of endothelial-intact preparations. Although the authors focused their analysis on result variability from different laboratories, it emerged that variations in arterial tone and stretch may influence the response of smooth muscle cells to endothelial-derived vasoactive factors.

In coronary preparations from guinea-pigs and rats, stretch influences the relative contribution of endothelial-derived vasorelaxant substances to smooth muscle cell hyperpolarization (Parkington *et al.*, 1993). Indeed, in unstretched guinea-pig coronary arteries, the hyperpolarization of smooth muscle cells induced by ACh is only slightly reduced by co-incubation with the eNOS inhibitor ω -nitro-L-arginine methylester (L-NAME) and indomethacin. Indeed, exogenous NO and PGI₂ are without effect. In contrast, in stretched preparations, exogenous NO and PGI₂ induce hyperpolarization and co-incubation with L-NAME, and indomethacin abolishes the slow component of ACh-induced hyperpolarization. Delayed hyperpolarization is also induced by arachidonic acid, and indomethacin is able to antagonize its effect, suggesting that a metabolite of arachidonic acid may account for this effect. Similar results are also obtained in rat coronary arteries, although PGI₂ is unable to induce hyperpolarization (Parkington *et al.*, 1993). These data indicate that the contribution of different vasorelaxant products may vary according to the resting tension.

Since the role of stretch in endothelial-derived vascular tone regulation has been only partially explored, we decided to investigate the ACh effect on contractile tone in rat aortic strips stretched at different resting tensions. Our data indicate that, according to stretch, the relative contribution of NO and arachidonic acid metabolites on ACh-induced relaxation varies. At low stretch, ACh can either relax or contract aortic preparations, considering that relaxation is obtained when COX products are present and contraction is obtained when COX is inhibited. Moreover, in this condition a stored, depletable NO pool is released independently of eNOS inhibition. At high stretch, NO relaxation predominates and COX inhibition does not influence the ACh-induced relaxation.

Methods

This investigation conforms to European Community rules for the care and use of laboratory animals.

Microscopy

Helical preparations of rat thoracic aorta were fixed by immersion in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for 6 h at room temperature, postfixed in 1% OsO₄ in 0.1 M phosphate buffer (pH 7.4) dehydrated in acetone

series, passed through propylene oxide, and embedded in epoxy resin (Epon 812, Fluka, Buchs, Switzerland). Semithin sections, 2 μ m thick, were cut with an ultramicrotome, stained with toluidine blue-sodium tetraborate, viewed and photographed with a light microscope. Semithin sections were used because they allow for a clear-cut identification of the components of the arterial tunica intima, especially the endothelium.

Functional studies

Male Wistar rats (300–350 g) were anaesthetized and killed by cervical dislocation. Thoracic aortas between the aortic arch and diaphragm were isolated and placed in cold gassed (5% CO₂ in O₂) Krebs-Henseleit solution (K-H) of the following composition (mM): NaCl 110, NaHCO₃ 25, KCl 4.8, KH₂PO₄ 1.2, MgSO₄ 1.2, D(+)glucose 11, CaCl₂ 2.5. The aorta was cleaned from adhering fat and connective tissue and cut into helical strips (3 mm width, 20 mm length). The strips were mounted in an organ bath (2 ml) with one end connected to a tissue holder and the other to a force transducer (Battaglia-Rangoni), then the strips were perfused with pre-warmed (37°) K-H, gassed with 5% CO₂ in O₂. The resting tension was set at 0.7, 1.2 or 2.5 g and the tissue was equilibrated for 60 min. These resting tensions were arbitrarily chosen in the range in which phenylephrine, prostaglandin F_{2 α} (PGF_{2 α}) and ACh induced, in control conditions, a similar dose-response curve (see below). Isometric contractions were recorded. After equilibration, dose-response curves to phenylephrine or PGF_{2 α} were performed and the maximal contraction obtained was expressed as 100% of contractile response. Using the maximal contracting dose of phenylephrine or PGF_{2 α} (100%), the cumulative dose-response curve of ACh was done. In several experiments, after extensive washing in the absence (control) or presence of eNOS inhibitors, 10⁻⁷ M of phenylephrine was reapplied and the ACh dose-response curve repeated. The complete procedure was reproduced several times until a superimposable ACh dose-response curve was obtained. All dose-response curves were performed by administering drugs as a bolus injection. In each preparation, the presence of a functional endothelial lining was demonstrated in control conditions by a near-complete relaxation to ACh of precontracted strips. To further assess that the manipulation of aortic preparations had not caused a loss of the endothelial layer, light microscopic examination was carried out on aortic helical strips prepared as for the functional experiments. The relaxation or contraction induced by ACh was expressed as a decrease or an increase in the contractile force from the maximal contraction. Experiments were mostly performed on endothelium-intact preparations. In several cases, the endothelium was removed by rubbing the cells with cotton gauze, and the absence of endothelium was determined as described before, where ACh was unable to induce relaxation.

