

The endothelium-dependent relaxation of rabbit aorta: effects of antioxidants and hydroxylated eicosatetraenoic acids

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Acetylcholine (ACh) induced concentration-dependent relaxations of rabbit aortic strips precontracted with noradrenaline. The relaxations were abolished if the endothelium of the strips was disrupted. Three different antioxidants (butylated hydroxytoluene, dithiothreitol and α -tocopherol) reversed the endothelium-dependent vasodilatation in a concentration-dependent manner. However, the antioxidant ascorbic acid did not alter the vasodilatation. The hydroxylated eicosatetraenoic acids 5-HETE, 12-HETE, 15-HETE and 5,12-diHETE had no effect on aortic strips under basal or induced tension. These results suggest, that (non-cyclo-oxygenase) oxidation processes, insensitive to the action of ascorbic acid, represent a crucial step in the endothelium-dependent dilatation of rabbit aorta by ACh. The hydroxylated fatty acids tested are unlikely to mediate this relaxation.

Introduction Muscarinic receptor agonists like acetylcholine (ACh) induce relaxations of several isolated arteries (e.g. the rabbit aorta) by a mechanism dependent on the integrity of vascular endothelium; arteries devoid of endothelial cells contract to ACh (Furchgott & Zawadzki, 1980; Chand & Altura, 1981; De Mey & Vanhoutte, 1981; Singer & Peach, 1983). The endothelium-mediated relaxation can be reversed by the inhibitor of phospholipase A₂, mepacrine, and by a variety of lipoxygenase inhibitors but not by cyclo-oxygenase inhibitors (Furchgott & Zawadzki, 1980; Chand & Altura, 1981; De Mey, Claeyns & Vanhoutte, 1982; Singer & Peach, 1983; Förstermann & Neufang, 1984).

Since several lipoxygenase inhibitors have antioxidant properties (Duniec *et al.*, 1983) we tested the effect of four different antioxidants on the vasodilator effect of ACh. In addition we investigated whether hydroxylated eicosatetraenoic acids (HETEs) could mimic the effect of ACh and thus be potential mediators of the vasodilatation.

Methods Helically cut strips of rabbit aorta (2 × 15 mm, about 30 mg wet weight) were prepared

and set up in 3.5 ml vessel chambers as previously described (Förstermann *et al.*, 1984). In some experiments the endothelium of the strips was removed by careful abrasion of the intimal surface with a razor blade. Contractions were registered with an isotonic lever system (load 1.5 g). The bath medium (Krebs bicarbonate solution, composition (mM): NaCl 120.0, KCl 4.75, NaHCO₃ 25.0, KH₂PO₄ 1.2, MgSO₄ 1.2, CaCl 1.7 and glucose 6.4) was changed every 12 min throughout the experiment. Aortic strips were precontracted with noradrenaline (NA, 10⁻⁷ M) at 1 h intervals. Seven min after NA, acetylcholine (ACh, 10⁻⁸–10⁻⁵ M) was added to induce relaxations. Different concentrations of antioxidants were added to the bath fluid 36 min (3 wash-out periods) before the next contraction-relaxation period. When testing different hydroxylated eicosatetraenoic acids (HETEs, 10⁻⁷–10⁻⁶ M) for their direct effect on the precontracted strips, these fatty acids were added to the bath 7 min after NA (in place of ACh).

Noradrenaline tartrate (Sigma, Munich, FRG) was dissolved and diluted in 0.001 N HCl containing ascorbic acid 1 mg ml⁻¹. Acetylcholine chloride (Sigma, Munich, FRG) was dissolved and diluted in water. The methyl esters of 5- and 15-HETE were obtained from Paesel, Frankfurt, FRG. They were hydrolysed to the respective sodium salts with 0.001 N NaOH for 150 min at 37°C. Subsequently the pH was readjusted to 7.4 with HCl and the solution was diluted with water if appropriate. The effectiveness of the hydrolysis was tested by thin layer chromatography of the resulting compound (plates: Merck Kieselgel 60 F₂₅₄, solvent system: ether: *n*-hexane: acetic acid; 60:40:1, v/v). 12-HETE (generous gift from Dr Nugteren, Unilever, Vlaardingen, Netherlands) and 5,12-diHETE (leukotriene B₄, LTB₄), Paesel, Frankfurt, FRG) were obtained and used as the free acids.

Ascorbic acid and butylated hydroxytoluene (BHT, 2,6-di-tertbutyl-*p*-cresol) were purchased from Roth, Karlsruhe, FRG. DL-Dithiothreitol (DTT) and DL- α -tocopherol were bought from

Sigma, Munich, FRG). BHT was dissolved in ethanol and added to the organ bath in $10\ \mu\text{l}$ immediately after wash-out (every 12 min). The ethanol concentration which resulted in the bath (0.28%) did not influence the ACh-effect when tested alone. α -Tocopherol was freshly emulsified every 12 min by ultrasonication immediately before use. The emulsion with the stated final concentration of tocopherol was that added to the organ bath.

All concentrations of drugs given refer to the active bases or acids respectively.

Results ACh induced concentration-dependent relaxations of the aortic strips precontracted with NA ($10^{-7}\ \text{M}$). About half-maximal relaxations were obtained with $10^{-7}\ \text{M}$ ACh and a maximal effect (relaxation to $55 \pm 6\%$, mean \pm s.e. mean, $n=7$, of the precontraction level) was seen with $10^{-6}\ \text{M}$ ACh. In strips without endothelium ACh had no effect or, at concentrations $>10^{-6}\ \text{M}$, slightly contracted the tissues.

