The non-obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine

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The concept of Endothelium Derived Relaxing Factor (EDRF), put forward by Furchgott in the earlier 80's of the past century[1, 2], implies that nitric oxide (NO) produced by NO synthase (NOS) in the endothelium in response to acetylcholine (ACh) passively diffuses to the underlying vascular smooth muscle cells (VSMC) thereby reducing vascular tension. It was thought that VSMC do not express NOS by themselves, but to the time of those studies immunohistochemical techniques were not what they are now. State-of-the-art immunohistochemistry permits nowadays to localize NOS both to the endothelium and to VSMC[3, 4]. However, the principal question remained unanswered, is the NO generation by VSMC physiologically relevant? We hypothesized that the destruction of the vascular wall anatomical integrity by rubbing the blood vessel intimal surface may increase vascular superoxides that, in turn, reduce NO bioactivity. To address this issue, we examined ACh-induced vasorelaxation in endothelium-deprived blood vessels under protection against oxidative stress and found that superoxide scavengers - tempol and N-acetyl-L-cysteine - restored vasodilatory responses to ACh in endothelium-deprived blood vessels without influencing the vascular wall tension in intact blood vessels. Herewith we provided the first evidence that VSMC can release NO in amounts sufficient to account for the vasorelaxatory response to ACh. In contrast to the commonly accepted concept of the obligatory role of endothelial cells in the relaxation of arterial smooth muscle, the local NO generation by VSMC may modulate vascular functions in an endothelium-independent manner.

In the article "The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by ACh" published in Nature in 1980, Furchgott and Zawadzki reported that rubbing off the endothelial layer rendered blood vessels insensitive to ACh[1]. It was concluded, that the endothelial cells when stimulated by ACh, released a nonprostanoid, diffusible factor

(later termed EDRF for endothelium-derived relaxing factor) that acted on the subjacent VSMC to produce relaxation, whereby VSMC were regarded as passive recipients of NO from endothelial cells. Later, it was reported that the NO release accounts for the biological activity of EDRF[2].

However, aspects of the anatomical integrity of the organ (i.e., blood vessel) subjected to experiments with rubbing the blood vessel intimal surface were neglected. More recently it was, however, found that the destruction of the vascular wall integrity in the process of endothelial denudation destroys myoendothelial gap junctional communications in VSMC[5] and impairs K^+-induced vasorelaxation (background-K^+ channel activation)[6]. What is more, endothelial denudation was reported to increase the concentration of vascular superoxides[7, 8] that, in turn, impair vasodilatory responses to exogenous and endogenous nitrovasodilators[9]. Known as NO scavengers, superoxides drastically reduce NO bioactivity and NO bioavailability[10-12], while the intact endothelium protects VSMC from the superoxide attack[13, 14]. In addition to NO scavenging, superoxides can also directly exert a vasoconstrictor action[15, 16]. Therefore, the objective of the present study was to elucidate the role of superoxides associated with vascular dysfunction induced by destroying the anatomical integrity of the blood vessel.

In these experiments, thoracic aorta rings, mesenteric artery rings and pulmonary artery rings from intact and denudated blood vessels of rat were first subjected to morphological and immunohistochemical control to confirm the absence of the endothelial layer in endothelium-deprived blood vessels and to demonstrate NOS expression in blood vessels under study. The specificity of anti-NOS antibodies used in this study has been earlier confirmed by us with Western blotting of rat and porcine blood vessels[3, 4], rat and human skeletal muscles[17, 18] and rat myocardium[19]. For immunohistochemical assay in this study, we employed highly sensitive chain polymer-conjugated technology (EnVision System) developed by DakoCytomation and found all three NOS isoforms expressed not only in the intima but also in media of the blood vessels under study. As an example, Figure 1 shows strong expression of NOS3 in the thoracic aorta (**Fig. 1a**), mesenteric artery (**Fig. 1b**) and pulmonary artery (**Fig. 1c**), in both intimal and medial cells. Inserts in this layout (**Fig. 1**) demonstrate the complete removal of the endothelial layer after denudation. The NOS expression by cells in the media of blood vessels was also confirmed by us earlier with Western blotting showing the presence of characteristic immunoreactive protein bands for NOS1, NOS2 and NOS3 not only in the intact porcine carotid artery and rat aorta, but also in

the blood vessels devoid of endothelium[3, 4]. Further evidence for NOS expression in VSMC can also be drawn from more recent publications[20-22].