6-keto prostaglandin F_{1 α} (6-keto PGF_{1 α}) release 6-keto PGF_{1 α} was measured, using a commercial radioimmunoassay kit (Amersham Pharmacia Biotech, Buckinghamshire, U.K.), in the lyophilized perfusion medium obtained by closing the organ bath for 4 min. Standard concentrations of 6-keto PGF_{1 α} were also dissolved in K-H and lyophilized analogously to unknown samples. Determinations were carried out according to the instructions provided by the manufacturer.

Materials

Acetylcholine (ACh), phenylephrine, prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), indomethacin, ibuprofen sodium salt, 5,8,11-triynoic acid (ETI), 2-phenyl-4,4,5-tetramethyl-imidazoline-1-oxyl-3-oxide (PTIO), ω -monomethyl-L-arginine monoacetate salt (L-NMMA) and L-N⁵-(1-iminoethyl)ornithine dihydrochloride (L-NIO) were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.), ω -nitro-L-arginine methylester (L-NAME), 6-anilino-5,8-quinolinequinone (LY83583) were purchased from Calbiochem (La Jolla, CA, U.S.A.). All other reagents were of analytical grade. 3-(1-(4-chlorobenzyl)-3t-butyl-thio-5-isopropylindol-2-yl)-2,2-dimethylpropanoic acid (MK-886) was the kind gift of Merck Sharp & Dohme, Italy (Rome).

Mathematical and statistical methods

Statistical comparisons between data groups were performed using paired Student's *t*-test. Median effective concentrations (EC_{50} s) were calculated using the Implot (version4) program (GraphPad Software, San Diego, CA, U.S.A.). A *P* value <0.05 was considered significant.

Results

Morphology

Histological examination of semithin sections of aortic strips revealed the presence of large tracts of the aortic wall provided with a continuous layer of endothelial cells (not shown).

Functional studies

Phenylephrine caused a rapid onset, dose-dependent contraction of aortic strips independent of the resting tension; therefore, as shown in Figure 1, the dose-response curves were superimposable, obtaining maximal contraction at 10^{-7} M phenylephrine. Hence, in all experiments 10^{-7} M phenylephrine was used to precontract aortic strips.

In phenylephrine precontracted aortic strips, administration of ACh induced a dose-dependent relaxation (Figure 2, panels A and B), 10^{-6} M ACh being sufficient to induce a complete relaxation of endothelium-bearing preparations. As predicted, in the endothelial-denuded preparations, ACh was

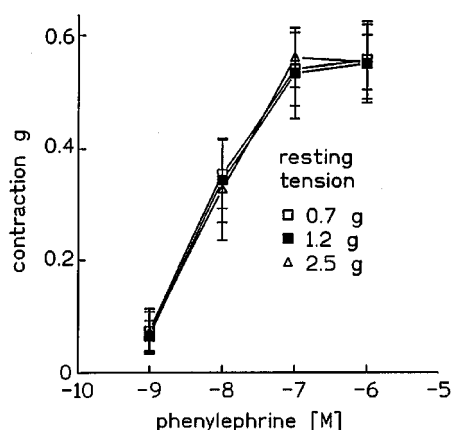


Figure 1 Effect of phenylephrine on isolated rat thoracic aortic strips. Cumulative dose-response curves of phenylephrine were performed at 0.7, 1.2 and 2.5 g resting tension. Values are the mean \pm s.e. mean of at least four experiments.

