Mechanisms of action of lipoxygenase and cytochrome P-450-mono-oxygenase inhibitors in blocking endothelium-dependent vasodilatation

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1   Acetylcholine, ionophore A23187 and melittin induced endothelium-dependent relaxations of preconstricted strips of rabbit aorta. These relaxations are likely to be mediated by endothelium-derived relaxing factor (EDRF).

2   Relaxations in response to acetylcholine (1 μM) were inhibited by the following lipoxygenase inhibitors, with the approximate IC_{50} values indicated in parentheses: gossypol (1.5 μM), nordihydroguaiaretic acid (NDGA, 5 μM), AA 861 (20 μM), phenidone (30 μM), quercetin (40 μM), BW 755C (300 μM), and piriprost (500 μM); with cirsiliol 50% inhibition was not achieved. Acetylcholine-induced relaxations were also blocked by the cytochrome P-450-mono-oxygenase inhibitors proadifen (SKF 525A, 4 μM), metyrapone (300 μM), and cimetidine (300 μM); 7,8 benzoflavone had no effect up to 100 μM.

3   The more potent inhibitors were also tested against relaxations induced by A23187 (0.1 μM) and melittin (1 μM) and produced partial inhibition of these relaxations.

4   The mechanism of action of the more potent inhibitors was investigated in a bioassay system. EDRF was produced in columns filled with cultured human endothelial cells. The factor was bioassayed with endothelium denuded segments of rabbit femoral artery. When added to effluent of the column, NDGA, AA861, proadifen and metyrapone inhibited the EDRF-induced vasodilatation, whereas gossypol had no effect. Gossypol, however, blocked EDRF production when infused through the column.

5   The more potent inhibitors were also tested to determine their effect on purified soluble guanylate cyclase. While gossypol, NDGA and proadifen had no appreciable effects, basal and nitroprusside (50 μM)-stimulated guanylate cyclase activity was inhibited by AA861 and metyrapone.

6   These data suggest that many of the above compounds inhibit EDRF by mechanisms other than lipoxygenase- or cytochrome P-450-mono-oxygenase inhibition.

Introduction

Vascular relaxation in response to a number of 'indirect' vasodilators such as acetylcholine (ACh) is mediated by a vasodilator substance produced by vascular endothelium and referred to as endothelium-derived relaxing factor (EDRF) (Furchgott, 1984). EDRF is a powerful, but short-lived humoral substance (Griffith *et al.*, 1984; Förstermann *et al.*, 1984; Cocks *et al.*, 1985). Vasodilatation induced by EDRF

is correlated with increased levels of guanosine 3′:5′-cyclic monophosphate (cyclic GMP) in vascular smooth muscle (for review see Ignarro & Kadowitz, 1985; Murad, 1986), and this is likely to be due to the activation of soluble guanylate cyclase (Förstermann *et al.*, 1986a, Mülsch *et al.*, 1987). A recent study indicates that EDRF may be identical with nitric oxide (Palmer *et al.*, 1987). However, the mechanisms involved in the production of this factor are still largely unclear.

Among the substances found to inhibit endothe-

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lium-dependent relaxations were lipoxygenase inhibitors (Furchgott & Zawadski, 1980; Singer & Peach, 1983; Förstermann & Neufang, 1984) and inhibitors of cytochrome P-450 mono-oxygenases (Singer *et al.*, 1984; Pinto et al., 1986). Consequently, it had been suggested by these authors that EDRF could be a lipoxygenase- or cytochrome P-450 mono-oxygenase product of arachidonic acid. However, more recently, some lipoxygenase inhibitors have been found to destroy EDRF after its release from the endothelial cell (Griffith *et al.*, 1984) or to inactivate EDRF through the formation of superoxide anions (Moncada *et al.*, 1986). During recent years a number of new compounds have been developed or discovered that inhibit lipoxygenases, probably in a more specific manner (for review see Bach, 1984; Yamamoto *et al.*, 1984). Therefore, we sought to investigate the effects of these novel lipoxygenase inhibitors and of several cytochrome P-450 mono-oxygenase inhibitors on endothelium-dependent relaxations induced by different vasodilators.

The more potent inhibitors were then tested in a bioassay system (Förstermann *et al.*, 1984, 1986a) for their interference with EDRF after release.

Further, as direct activation of soluble guanylate cyclase is the likely mechanism of action of EDRF (Förstermann *et al.*, 1986b, Mülsch *et al.*, 1987), the effects of these lipoxygenase and cytochrome P-450 inhibitors were also tested on the activity of the purified enzyme.