To gain evidence for the role of NOS in the regulation of vascular tension and to elucidate the role of superoxides in impairing vasodilatory responses, we examined ACh-induced vasorelaxation in endothelium-deprived blood vessels in the presence of superoxide scavengers - tempol and N-acetyl-L-cysteine (NAC).

In thoracic aorta rings with intact endothelium, cumulative addition of ACh (10^{-10}-$3x10^{-5}$ M) produced concentration-dependent relaxation. The maximum relaxation was 85.59 ± 4.69 % (**Fig. 2**). In rings with denuded endothelium, ACh-induced relaxation was held back with the maximum relaxation 15.85 ± 4.31 % ($p<0.01$). Pre-treatment of denuded rings with NAC (10^{-4} M) significantly restored ACh-induced relaxation to the level of 33.75 ± 7.25% ($p<0.05$) (**Fig. 2,** left panel). Tempol ($3x10^{-3}$ M), a superoxide dismutase mimetic, also reversed the ACh-mediated relaxations in endothelium-deprived aortic ring preparations with the maximum relaxation of 34.58 ± 6.65% ($p<0.05$), whereas pre-treatment of thoracic aorta intact rings with tempol but insignificantly inhibited ACh-induced relaxation (**Fig. 2,** right panel).

Cumulative addition of ACh (10^{-9}-$3x10^{-5}$ M) relaxed phenylephrine-precontracted intact mesenteric artery rings with a maximum relaxation of 74.0 ± 8.04% (**Fig. 3,** left panel). Compared to intact mesenteric artery rings, endothelial denudation resulted in a significant depression of ACh-induced relaxation (20.2 ± 3.23%, $p<0.01$), but pre-treatment of endothelium-denuded rings with tempol ($3x10^{-3}$ M) restored ACh-induced relaxation up to 51.6 ± 6.2% ($p<0.01$). Important, tempol pre-treatment of intact mesenteric artery rings did not affect the vasorelaxatory response to ACh.

In pulmonary artery rings with intact endothelium, ACh-induced relaxation amounted to 89.9 ± 4.12% (**Fig. 3,** right panel). Like in intact mesenteric artery rings, ACh-induced relaxation was not affected by tempol pre-treatment. After endothelial denudation, ACh-induced relaxation was held back, and the maximum relaxation decreased to 20.0 ± 7.76% ($p<0.01$). However, tempol ($3x10^{-3}$ M) pre-treatment of endothelium-deprived rings restored ACh-induced relaxation to the level of 57.4 ± 8.81% ($p<0.01$).

Thus, our results indicate that superoxides induced by destruction of the vascular wall integrity play crucial role in impaired vasodilatory responses to ACh and explain, why rubbing off the endothelial layer rendered blood vessels insensitive to ACh in the experiment of Furchgott and Zawadzki[1].

To summarize, our data provided the first evidence that VSMC can release NO in amounts sufficient to account for the vasorelaxatory response to ACh, which implies an autocrine fashion of NO signaling in the control of the vascular tension. A better understanding of the NO regulatory networks in the vasculature may contribute to development of novel drug and gene therapies for the treatment of cardiovascular diseases.

METHODS

Animals. All animal experiments were performed in accordance with the guidelines of the Institutional Animal Care Committee, Institute of Normal and Pathological Physiology, Bratislava. Male Wistar rats (350-450 g; n=11) were housed under a 12 h light-12 h darkness cycle, at a constant humidity and temperature, with free access to standard laboratory rat chow and drinking water.

Reagents. We purchased phenylephrine, ACh, and N-acetyl-L-cysteine from Sigma and tempol (4-Hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl) from Merck.

Histology. Tissue probes of the thoracic aorta, mesenteric artery and pulmonary artery were fixed in buffered 4% formaldehyde and routinely embedded in paraffin. Light microscopy of paraffin sections stained with hematoxylin-eosin confirmed complete removal of the blood vessel intimal layer after endothelial denudation.