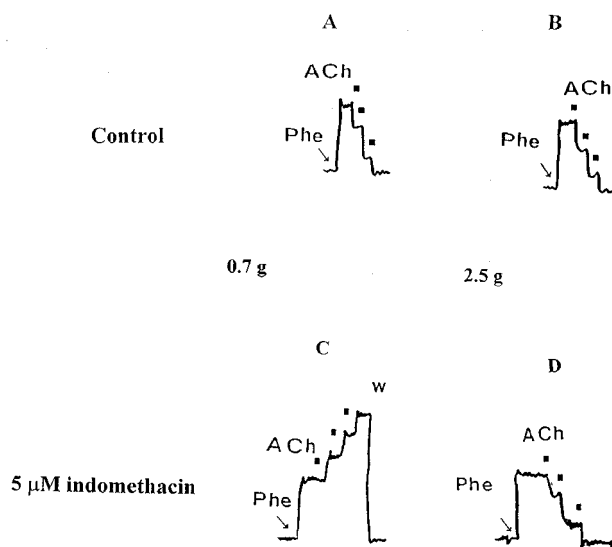


Figure 2 Effect of ACh on phenylephrine-precontracted rat aortic strips. Cumulative dose-response curves of ACh were performed in control conditions (A and B) and in the presence of $5 \mu\text{M}$ indomethacin (C and D). Aortic strips were stretched at a resting tension of 0.7 g (A and C panels) and 2.5 g (B and D) and precontracted with 10^{-7} M phenylephrine (Phe). Black squares indicate ACh (10^{-7} – 3×10^{-7} – 10^{-6} M) administration. Typical traces are shown. W = washing.

ineffective independent of applied resting tension, while Na nitroprusside was still able to relax aortic strips (not shown). No differences in the dose-response curve of ACh were observed in relation to resting tension (Table 1). Moreover, in the presence of an intact endothelium, the Na nitroprusside relaxant effect was also not different in preparations stretched at 0.7 g and 2.5 g (EC_{50} s: 44.1 ± 4.01 and 41.8 ± 3.80 nM respectively at 0.7 g and 2.5 g).

The role of NO-mediated relaxation dependent on the activation of eNOS at the different tensions was tested by inhibiting the enzyme with $100 \mu\text{M}$ L-NAME (Figure 3). At the lower resting tension, L-NAME ($100 \mu\text{M}$, 30 min preincubation) did not reduce the endothelium-dependent vasorelaxation induced by ACh (Figure 3A) and ACh EC_{50} s were not significantly different (Table 1). Similar results were obtained by preincubating aortic strips with $200 \mu\text{M}$ L-NAME for 60 min.

We also tested the effect of two other eNOS inhibitors, namely L-NMMA ($100 \mu\text{M}$, 60 min preincubation) and L-NIO ($25 \mu\text{M}$, 60 min preincubation). As reported in Table 2, at low resting tension ACh EC_{50} s were not significantly different when they were calculated on the 1st curve performed in the presence of the indicated eNOS inhibitor. However, by repeating ACh dose-response curves several times on the same preparation maintained in a continuous perfusion of eNOS inhibitors, the ACh relaxant effect decreased and on the 4th curve the ACh EC_{50} s were up to 4 fold higher than those calculated on the 1st curve (Table 2).

When resting tension was set at 2.5 g, the preincubation with $100 \mu\text{M}$ L-NAME for 30 min shifted the ACh dose-response curve to the right (Figure 3B), as demonstrated by the increase in ACh EC_{50} s (Table 1). Similarly, the ACh dose-response curve was shifted to the right after preincubation with either $100 \mu\text{M}$ L-NMMA or $25 \mu\text{M}$ L-NIO (Table 2). By repeating ACh dose-response curves several times on the same preparation maintained in the constant presence of eNOS inhibitors, ACh, EC_{50} s were increased,

although these increases were of a lesser extent than those observed at a lower resting tension (Table 2).

At an intermediate resting tension (1.2 g), the ACh EC_{50} calculated in the presence of $100 \mu\text{M}$ L-NAME was significantly increased from 0.25 ± 0.015 (control) to $0.49 \pm 0.012 \mu\text{M}$ ($P < 0.001$ vs control).