Methods

Organ bath experiments with rabbit aortic strips

Rabbits of either sex (2.5–3.5 kg body weight) were killed by a blow on the head and exsanguinated from the carotid arteries. The thoracic aorta and the femoral arteries (for the bioassay experiments, see below) were carefully removed from the animal and placed into modified Krebs solution (composition see below) at room temperature. Transverse strips (cut open rings) of about 2 mm width were prepared from the aorta. They were suspended in 5 ml organ baths containing oxygenated (95% O_2, 5% CO_2) modified Krebs solution, 37°C, pH 7.4, composed as follows (mM): Na^+ 145.0, K^+ 5.95, Ca^{2+} 1.7, Mg^{2+} 1.2, Cl^- 128.15, HCO_3^- 25.0, $H_2PO_4^-$ 1.2, SO_4^{2-} 1.2, glucose 10.6 and disodium EDTA 0.025. Tension was recorded using an isometric force transducer as described previously (Förstermann *et al.*, 1986a); resting force was 2 g. The bath fluid was changed every 12 min. The preparations were exposed at hourly intervals to noradrenaline (0.1 μM) to induce half maximal contractions. Contractions to noradrenaline generally reached a stable plateau tension after about 6 min. Then acetylcholine (1 μM) was added to each strip as a functional test of endothelial integrity. Strips showing less than 40% relaxation were considered endothelium-damaged and discarded from the study (the average response to 1 μM acetylcholine was about 60% relaxation, cf. legend to Figure 1a and b). During the actual experiment maximum effective concentrations (as previously determined) of ACh (1 μM), Ca^{2+}-ionophore A23187 (0.1 μM) or melittin (1 μM) were added to preconstricted strips in order to induce relaxation. When two control relaxations with one of the vasodilators had given reproducible results, the tissue was exposed to increasing concentrations of inhibitors. Addition of each concentration of inhibitor was started at three wash-out periods (36 min) before the next contraction-relaxation period.

Culture of human endothelial cells

Umbilical cords were obtained at birth and transported to the laboratory in sterile Cord buffer (composition, mM: Na^+ 145.2, Mg^{2+} 4.0, Cl^- 148.0, HPO_4^{2-} 2.0, $H_2PO_4^-$ 1.2, glucose 11.0; pH 7.2). Time until preparation of endothelial cells never exceeded 3 h. Collagenase (Gibco, Karlsruhe, FRG; 0.01% in Cord buffer) was injected and the umbilical cord was incubated for 30 min at 37°C. The endothelial cells were flushed out of the vein with sterile Cord buffer and collected by centrifugation (300 g, 10 min). The cells were grown in gelatine coated culture flasks; 0.1% gelatine in H_2O was incubated for 1 h at 37°C in culture flasks (Corning, New York, U.S.A.) and then washed away. The culture medium was a 1:1 (v/v) mixture of RPMI medium (Gibco) and M 199 medium with Earle-salts containing 25 mM HEPES buffer (Gibco). Foetal calf serum (10%, v/v, Boehringer, Mannheim FRG) 1.0 g l^{-1} ampicillin and 0.5 g l^{-1} oxacillin (Lucipen, Hoechst, Frankfurt, FRG) were added. Cells were incubated at 37°C under 5% CO_2. Confluent cells were harvested with trypsin and subcultured. Cells of passage 1–3 were seeded onto microcarrier beads (Biosilon, Nunc, Roskilde, Denmark; precoated with gelatine). They were cultured in a microcarrier stirring system (Techne, Cambridge, U.K.) until 70–80% confluence was reached on the beads.

Bioassay experiments

Bioassay experiments were performed as described previously (Förstermann *et al.*, 1986a). Briefly, 300 μl microcarrier bead (covered with approximately 6 × 10^6 endothelial cells) were filled into a column and superfused with Krebs solution containing indomethacin (10 μM). The solution was gassed with 75% N_2, 20% O_2, 5% CO_2; flow rate was 1 ml min^{-1}. EDRF production was estimated in terms of dialatation of an endothelium-denuded segment of rabbit femoral

artery. The column effluent entered the vessel after a transit time of 5 s. Thimerosal (5 μM) was infused through the column (t.c., Figure 3). This compound induces a longlasting stimulation of EDRF production (Förstermann *et al.*, 1986a).

Inhibitors could be infused directly into the lumen (i.l.) of the detector artery through the cannula connecting the column and the detector. Thus they exhibited their effects on the factor released from the endothelial cells rather than on the cells themselves (cf. Figure 3a). Interaction time with EDRF was approximately 4 s. Alternatively, inhibitors could be added to the medium infused through the endothelial cell column (t.c., Figure 3a). Approximate IC_{80} concentrations of inhibitors (as judged from the experiments on aortic strips, Figure 1 and Table 1) were tested in the bioassay system for their interaction with EDRF.

Determination of activity or soluble guanylate cyclase

Soluble heme-containing guanylate cyclase was purified from bovine lung to apparent homogeneity by using the method described by Gerzer *et al.* (1981) with slight modifications. The activity of purified guanylate cyclase was determined in test tubes by measuring the formation of [^{32}P]-cyclic GMP from [α-^{32}P]-GTP. The enzyme reaction was started by the addition of 10 μl [α-^{32}P]-GTP (0.1 mM final concentration, 0.2 μCi) to a 90 μl incubation mixture containing triethylamine-HCl buffer (30 mM, pH 7.4), $MgCl_2$ (3 mM), glutathione (3 mM), EGTA (0.1 mM), bovine γ-globulin (0.1 mg ml^{-1}) and purified soluble guanylate cyclase (0.02 μM). All concentrations given are final concentrations. Incubation time was 10 min at 37°C. The enzyme reaction was stopped by addition of 50 mM zinc acetate and 55 mM sodium carbonate. Isolation of [^{32}P]-cyclic GMP and calculation of specific guanylate cyclase activity was performed as described previously (Mülsch *et al.*, 1987). Various concentrations of different lipoxygenase- and cytochrome P-450 mono-oxygenase inhibitors were tested on the basal enzyme activity and on the enzyme stimulated with sodium nitroprusside (50 μM).