Antibodies and immunohistochemical techniques. 4-μm sections of the paraffin blocks were dewaxed in xylene, rehydrated in graded alcohols, and pre-treated for antigen retrieval in 10 mmol/L citric acid, pH 6.0, in a pressure cooker as described earlier[3, 4]. After blocking non-specific binding sites with BSA-c basic blocking solution (1:10 in PBS, Aurion, Wageningen, The Netherlands), sections were immunoreacted with primary antibodies over night at 4°C. Characterization of rabbit primary polyclonal antibodies recognizing NOS1, NOS2 and NOS3 (Transduction Laboratories, Lexington, KY, USA; and Santa Cruz Biotechnology, Santa Cruz, California) including Western blotting procedure were described elsewhere[3, 4]. Primary anti-NOS1-3 antibodies were diluted to a final concentration of 2.0 μg ml^{-1}. After immunoreacting with primary antibodies and following washing in PBS, the sections were treated for 10 min with methanol containing 0.6% H_2O_2 to quench endogenous peroxidase. Bound rabbit primary antibodies were detected using DAKO EnVision-HRP system and NovaRed substrate kit (Vector Laboratories, Burlingame, CA, USA),

counterstained with Ehrlich hematoxylin for 30 sec and mounted with an aqueous mounting medium GelTol (Immunotech, Marseille, France).

The exclusion of the primary antibody from the immunohistochemical reaction, substitution of primary antibodies with the rabbit IgG (Dianova) at the same final concentration, or preabsorption of primary antibodies with corresponding control peptides resulted in lack of immunostaining.

Visualization and image processing. Immunostained sections were examined on a Zeiss microscope "Axio Imager Z1". Microscopy images were captured using AxioCam 12-bit camera and AxioVision single channel image processing (Carl Zeiss Vision GmbH, Germany). Resulting images were imported as JPEG files into PhotoImpact 3.0 (Ulead Systems, Inc. Torrance, CA, USA) for analysis on Power PC followed with printing on a color printer Hewlett Packard DeskJet 970Cxi. Images shown are representative of at least 3 independent experiments which gave similar results.

Functional *in vitro* study. Rats were anaesthetized with diethylether, decapitated and exsanguinated. The thoracic aorta, mesenteric artery and pulmonary artery were immediately removed, cleaned of adhering fat and connective tissue and cut into 2-4 mm wide rings. In one part of rings care was taken to avoid abrasion of the intimal surface to maintain the integrity of the endothelial layer. In the second part of rings endothelial cells were removed by gently rubbing the intimal surface with cotton-covered wire. The rings were vertically fixed between two stainless steel triangles in 20 ml incubation organ bath with Krebs solution of the following millimolar composition: NaCl 118; KCl 5; $NaHCO_3$ 25; $MgSO_4$ 1.2; KH_2PO_4 1.2; $CaCl_2$ 2.5; glucose 11; ascorbic acid 1.1; $CaNa_2EDTA$ 0.032, and bubbled with a 95% O_2 and 5% CO_2 gas mixure. The vessel segments were allowed to equilibrate for 1 hour at a resting tension of 1g and the changes of isometric tension were recorded as described previously (Török et al. 1993)[23]. Krebs solution containing 80 mM KCl was prepared by replacing NaCl with equimolar KCl and after an equilibration period the rings were stimulated until a sustained response was obtained, in order to test their contractile capacity. The presence of functional endothelium was assessed in all preparations by determining the ability of ACh (10^{-5} M) to induce relaxation of rings pre-contracted with phenylephrine. For relaxation studies, the rings were pre-contracted with maximum concentration of phenylephrine (10^{-5} M) and cumulative concentration-response curves for ACh (10^{-10}-$3x10^{-5}$ M) were obtained. After washout the rings of aorta were preincubated with N-acetylcysteine

(10^{-4} M; 20 min) or tempol ($3x10^{-3}$ M; 20 min). The rings of mesenteric artery and pulmonary artery with tempol only, and the relaxant responses to ACh were determined. Relaxation was expressed as a percentage of phenylephrine-induced contraction.

Statistical analysis. Data are given as means ± S.E.M. For the statistical evaluation of differences between groups, one-way analysis of variance (ANOVA) was used and followed by Bonferroni´s post-hoc test. The differences of means were considered as significant at *P* value < 0.05.