In order to better investigate the role of NO in the vasorelaxant effect of ACh, we performed experiments using either a guanylyl cyclase inhibitor (LY83583) or a NO trapping agent (PTIO). As shown in Figure 4, the inhibition of guanylyl cyclase or the trapping of NO prevented the vasorelaxant effect of ACh. LY83583 ($10 \mu\text{M}$) totally abolished the vasorelaxation induced by ACh, whereas $10 \mu\text{M}$ PTIO blocked it by 96%. At high resting tension,

the ACh dose-response curve performed in the presence of L-NAME was not modified by co-incubation with either $10 \mu\text{M}$ PTIO or $10 \mu\text{M}$ LY83583.

The role of COX metabolites in the vasorelaxation induced by ACh was then investigated by blocking COX isoenzymes with $5 \mu\text{M}$ indomethacin. In these conditions (pre-incubation with $5 \mu\text{M}$ indomethacin for 30 min), the contractile effect of phenylephrine was not modified at the assayed resting tensions. ACh dose-dependently relaxed phenylephrine-precontracted aortic strips only when resting tension was set at 2.5 g (Figure 2D). At the lower tension, ACh induced a strong, long-lasting dose-dependent contraction (Figure 2C). This increase in contraction was up to 2 fold the phenylephrine-induced one at 10^{-6} M ACh. Simultaneous incubation with $5 \mu\text{M}$ indomethacin and $100 \mu\text{M}$ L-NAME did not modify the contractile dose-response curve of ACh. When preparations were stretched at 0.7 g and incubated with ibuprofen (200 and $10 \mu\text{M}$), another well known COX-inhibitor avoiding lipoxygenase cross-inhibition, 10^{-6} M ACh induced a dose-dependent vasoconstriction. In this condition, 10^{-6} M ACh induced an increase in contraction twice as much as phenylephrine alone ($206 \pm 19.3\%$ and $203 \pm 6.2\%$ in the presence of 200 and $10 \mu\text{M}$ ibuprofen respectively). When aortic strips were stretched at an intermediate resting tension (1.2 g) and in the presence of $5 \mu\text{M}$ indomethacin, ACh also induced a strong contraction; 10^{-6} M ACh was able to increase up to twice as much as phenylephrine alone ($197 \pm 22\%$). The ACh contractile effect observed in the

Table 1 Acetylcholine median effective concentrations (EC_{50} s) that induce relaxation in 10^{-7} M phenylephrine-precontracted rat aortic strips

	EC_{50} (μM)	
	Resting tension	
	0.7 g	2.5 g
Control	0.25 ± 0.024 (5)	0.25 ± 0.017 (8)
$100 \mu\text{M}$ L-NAME	0.28 ± 0.017 (5)	$0.85 \pm 0.09^*$ (8)

Values are the mean \pm s.e.mean of the number of experiments in brackets. * $P < 0.001$ vs control.

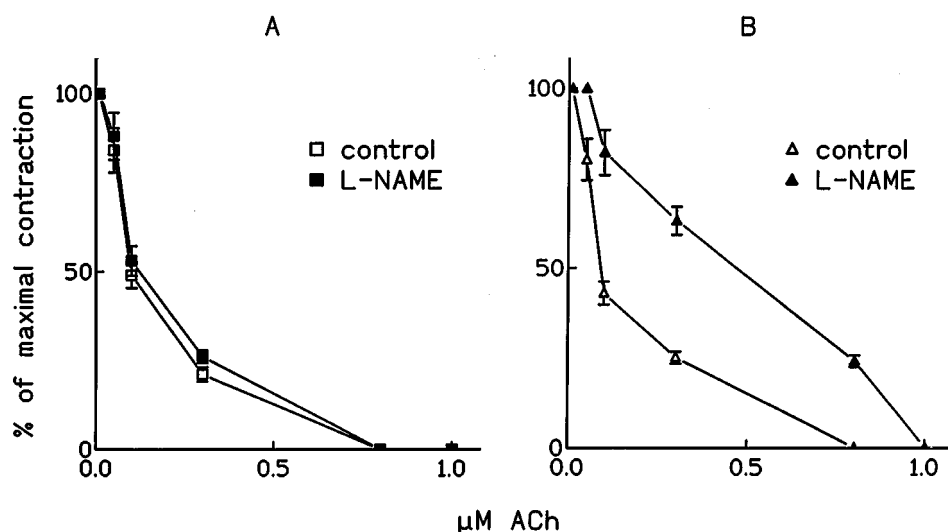


Figure 3 Effect of L-NAME on ACh-induced relaxation. Cumulative dose-response curves of ACh either in control conditions or after 30 min preincubation with $100 \mu\text{M}$ L-NAME were performed on 10^{-7} M phenylephrine-precontracted rat thoracic aortic strips stretched at different resting tensions (A: 0.7 g and B: 2.5 g). The contraction obtained after phenylephrine is set as 100%. Values are the mean \pm s.e.mean of at least four experiments.