Drugs and solutions

Noradrenaline (Sigma, Munich, FRG) was dissolved and diluted in 1 mM HCl containing 1 mg ml^{-1} ascorbic acid and injected into the organ baths in 1% (v/v). Acetylcholine and thimerosal (both Sigma) were dissolved in water, melittin (Sigma) in phosphate buffered saline containing 1 g l^{-1} gelatine and A23187 (Sigma) in dimethyl-sulphoxide (DMSO). A23187 was injected or infused in 0.2% (v/v). The resulting DMSO concentration of 0.2% had no effect on the contraction-relaxation processes.

The inhibitors used were: gossypol (1–10 μM), nor-

dihydroguaiaretic acid (NDGA, 3–30 μM), 2,3,5-trimethyl-6-(12-hydroxy-5,10-dodecadiynyl)-1,4-benzoquinone (AA861, 3–100 μM), phenidone (3–30 μM), quercetin (10–100 μM), cirsiliol (3–100 μM), 3-amino-1-(m-(trifluoromethyl)phenyl)-2-pyrazoline (BW 755C, 10 μM–1 mM), piriprost (0.1–1 mM), proadifen (SKF525A, 3–100 μM), metyrapone (10 μM–1 mM), cimetidine (10 μM–1 mM), 7,8-benzoflavone (3–100 μM), ranitidine (10 μM–1 mM), and indomethacin (1–30 μM). The appropriate concentrations were determined in preliminary experiments. Gossypol (Sigma), AA861 (Takeda, Osaka, Japan), cirsiliol (Kyoto Pharmaceutical University, Kyoto, Japan) and 7,8-benzoflavone were dissolved and diluted in DMSO. Quercetin (Sigma) was dissolved and diluted in ethanol. Proadifen (Smith Kline & French, Welwyn, U.K), BW 755C (Wellcome, Beckenham, U.K.), and phenidone (Sigma) were dissolved in distilled water. Indomethacin (Sharp & Dohme, Munich, FRG) was dissolved in phosphate buffer. These inhibitors were diluted in Krebs solution. The concentration of organic solvents never exceeded 0.2% (v/v) in the bathing fluids and had no effect on the contraction-relaxation process. NDGA and metyrapone (both Sigma), cimetidine (Smith, Kline & Dauelsberg, Göttingen, FRG) and ranitidine (Glaxo, London, U.K.) were dissolved directly in prewarmed (37°C) Krebs solution immediately before use. Solutions of NDGA were used for no longer than 1 h as the compound is subject to spontaneous oxidation. Piriprost (Upjohn, Kalamazoo, MI, U.S.A.) was kept in the dark at −70°C under argon and was dissolved freshly in prewarmed (37°C) Krebs solution (pH 7.4) immediately before use. The vehicles of the inhibitors were always present during control relaxations.

Calculations and statistics

Relaxations of aortic strips (−a) starting from the noradrenaline-induced contraction plateau (b) were always calculated as −a/b (%). If a relaxation was converted into a contraction, which was occasionally observed after the highest concentrations of gossypol, NDGA or proadifen, this was taken as 0% relaxation. Values are presented as means ± s.e. Statistical significance was judged by applying Student's *t* test for paired values to groups of data. A *P* value less than 0.05 was considered significant.

Results

Relaxations of rabbit aortic strips in response to acetylcholine, ionophor A23187 or melittin were strictly dependent on an intact endothelium as previously established (Furchgott & Zawadzki, 1980; Furchgott, 1984; Förstermann & Neufang, 1985).

Effect of lipoxygenase inhibitors on acetylcholine-induced relaxation of rabbit aorta

Endothelium-dependent relaxations of rabbit aortic strips in response to a maximum effective concentration of acetylcholine (1 μM) were inhibited in a concentration-dependent fashion by several lipoxygenase inhibitors. The most potent compound was gossypol, a polyphenolic antioxidant from cotton plant (Hamasaki & Tai, 1985). It was about 3 times more potent than the 'classical' lipoxygenase inhibitor NDGA (Figure 1a). So far, NDGA has been considered the most potent inhibitor of EDRF-mediated relaxations (cf. Furchgott 1984; Förstermann & Neufang, 1984). Gossypol (up to 30 μM) did not alter the noradrenaline-induced precontraction level. Also AA861, a benzoquinone derivative described as a selective 5-lipoxygenase inhibitor (Yoshimoto *et al.*, 1982), proved to be a relatively potent inhibitor of acetylcholine relaxations (Figure 1a).