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AUTHOR CONTRIBUTIONS

I.B., J.N and W.B. formulated the hypothesis and initiated the study; S.C. and F.K. performed experiments with endothelial denudation and with ACh stimulation of blood vessel rings; I.B. and V.S. performed immunohistochemical stainings, microscopy and image processing; W.B. and F.K. provided the main funding for the research; I.B. wrote the manuscript.

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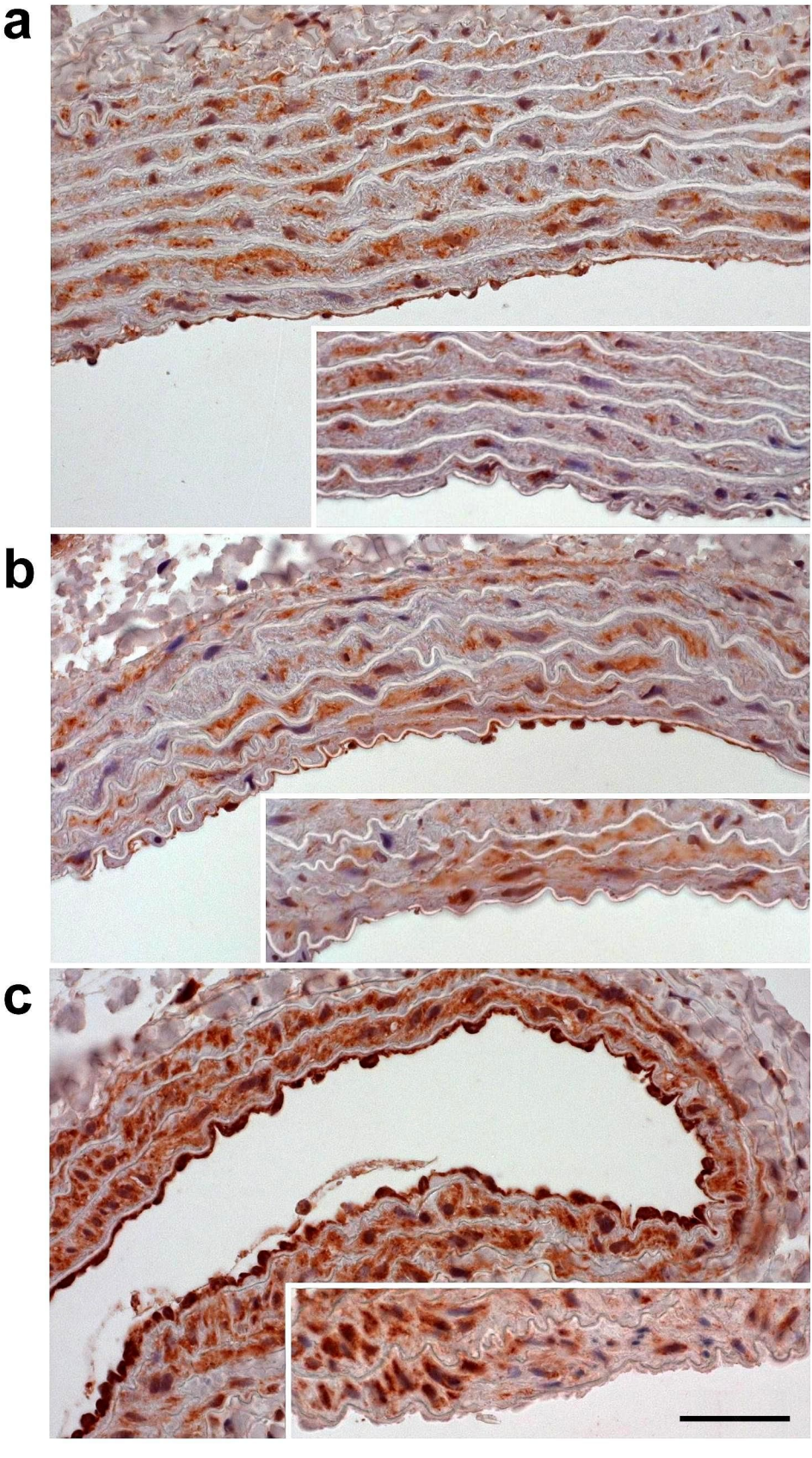
FIGURE LEGENDS

Figure 1 Expression of NOS3 in (**a**) thoracic aorta, (**b**) mesenteric artery and (**c**) pulmonary artery, in both intimal and medial cells. Inserts demonstrate the complete removal of the endothelial layer after denudation. 50 μm scale bar for entire layout.