Table 2 Acetylcholine median effective concentrations (EC_{50} s) that relax phenylephrine-precontracted rat aortic strips calculated on the 1st and 4th repeated curve

	EC_{50} (μM)			
	0.7 g		2.5 g	
	1st	4th	1st	4th
Control	0.26 ± 0.022	0.27 ± 0.028	0.25 ± 0.017	0.27 ± 0.028
$25 \mu\text{M}$ L-NIO	0.28 ± 0.030	$1.12 \pm 0.187^{***}$	$0.81 \pm 0.075^*$	$1.10 \pm 0.123^{***}$
$100 \mu\text{M}$ L-MMA	0.26 ± 0.025	$1.14 \pm 0.147^{***}$	$0.73 \pm 0.012^*$	$0.91 \pm 0.082^{***}$

ACh dose-response curves were repeatedly performed on 10^{-7} M phenylephrine-precontracted rat aortic strips after washing in the continuous presence of the indicated eNOS inhibitor. For more details, see Methods. Values are the mean \pm s.e.mean of at least three experiments. * $P < 0.001$ vs control, ** $P < 0.001$ vs 1st curve of same treatment.

presence of COX inhibitors was, however, absent when endothelium was mechanically removed. In the absence of endothelium, 10^{-4} M ACh was also unable to induce contraction or relaxation in either the presence or absence of indomethacin.

When aortic strips were precontracted with the maximal contracting dose of $\text{PGF}_{2\alpha}$ (10^{-5} M), ACh induced a dose-dependent relaxation independent of resting tension (Figure 5A and B). When preparations were stretched at 0.7 g and preincubated with 5 μM indomethacin, ACh induced a dose-dependent contraction (Figure 5C), while when resting tension was at 2.5 g, ACh also induced relaxation in the presence of indomethacin.

In order to explore the nature of contractile agents released by endothelium in the presence of 5 μM indomethacin at 0.7 g resting tension, the effects of two lipoxygenase inhibitors were tested. Both ETI (20 μM) and MK-886 (1 μM) antagonized the contractile effect of 10^{-6} M ACh (Table 3), but did not restore its relaxant effect. Similarly, at a very high dose, indomethacin (100 μM) prevented the contractile effect of 10^{-6} M ACh observed in its presence at a low dosage (5 μM

indomethacin), and the administration of ACh did not modify the contracting tone induced by phenylephrine. Moreover, since the increase in contraction may be dependent on the calcium influx through the L-type calcium channel, we tested the effect of the L-type calcium channel blocker verapamil (1 μM) on the extra contraction induced by ACh in the presence of indomethacin. While the contractile effect of phenylephrine was not influenced by 1 μM verapamil, it prevented the contractile effect of ACh (Table 3). Lipoxygenase inhibitors also blocked the ACh-induced extra contraction when resting tension was 1.2 g.

In a $\text{PGF}_{2\alpha}$ precontracted preparation, preincubation with 100 μM indomethacin also abolished the extra contraction induced by ACh at 0.7 g resting tension.

We then evaluated the effect of a simultaneous inhibition of COX and NO-dependent relaxation. When resting tension was set to 0.7 g, and 10 μM PTIO and 5 μM indomethacin were simultaneously perfused, the ACh contractile dose-response curve was only marginally modified, having ACh EC_{50} s not significantly different (0.41 ± 0.13 μM , indomethacin alone vs 0.35 ± 0.14 μM , indomethacin plus PTIO). When resting tension was set to 1.2 g, the ACh dose-response curve was shifted to the left by pre-incubating with 5 μM indomethacin and 100 μM L-NAME; however, 3×10^{-7} M ACh induced the maximal contraction. When resting tension was set to 2.5 g, the simultaneous perfusion of 5 μM indomethacin and 100 μM L-NAME totally prevented the relaxant effect of ACh. Still, ACh did not induce a significant increase in contractions (not shown).