Other lipoxygenase inhibitors, namely phenidone (Blackwell & Flower, 1978), quercetin (Hope *et al.*, 1983), cirsiliol (Yoshimoto *et al.*, 1983), BW 755C (Higgs *et al.*, 1979), and piriprost (Bach *et al.*, 1982, Bach, 1984) were less potent. Their effects on acetylcholine-induced relaxations and on the noradrenaline-induced contraction plateau are summarized in Table 1.

Effect of cytochrome P-450-mono-oxygenase inhibitors on acetylcholine-induced relaxations of rabbit aorta

The inhibitor of cytochrome P-450-dependent mixed functional oxidase, proadifen, (Mannering, 1971), proved to be a relatively potent inhibitor of endothelium-dependent relaxations induced by acetylcholine (1 μM) (Figure 1b). Half maximum inhibition was achieved with about 4 μM of the compound. In contrast, two other cytochrome P-450-mono-oxygenase inhibitors, namely metyrapone and cimetidine (Hildebrand, 1972; Pelkonen & Puurunen, 1980) were much less effective against the acetylcholine relaxation. Half maximum inhibition was seen with approximately 300 μM of either compound (Figure 1b). Ranitidine (as control for cimetidine) gave 50% inhibition at 1 mM. 7,8-Benzoflavone, another inhibitor of cytochrome P-450 mixed functional oxidase (Diamond & Gelboin, 1969) had no effect on the acetylcholine relaxation up to 100 μM. The effects of these inhibitors are listed in Table 1.

Effect of inhibitors on relaxations induced by the Ca^{2+}-ionophore A23187 and melittin

Of the above mentioned inhibitors, the more potent ones (gossypol, NDGA, AA861 and proadifen) were also tested against maximum relaxations induced by the Ca^{2+}-ionophore A23187 (0.1 μM) and by melittin

(1 μM). At concentrations that produced virtually complete inhibition of the acetylcholine-induced relaxation all compounds tested also significantly attenuated the relaxations in response to A23187 or melittin (Figure 2a and b). However, generally no complete inhibition could be attained. Proadifen, especially, was much less effective against these relaxations.

Bioassay experiments

The mechanisms of action of the more potent inhibitors from both groups were studied in our
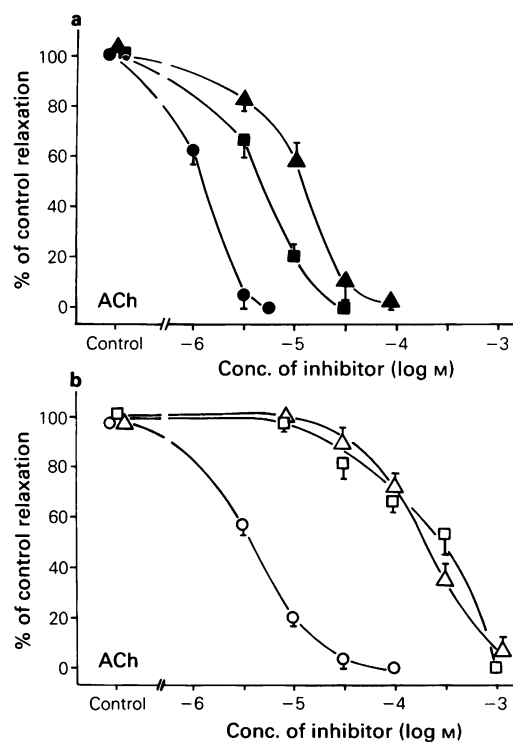

Figure 1 Effect of (a) three lipoxygenase inhibitors and (b) three cytochrome P-450 mono-oxygenase inhibitors on endothelium-dependent relaxations of rabbit aortic strips induced by a maximum effective concentration of acetylcholine (ACh, 1 μM). The vascular preparations were preconstricted with noradrenaline (0.1 μM). Control relaxations with ACh (100%) were induced in normal Krebs solution in the presence of the vehicle of the inhibitor. Then increasing concentrations of inhibitor were added. The mean relaxation induced by ACh under control conditions was for (a) 66 ± 3% (*n* = 19) and for (b) 59 ± 3% (*n* = 20) of the noradrenaline contraction plateau. The inhibitors were (a) gossypol (●, *n* = 6), nordihydroguaiaretic acid (■, *n* = 6), compound AA861 (▲, *n* = 7); (b) proadifen (○, *n* = 8), cimetidine (□, *n* = 6) and metyrapone (△, *n* = 6). Values plotted are means with vertical lines representing the s.e.mean.