Figure 2 (**Left panel**) Effect of NAC (10^{-4}M) on the concentration-response curves to ACh in thoracic aorta endothelium-intact (E+) and endothelium-denuded (E-) rings. Tissues were exposed for 20 minutes to NAC before addition of phenylephrine. Data points are mean values and vertical lines represent S.E.M. * $p<0.05$; ** $p<0.01$; with respect to E+; + $p<0.05$; ++ $p<0.01$ with respect to E-. (**Right panel**) Effect of tempol ($3x10^{-3}$M) on the concentration-response curves to ACh in thoracic aorta endothelium-intact (E+) and endothelium-denuded (E-) rings. Tissues were exposed for 20 minutes to tempol before addition of phenylephrine.

Data points are mean values and vertical lines represent S.E.M. * $p<0.05$; ** $p<0.01$; with respect to E+; + $p<0.05$; ++ $p<0.01$ with respect to E-

Figure 3 (**Left panel**) Effect of tempol ($3x10^{-3}$M) on the concentration-response curves to ACh in mesenteric artery endothelium-intact (E+) and endothelium-denuded (E-) rings. Tissues were exposed for 20 minutes to tempol before addition of phenylephrine. Data points are mean values and vertical lines represent S.E.M. ** $p<0.01$; with respect to E+; + $p<0.05$; ++ $p<0.01$ with respect to E-. (**Right panel**) Effect of tempol ($3x10^{-3}$M) on the concentration-response curves to ACh in pulmonary artery endothelium-intact (E+) and endothelium-denuded (E-) rings. Tissues were exposed for 20 minutes to tempol before addition of phenylephrine. Data points are mean values and vertical lines represent S.E.M. ** $p<0.01$; with respect to E+; + $p<0.05$; ++ $p<0.01$ with respect to E-.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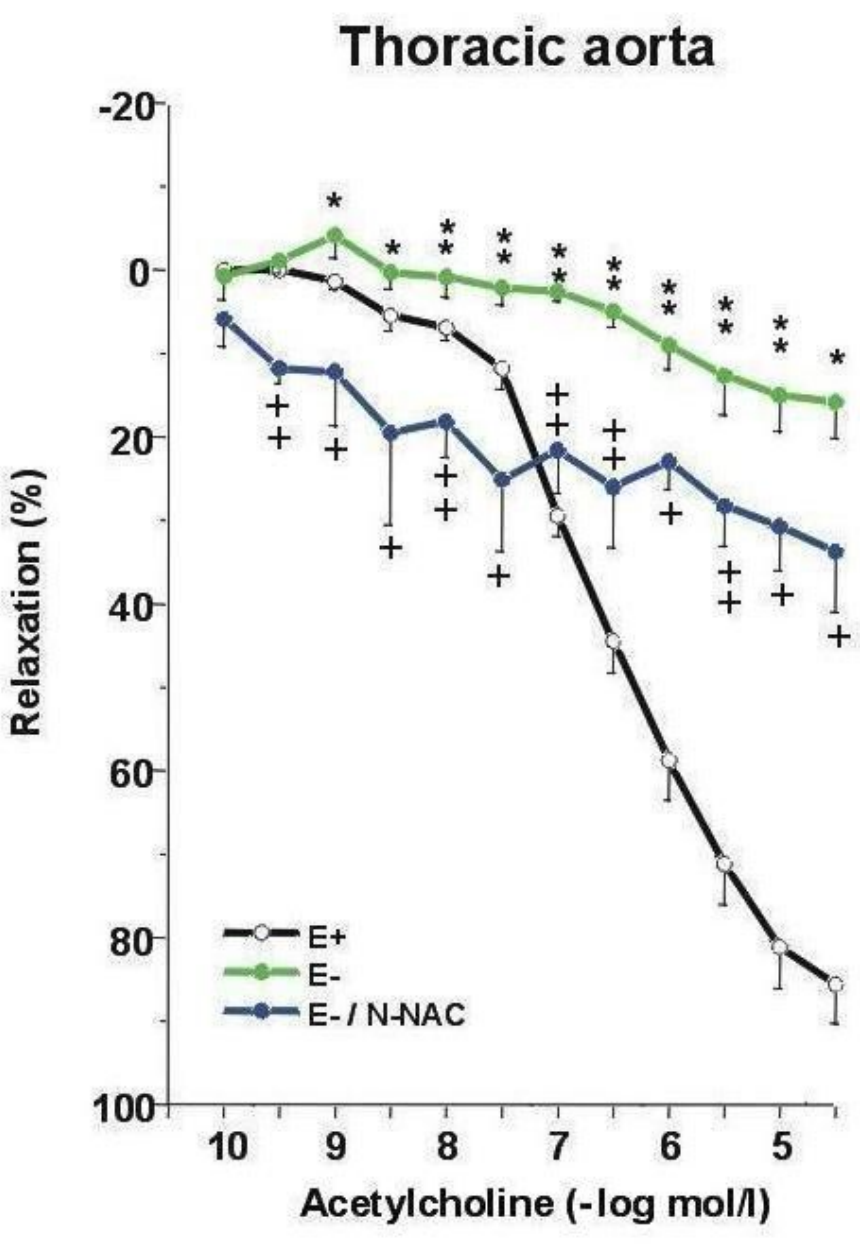