PGI₂ release

We also measured the release of PGI₂ in the organ bath by measuring its main metabolite 6-keto $\text{PGF}_{1\alpha}$ at the lower resting tension. As shown in Figure 6, after the administration of phenylephrine, a significant increase in PGI₂ was observed. The successive administration of ACh was unable to further increase the release of PGI₂ in the organ bath. After washing ACh and phenylephrine, the same aortic strips were preincubated with 5 μM indomethacin. As predicted, indomethacin strongly reduced the production of PGI₂. Nor the consecutive administration of phenylephrine and ACh, nor preincubation with ETI restored the release measured in control conditions.

Discussion

Our data show new aspects of the endothelial-mediated regulation of rat aorta tone primarily dependent on resting tension.

When resting tension is low (0.7 g), COX-derived metabolites, synergistically with NO, seem to play an important role in the relaxation induced by ACh. In these experimental conditions, a stored pool of NO is released, while NO neosynthesis is practically absent. Indeed, although eNOS inhibitors, namely L-NAME, L-NMMA and L-NIO, do not modify the dose-response curve of ACh, LY83583 abolishes ACh-induced relaxation of phenylephrine-precontracted aortic strips, indicating a relevant role for guanylyl cyclase. Moreover, the finding that PTIO strongly reduces ACh-induced relaxation suggests that ACh can mobilize a stored NO pool, which, synergistically with PGI₂, can relax precontracted strips. This stored NO pool is, however, limited. Indeed, by repeating the ACh dose-response curve several times in the continuous presence of eNOS inhibitors,

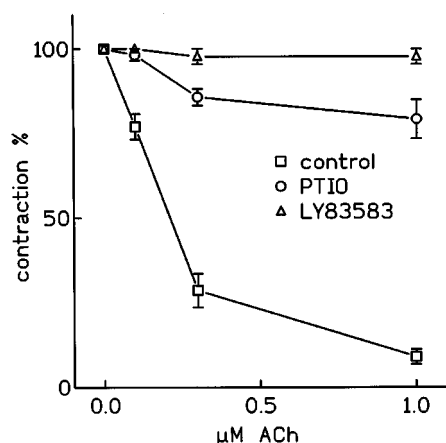


Figure 4 Effect of LY83583 and PTIO on ACh-induced relaxation. Cumulative dose-response curves of ACh in control conditions, after 30 min preincubation with either 10 μM LY83583 or 10 μM PTIO were performed on 10^{-7} M phenylephrine-precontracted rat thoracic aortic strips stretched at 0.7 g resting tension. The contraction obtained after phenylephrine is set as 100%. Values are the mean \pm s.e.mean of at least four experiments.

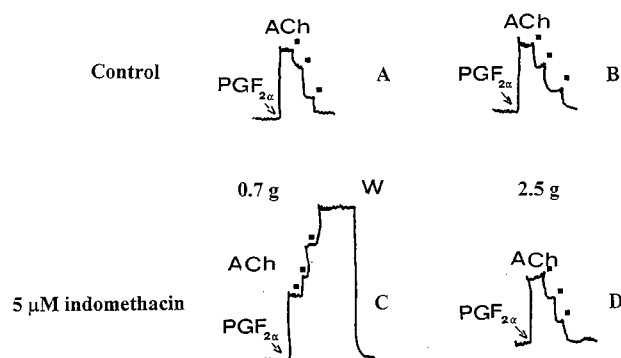


Figure 5 Effect of ACh on $\text{PGF}_{2\alpha}$ -precontracted rat aortic strips. Cumulative dose-response curves of ACh were performed in control conditions (A and B) and in the presence of 5 μM indomethacin (C and D). Aortic strips were stretched at a resting tension of 0.7 g (A and C) and 2.5 g (B and D) and precontracted with 10^{-5} M $\text{PGF}_{2\alpha}$ ($\text{PGF}_{2\alpha}$). Black squares indicate ACh (10^{-7} – 3×10^{-7} – 10^{-6} M) administration. Typical traces are shown. W = washing.