Table 1 Inhibition by different compounds of endothelium-dependent relaxation of rabbit aortic strips

Inhibitor	Classification	Approximate IC_{50} value	Full inhibition	Effect on noradrenaline induced tension
Gossypol	Lipoxygenase inhibitors and antioxidants	1.5 μM	5 μM[1]	No effect up to 30 μM[2]
NDGA		5 μM	30 μM	10% depression at 10 μM, 30% depression at 30 μM
AA 861		20 μM	About 95% inhibition at 100 μM[3]	No effect up to 30 μM, 15% depression at 100 μM
Quercetin		40 μM	About 90% inhibition at 100 μM[3]	20% depression at 30 μM, 30% depression at 100 μM
Cirsiliol		—	—[7]	20% depression at 30 μM, 30% depression at 100 μM[3]
Phenidone	Inhibitors of cyclo-oxygenase and lipoxygenase	30 μM	Not determined[4,8]	55% depression at 30 μM
BW 775C		300 μM	About 80% inhibition at 1 mM[5]	40% depression at 300 μM, 60% depression at 1 mM
Piriprost	Lipoxygenase inhibitor	500 μM	About 70% inhibition at 1 mM[5]	No effect up to 1 mM
Proadifen (SKF 525A)	Inhibitors of cytochrome P-450 mono-oxygenase	4 μM	100 μM	No effect up to 10 μM 40% depression at 100 μM
Metyrapone		300 μM	90% inhibition at 1 mM[5]	No effect up to 1 mM
Cimetidine		300 μM	About 90% inhibition at 1 mM[5]	No effect up to 300 μM 35% depression at 1 mM
7,8-Benzoflavone		—	—[9]	No effect up to 30 μM 25% depression at 100 μM[3]
Ranitidine	For comparison with cimetidine	1 mM	Not determined[5]	25% depression at 300 μM, 50% depression at 1 mM
Indomethacin	Inhibitor of cyclo-oxygenase	—	—[6,10]	No effect up to 30 μM

All strips were preconstricted with noradrenaline (0.1 μM); relaxation was induced with a maximum effective concentration of acetylcholine (1 μM). Concentration-effect curves for each inhibitor were generated in 5–9 different strips from rabbit aorta.

[1]Inhibition of relaxation was irreversible at concentrations > 10 μM.
[2]See Alheid *et al.* (1987).
[3]Higher concentrations were not tested due to limited solubility in water.
[4]Not determined as the depression of noradrenaline-induced tension was > 50% at IC_{50} of inhibitor.
[5]Concentrations above 1 mM were not tested.
[6]Prostacyclin production of rabbit aorta was completely blocked by 10 μM indomethacin (cf. Förstermann & Neufang, 1984; Förstermann *et al.*, 1986a).
[7]Less than 50% inhibition at 100 μM.
[8]No complete concentration-effect curve.
[9]No effect on relaxation up to 100 μM.
[10]No effect on relaxation up to 30 μM.

bioassay system (Figure 3a). When columns filled with human endothelial cells on microcarrier beads were superfused with Krebs solution containing thimerosal (5 μM, t.c.), the effluent of the column produced a marked dilatation of the preconstricted endothelium-denuded detector artery (Figure 3b). This dilatation was reversed by NDGA (10 μM), AA861 (30 μM), proadifen (10 μM) and metyrapone (0.5 mM) when infused into the cannula connecting the EDRF-generating column to the EDRF detector artery (i.l.

infusion, cf. Figure 3 and Table 2). Gossypol (3 μM) had little, if any, effect when infused i.l. However, it inhibited the relaxation when infused through the column (Table 2, confirming previous observations in our laboratories, Förstermann *et al.*, 1986a).

Guanylate cyclase experiments

The bioassay system used cannot differentiate between chemical destruction of EDRF by an inhibitor or inhibition of EDRF-effects on vascular smooth muscle cells. Therefore, the same inhibitors were tested for

their effect on soluble guanylate cyclase. This is likely to be the key-enzyme mediating smooth muscle effects of EDRF (Förstermann *et al.*, 1986b; Mülsch *et al.*, 1987). At concentrations that produced inhibition of EDRF-mediated relaxations (cf. Figure 1) gossypol, NDGA and proadifen had very little or no effect on the activity of guanylate cyclase under basal conditions or after activation with sodium nitroprusside (50 μM) (Figure 4 and Table 2). On the other hand, AA861 and metyrapone inhibited the enzyme both in the basal and activated states (Figure 4 and Table 2).

Discussion

In the present study we found that several lipoxygenase and cytochrome P-450 mono-oxygenase inhibitors blocked endothelium-dependent relaxations. It was demonstrated that the polyphenolic antioxidant and lipoxygenase inhibitor, gossypol, was the most potent EDRF inhibitor known so far. NDGA was about three times less potent. Of the cytochrome P-450-mono-oxygenase inhibitors, proadifen (SKF-525A) was found to be most potent, which is in accordance with previous findings (Singer *et al.*, 1984). However, part of the proadifen effect seems to be specific for acetylcholine relaxations as it was much less effective against A23187 and melittin relaxations.

The approximate IC_{50} values determined in the present study were about a factor three higher than those previously demonstrated for some of the lipoxygenase inhibitors (Förstermann & Neufang, 1984). This is due to the fact that in the present study we tested against the effect of a maximum effective concentration of acetylcholine (1 μM), whereas in the previous study an approximate EC_{50} of acetylcholine (0.1 μM) was used.

Experiments on vascular strips are useful to screen the inhibitory potency of a large number of compounds, but they do not allow conclusions about the mechanism of action of these inhibitors. Possible actions are: (i) inhibition of the production and/or release of EDRF, (ii) interaction with and inactivation of the factor after release (like haemoglobin, Martin *et al.*, 1985), or (iii) inhibition of the response of vascular smooth muscle to EDRF (like the inhibitor of soluble guanylate cyclase methylene blue, Ignarro & Kadowitz, 1985).