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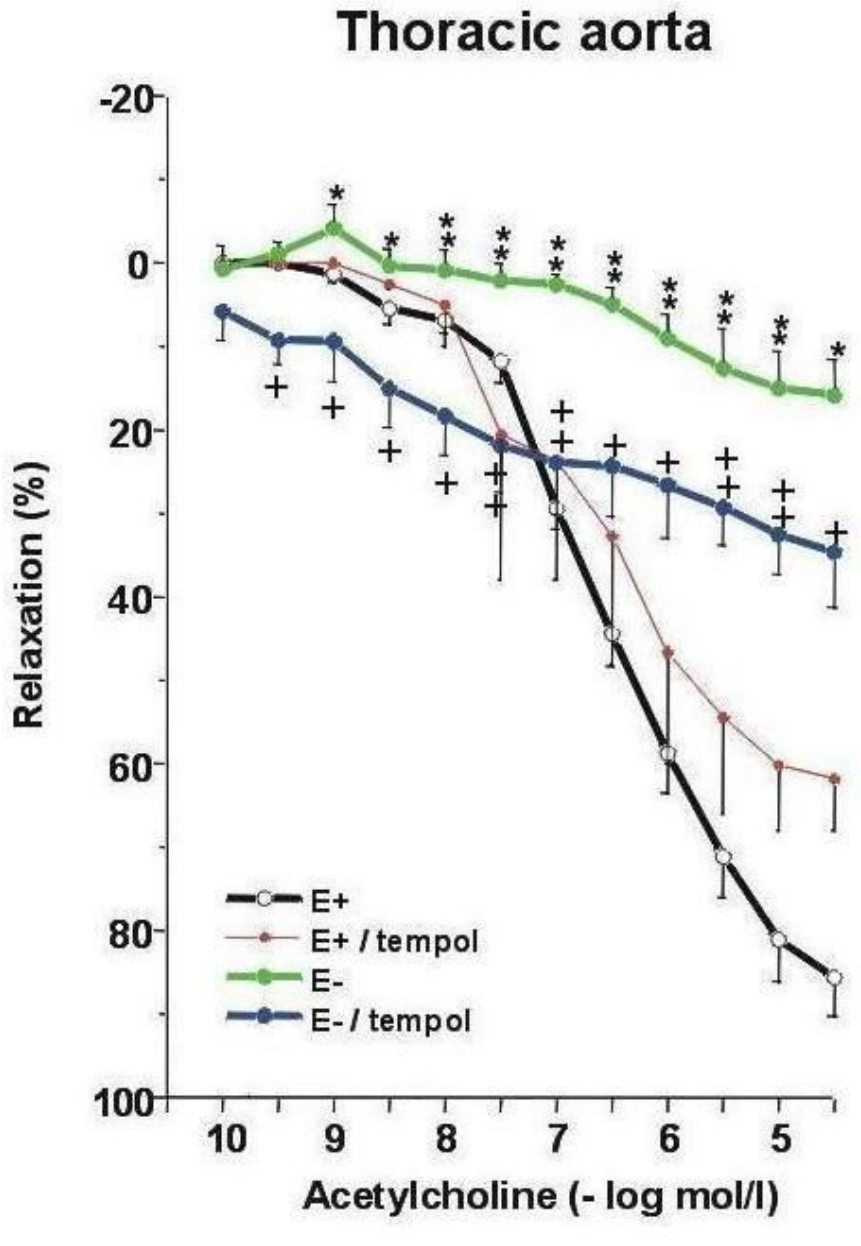
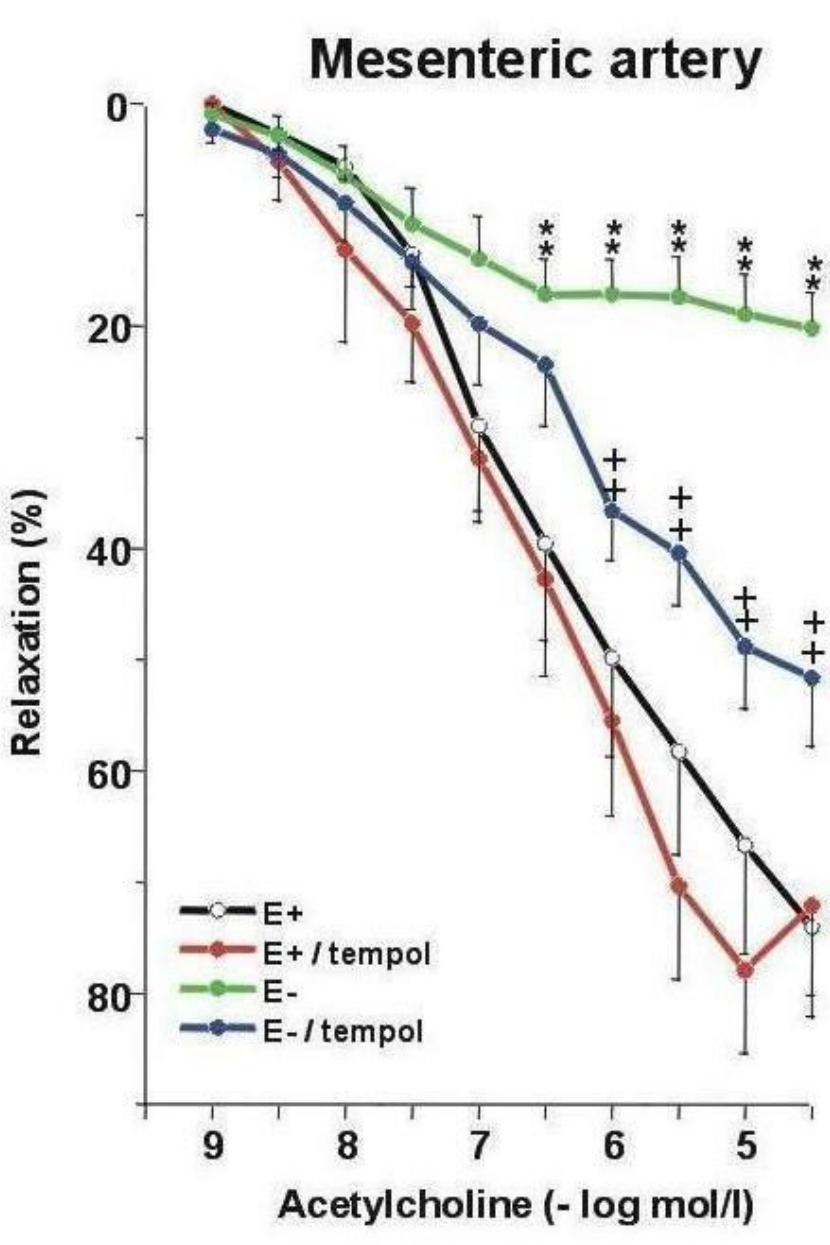
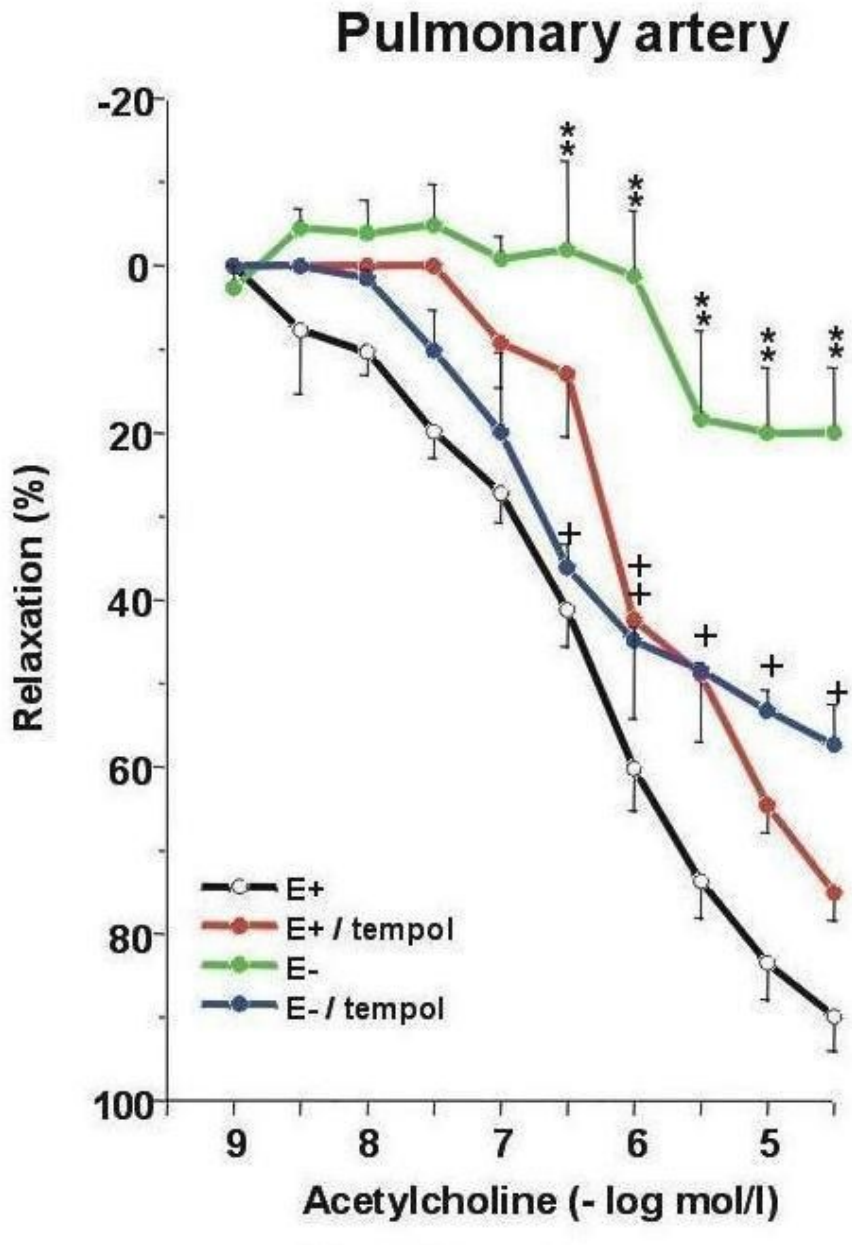
+p<0.05 ++p<0.01 vs. E-



*p<0.05 **p<0.01 vs. E+

+p<0.05 ++p<0.01 vs. E-


Mesenteric artery
Relaxation (%)
0
20
40
60
80
9
8
7
6
5
Acetylcholine (- log mol/l)
E+
E+ / tempol
E-
E- / tempol
**p<0.01 vs. E+
++p<0.01 vs. E-


Pulmonary artery
Relaxation (%)
-20
0
20
40
60
80
100
9
8
7
6
5
Acetylcholine (- log mol/l)
E+
E+ / tempol
E-
E- / tempol
**p<0.01 vs. E+
+p<0.05 ++p<0.01 vs. E-