Table 3 Effect of 10^{-6} M acetylcholine on rat aortic strips preincubated with $5 \mu\text{M}$ indomethacin in the absence (none) or presence of lipoxygenase inhibitors or verapamil

None	20 μM ETI	1 μM MK-886	% of contraction 100 μM Indomethacin	1 μM Verapamil
202 \pm 20	119 \pm 6*	113 \pm 6.7*	115 \pm 7.5*	100 \pm 3.5*

* $P < 0.001$ vs no lipoxygenase inhibitors (None). Rat aortic strips were precontracted with 10^{-7} M phenylephrine and preincubated with $5 \mu\text{M}$ indomethacin (30 min, 37°C) in the absence (None) or presence of either lipoxygenase inhibitors or verapamil. Contractile force developed by 10^{-7} M phenylephrine was set as 100%. Values are the mean \pm s.e.mean of at least four experiments. Experiments were performed at 0.7 g resting tension.

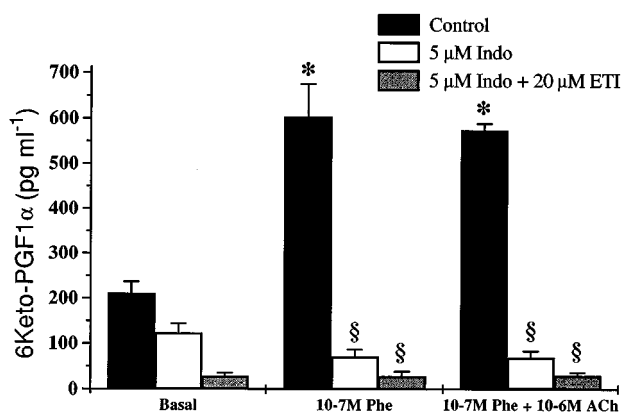


Figure 6 Release of 6-keto $\text{PGF}_{1\alpha}$ from isolated rat aortic strips. 6-keto $\text{PGF}_{1\alpha}$ was measured by RIA either under basal conditions, after administration of 10^{-7} M phenylephrine or after administration of 10^{-7} M phenylephrine and 10^{-6} M ACh on the supernatant obtained from the same isolated rat thoracic aortic strips preloaded at 0.7 g. Basal value indicates the level of 6-keto $\text{PGF}_{1\alpha}$ after reaching a steady-state tension in each experimental condition. For more details, see Methods. Values are the mean \pm s.e.mean of four experiments. * $P < 0.001$ vs basal conditions; § $P < 0.001$ vs control.

the relaxing effect of ACh is strongly reduced as demonstrated by the powerful increase in ACh EC_{50} s. A similar stored nitric oxide pool has been described in rabbit aortic smooth muscle (Venturini *et al.*, 1993), rat aortic non-endothelial cells when the aorta is treated for 18 h with LPS and L-Arginine (Muller *et al.*, 1996), *in vivo* in L-NAME treated rats (Davisson *et al.*, 1996) and, more recently, in human platelets (Hirayama *et al.*, 1999). The exact biological meaning of this pool is still unclear, but according to our data, it can be hypothesized that it plays a role when stretch is low. *In vivo* this condition may be mimicked when blood pressure is low and cells of resistance vessels are marginally stretched. In this condition, NO can be supplied without a constant eNOS activation. Moreover, as judged by the data obtained in the presence of LY83583, it seems that guanylyl cyclase activation is the main relaxant pathway in rat aortic preparations stretched at low resting tension.

The dosage of PGI_2 through its metabolite 6-keto $\text{PGF}_{1\alpha}$ shows that a consistent PGI_2 amount is released immediately after phenylephrine administration. The subsequent administration of ACh is unable to achieve a further increase in PGI_2 production in the perfusion medium. As expected, in the presence of $5 \mu\text{M}$ indomethacin, the production of PGI_2 is strongly inhibited and the small trend to a further decrease in PGI_2 can probably be attributed to the washing of small quantities of either PGI_2 or its metabolites trapped inside cells and tissue.

On the other hand, as shown in Figures 1 and 2, phenylephrine induces a consistent contraction, which is only marginally affected by COX inhibition. Therefore, the self-

produced PGI_2 is incapable of inducing a relevant relaxation. However, this self-produced PGI_2 seems to be essential, since, at low resting tension, relaxation disappears in the presence of COX inhibitors and it is not restored also when the ACh-induced extra contraction is prevented (see below).

Thus at low stretch, ACh can induce a release of stored NO, which synergistically with self-produced PGI_2 induces relaxation. A synergistic antiaggregatory effect of PGI_2 and NO has been originally described in human platelets: low doses of both compounds inhibit aggregation and induce disaggregation of human platelets, while either PGI_2 or NO alone at the same dose are ineffective (Radomski *et al.*, 1987).