Destruction of EDRF by an inhibitor after release can be demonstrated in bioassay experiments in which the EDRF-producing component and the EDRF-detector component are separated, and in which the inhibitor can be added between the two components. NDGA and phenidone have been found previously to destroy the EDRF produced by rabbit aorta segments (Griffith *et al.*, 1984). Interaction with the EDRF produced by cultured bovine endothelial cells has
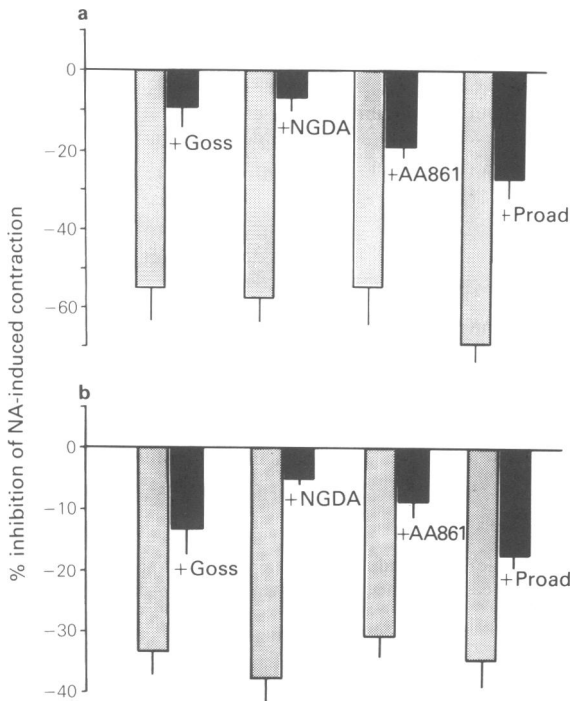

Figure 2   Effect of inhibitors on endothelium-dependent relaxations of rabbit aortic strips induced by (a) the Ca^{2+}-ionophore A23187 (0.1 μM) and (b) mellitin (1 μM). The response plotted is percentage change in the noradrenaline (NA, 0.1 μM)-induced plateau tension after addition of A23187 or melittin. Responses were obtained in eight separate groups of aortic strips. Control relaxations are depicted by the stippled columns (the vehicle of the inhibitor was present during this control relaxation). Solid columns represent responses after addition of the respective inhibitor: gossypol (Goss, 5 μM, $n = 6$), nordihydroguaiaretic acid (NDGA, 30 μM, $n = 5$), compound AA861 (30 μM, $n = 5$), proadifen (Proad, 100 μM, $n = 6$). Values plotted are means with vertical lines representing the s.e.mean. Relaxations to A23187 or mellitin were inhibited significantly ($P < 0.05$) by all four inhibitors.
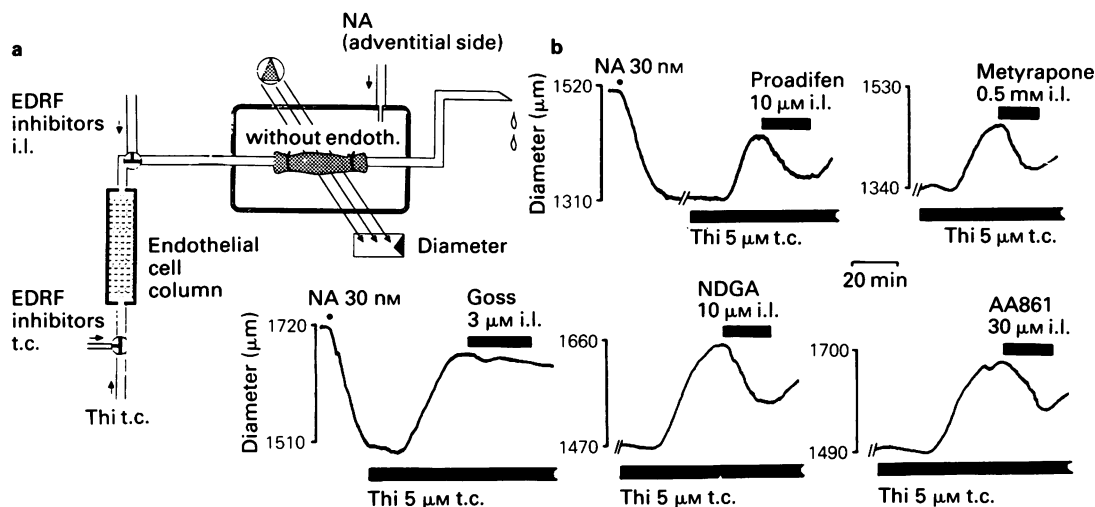
Figure 3 (a) Bioassay set-up consisting of a column containing approximately 6×10^6 human endothelial cells on microcarrier beads (EDRF-generating component) and an endothelium-denuded rabbit femoral artery (EDRF-detector component). The detector segment was constricted with noradrenaline (NA, 30–300 nM) added from the adventitial side. Thimerosal (Thi, 5 μM) was used to stimulate EDRF production in the column. EDRF inhibitors were infused directly into the lumen (i.l.) of the detector artery through the proximal end of the intervening cannula. Interaction time with EDRF was 4 s. Alternatively, inhibitors could be infused through the column (t.c.). (b) Effect of different lipoxygenase and cytochome P-450-mono-oxygenase inhibitors (infused i.l.) on the relaxant response of rabbit femoral artery (without endothelium) to EDRF generated by the endothelial cell column. Gossypol (Goss, 3 μM) had little, if any, effect when infused i.l., whereas the other inhibitors tested (NDGA, AA861, proadifen and metyrapone) reversed the relaxation. When the same concentrations of inhibitors were infused into the endothelium-denuded femoral artery without an endothelial cell column connected they had either no effect or produced small relaxations. Results presented are representative of at least three independent experiments.