The vasodilator effect of ACh was reversed in a concentration-dependent manner by the antioxidants BHT (10^{-5} – $10^{-4}\ \text{M}$), DTT (10^{-5} – $10^{-4}\ \text{M}$) and by emulsions of α -tocopherol (10^{-4} – $10^{-3}\ \text{M}$). BHT completely abolished the relaxations at $10^{-4}\ \text{M}$. The same concentration of DTT reversed the vasodilatation to $8\% \pm 3\%$ (mean \pm s.e. mean, $n=5$) of the control relaxation. α -Tocopherol reduced the relaxation to $17\% \pm 9\%$ of the control (mean \pm s.e. mean, $n=5$) at the highest concentration used ($10^{-3}\ \text{M}$) (Figure 1).

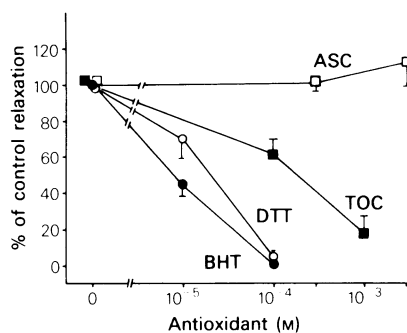


Figure 1 Effect of different antioxidants (ascorbic acid, ASC, $n=8$; α -tocopherol, TOC, $n=5$; dithiothreitol, DTT, $n=5$; butylated hydroxytoluene, BHT, $n=8$) on the relaxation by acetylcholine ($10^{-7}\ \text{M}$) of rabbit aortic strips precontracted with noradrenaline ($10^{-7}\ \text{M}$). Control relaxations of the strips to $10^{-7}\ \text{M}$ acetylcholine in the absence of any drug were taken as 100%. The mean control relaxation corresponds to $76\% \pm 2\%$ (mean \pm s.e. mean, $n=26$) of the initial contraction plateau induced by noradrenaline.

In contrast, ascorbic acid (3×10^{-4} – $3 \times 10^{-3}\ \text{M}$) had no effect on the relaxations induced by ACh (Figure 1). Higher concentrations of the acid could not be used, since they lowered the pH of the bath medium and markedly diminished the precontraction level.

The vasodilator effect of ACh could not be mimicked by any of the different HETEs tested (5-HETE, 12-HETE, 15-HETE, and 5,12-diHETE). None of these hydroxylated fatty acids had a significant effect on precontracted vascular strips or on strips under baseline tension at the concentrations tested (10^{-7} – $10^{-6}\ \text{M}$).

Discussion The present work confirms the observations that an intact endothelium is essential for the relaxation of rabbit aorta by ACh (Furchgott & Zawadzki, 1980; Chand & Altura, 1981; De Mey & Vanhoutte, 1981). It has previously been shown that this vasodilator effect can be inhibited by mepacrine, an inhibitor of phospholipase A_2 (Furchgott & Zawadzki, 1980). These findings suggested that liberation of arachidonic acid (or some other unsaturated fatty acid) might be the first step in the sequence of events leading to endothelium-dependent vasodilatation.

Obviously cyclo-oxygenase products of arachidonic acid do not act as mediators of the ACh-effect, since cyclo-oxygenase inhibitors have no influence on the action of ACh (Furchgott & Zawadzki, 1980; Chand & Altura, 1981; Förstermann & Neufang, 1984). The finding of the present study, that different compounds with antioxidant properties (BHT, DTT, α -tocopherol) inhibit the ACh-induced vasodilatation, indicates, that other oxidation processes (probably of arachidonic acid) represent a crucial biochemical step in endothelium-mediated relaxation.

Ascorbic acid, however, had no effect on the relaxation provoked by ACh, although this compound is also an antioxidant and in high concentrations (as used here) can inhibit lipid peroxidation (Wills, 1969). The reason for this ineffectiveness is not clear. It is possible that ascorbic acid, despite the high concentrations used, did not reach its site of action. The second possibility is that the oxidation processes involved in the effect of ACh are not sensitive to the antioxidant properties of ascorbic acid. Since several lipoxygenase inhibitors have previously been found to reverse the relaxation, it has been suggested that a lipoxygenase product might be the mediator substance released by endothelial cells (Furchgott & Zawadzki, 1980; Chand & Altura, 1981; De Mey *et al.*, 1982; Singer & Peach, 1983; Förstermann & Neufang, 1984). However, products of the recently discovered 'epoxygenase' pathway of arachidonic

acid (Capdevila *et al.*, 1981; Morrison & Pascoe, 1981; Chacos *et al.*, 1982) may also be involved. The dual inhibitor of cyclo-oxygenase and lipoxygenase 5,8,11,14-eicosatetraenoic acid has also been shown to inhibit this pathway (Morrison & Pascoe, 1981).

It is concluded from the present data that an oxidized (but not cyclo-oxygenase) derivative of arachidonic acid, produced by endothelial cells, is

likely to mediate the ACh-induced relaxation of rabbit aorta. 5-, 12-, 15-HETE and 5,12-diHETE are obviously not involved in this vasodilator effect.

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