Our data also show that at a lower resting tensions (0.7 g and 1.2 g), when COX metabolism is inhibited, ACh strongly contracts phenylephrine-precontracted vessels. In this condition, the arachidonic acid metabolic pathway through lipoxygenase can predominate and lipoxygenase-derived contracting factors account for this paradoxical effect of ACh. Although we are unable to measure by radioimmunoassay an in-the-range leukotriene amount (D4, C4 and E4 leukotrienes, that, according to Kähler *et al.* (1993), can be synthesized by bovine aortic endothelial cells), the finding that lipoxygenase inhibitors block the ACh-induced extra contraction strongly suggests this hypothesis. Also, the suppression of ACh-induced contraction by $100 \mu\text{M}$ indomethacin is in line with this interpretation: the high dose of indomethacin can also inhibit lipoxygenase (Siegel *et al.*, 1979). Moreover, since MK-886 is a specific inhibitor of 5-lipoxygenase (Gillard *et al.*, 1989), this isoform plays a major role in our preparations. A similar diversion of arachidonic acid to the lipoxygenase pathway has been observed in canine saphenous veins: the maximal contraction evoked by ACh and high extracellular potassium concentration (Rimele & Vanhoutte, 1983) and bradykinin (Marsault *et al.*, 1997) is enhanced in the presence of indomethacin in comparison to that obtained in control conditions. Since in all cases the control contraction is restored in the presence of indomethacin and nordihydroguaiaretic acid, an inhibitor of lipoxygenase, it has been argued that the inhibition of COX, turning arachidonic acid metabolism to lipoxygenase, produces lipoxygenase vasoconstrictor metabolites (Rimele & Vanhoutte, 1983; Marsault *et al.*, 1997). Lipoxygenase metabolites could increase intracellular calcium by directly activating voltage-sensitive calcium channels (Vanhoutte *et al.*, 1985). Indeed, in canine saphenous veins, diltiazem inhibits the increase in tension due to indomethacin preincubation during ACh- and potassium-induced contractions (Rimele & Vanhoutte, 1983).

In our experiments, verapamil prevents the ACh-induced contraction, suggesting that the paradoxical effect of ACh (observed at low resting tension in the presence of $5 \mu\text{M}$ indomethacin and blocked by lipoxygenase inhibitors) is mediated by an influx of extracellular calcium through L-type, voltage-dependent calcium channels. Therefore, a

relevant target for lipoxygenase products can be L-type calcium channels. Since the ACh contractile effect is abolished in the absence of endothelium, the contractile lipoxygenase metabolite(s) seem(s) to be produced by endothelial cells.

At high resting tension, the NO pathway predominates and COX metabolites seem to influence ACh-induced relaxation only marginally. Indeed, indomethacin does not modify the relaxant effect of ACh, while L-NAME, L-NMMA and L-NIO do. These data suggest that the 'de novo' synthesis of NO is mainly responsible for ACh-induced relaxation when a high resting tension is applied. Indeed, the inhibition of guanylyl cyclase by LY83583 or NO trapping by PTIO does not potentiate the L-NAME inhibitory effect. Moreover, by repeating the ACh dose-response curve on the same preparation in the continuous presence of eNOS inhibitors, the ACh EC₅₀s are increased, but these increases are quite limited.

The residual relaxation obtained at high ACh doses (10⁻⁶ M) in the presence of L-NAME is prevented by 5 µM indomethacin, but ACh never induces contractions. This result suggests that the lipoxygenase products responsible for ACh-induced contractions are either not produced or the mechanism controlling the contractions is inactivated.

At an intermediate tension (1.2 g), the ACh dose-response curve performed in the presence of the eNOS inhibitor L-NAME is shifted to the right, as shown when preparations are stretched at high resting tension. However, in the presence of 5 µM indomethacin, ACh strongly contracted aortic strips and the preparations behave as those stretched at low resting tension. Therefore, although we do not explore a linear range of resting tensions, it seems that the stretch-dependent behaviour of aortic preparations modifies gradually.

These stretch-dependent behaviours are not dependent on a different sensitivity to NO, since Na nitroprusside relaxation is not influenced by resting tension.

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