Table 2 Mechanism of action of several compounds in inhibiting EDRF-mediated vasodilatation

Compound	Classification	Inhibition of EDRF production	Interaction with EDRF after release (bioassay)[1]	Inhibition of soluble guanylate cyclase
Gossypol	Lipoxygenase inhibitors and antioxidants	+	3 μM	−	−
NDGA		?[2]	10 μM	+	−
AA 861		?[2]	30 μM	+	+
Proadifen (SKF 525A)	Cytochrome P-450-mono-oxygenase inhibitors	?[2]	10 μM	+	−
Metyrapone		?[2]	0.5 mM	+	+

+ Mechanism established, − not a mechanism of action
[1] Concentrations that caused about 80% inhibition in the strip experiments (see Figure 1) were tested in our bioassay system.
[2] Mechanism could not be proven as compound interacts with EDRF after release and/or inhibits soluble guanylate cyclase.
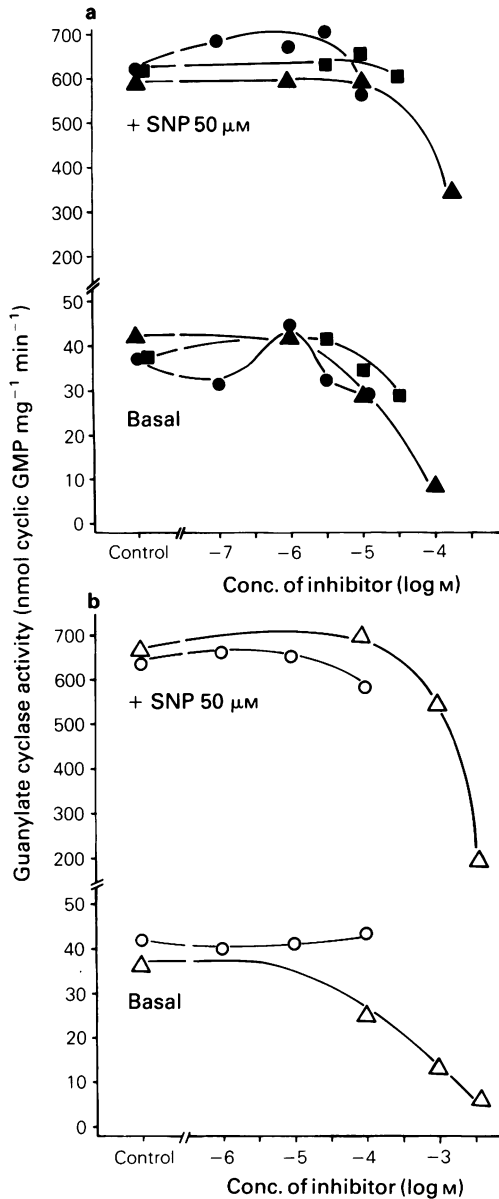
Figure 4 Effect of inhibitors of (a) lipoxygenase and (b) cytochrome P-450 mono-oxygenase on the activity of purified soluble guanylate cyclase under basal conditions (lower panels) or after stimulation of the enzyme with sodium nitroprusside (SNP, 50 μM; upper panels). The inhibitors were (a) gossypol (●), nordihydroguaiaretic acid (■), and compound AA861 (▲) and (b) proadifen (○) and metyrapone (△); concentrations were the same that produced partial to complete inhibition of endothelium-mediated relaxations (see Figure 1). Values shown are means of duplicate or triplicate determinations which were always within 10% of each other.

recently been shown for NDGA and metyrapone (Lückhoff *et al.*, 1987). The present study confirms these findings and demonstrates that such interactions can explain the inhibitory effect of NDGA, compound AA861, proadifen (SKF 525A) and metyrapone on EDRF-mediated relaxations.

However, the bioassay does not determine whether the compounds also inhibit EDRF production. The more marked inhibitory effect that was generally observed when the compounds were infused through the EDRF-producing endothelial cell column could be due to inhibition of EDRF production or to the longer interaction time with the factor.

Further, the bioassay system cannot readily differentiate between destruction of EDRF by an inhibitor or blockade of its action at the smooth muscle of the detector artery. For this reason the more potent inhibitors were tested to determine their effects on purified soluble guanylate cyclase. EDRF-mediated relaxations are associated with increases in cyclic GMP in vascular smooth muscle (for review see Ignarro & Kadowitz, 1985; Murad, 1986). Similarly, nitrovasodilators (like sodium nitroprusside and organic nitrates) are known to produce smooth muscle relaxation by increasing cellular levels of cyclic GMP. These compounds or their active intermediates are stimulators of soluble guanylate cyclase (Ignarro & Kadowitz, 1985; Murad, 1986). Recent evidence from our laboratories indicates that EDRF is likely to have a similar mechanism of action: the EDRF produced by native and cultured endothelial cells was found to be a direct stimulator of purified soluble guanylate cyclase (Förstermann *et al.*, 1986b; Mülsch *et al.*, 1987). This is in accordance with the recent proposal by Palmer *et al.* (1987) that EDRF and nitric oxide (the active principle of nitrovasodilators) are identical.

Thus, compounds that inhibit soluble guanylate cyclase are likely to be inhibitors of the effect of EDRF on vascular smooth muscle (as has been shown for methylene blue; Ignarro & Kadowitz, 1985). Compound AA861 and metyrapone inhibited basal and nitroprusside (50 μM)-activated guanylate cyclase activity in concentrations that also blocked EDRF-mediated relaxations. Consequently, inhibition of the action of EDRF could be the basis of their inhibitory effect.

Recently, Clark & Linden (1986) have demonstrated an inhibitory effect of NDGA on their preparation of guanylate cyclase from dog aorta (IC$_{50}$: 36–126 μM). As 30 μM NDGA was the maximum effective concentration in inhibiting endothelium-mediated relaxation (Figure 1a), we did not test higher concentrations on our guanylate cyclase preparation (Figure 4a). Up to 30 μM, NDGA produced no appreciable inhibition of enzyme activity (Figure 4a). The reason for this discrepancy may be differences in the enzyme preparation used; Clark & Linden (1986) used only

partially purified enzyme and may have been unable to exclude interference from protein contaminants.

Gossypol showed little or no interaction with EDRF after release and was also ineffective in the guanylate cyclase assay. Therefore, it seems likely that gossypol is a potent inhibitor of the production and/or release of EDRF.

The above data demonstrate that the effectiveness of lipoxygenase or cytochrome P-450-mono-oxygenase inhibitors in blocking EDRF-mediated relaxation cannot be taken as evidence for the involvement of the respective enzymes in the production of the factor.

In the case of lipoxygenase inhibitors, this is also suggested by the lack of correlation between the reported inhibitory potencies of the different compounds against lipoxygenases and their potencies as EDRF inhibitors (Table 1). For example, phenidone was about 5 times less potent than BW 755C against platelet 12-lipoxygenase (cf. Blackwell & Flower, 1978; Higgs et $al.$, 1979), but it is about 10 times more potent as an EDRF inhibitor (Table 1). Gossypol and cirsiliol were about equipotent in inhibiting 5- and 12-lipoxygenases from leukaemia cells or leukocytes (c.f. Hamasaki & Tai, 1985; Yoshimoto et $al.$, 1983), but gossypol was the most potent EDRF inhibitor ($IC_{50} \sim 1.5 \mu M$), whereas circiliol produced less than 50% inhibition at 100 μM (Table 1).

Similar discrepancies exist for the inhibitors of cytochrome P-450-dependent oxygenation. Proadifen and 7,8-benzoflavone were both found to inhibit endothelial aryl-hydrocarbon hydroxylase activity (Abraham et $al.$, 1985), but proadifen is a relatively potent EDRF inhibitor ($IC_{50} \sim 4 \mu M$), whereas 7,8-benzoflavone produced no inhibition at all up to 100 μM (Table 1). In comparison with cimetidine, ranitidine is far less potent at interacting with the cytochrome P-450 system (Breen et $al.$, 1982; Bast et $al.$, 1984). Nevertheless, ranitidine also inhibited endothelium-mediated relaxations in response to acetylcholine (Table 1).

In conclusion, the present data demonstate that the inhibitory potency of many lipoxygenase- and cytochrome P-450-mono-oxygenase inhibitors against EDRF mediated relaxations is due to properties other than inhibition of these enzymes. Therefore, these compounds are of limited value for the characterization of the mechanism of EDRF production.

References

ABRAHAM, N.G., PINTO, A., MULLANE, K.M., LEVERE, R.D. & SPOKAS, E. (1985). Presence of cytochrome P-450 dependent monooxygenase in intimal cells of the hog aorta. $Hypertension$, 7, 899–904.

ALHEID, U., DUDEL, C. & FÖRSTERMANN, U. (1987). Selective inhibition by gossypol of endothelium-dependent relaxations augments relaxations by glyceryl trinitrate. $Br.$ $J.$ $Pharmacol.$, 92, 237–240.

BACH, M.K. (1984). Inhibitors of leukotriene synthesis and action. In $Leukotrienes$, ed. Chakrin, L.W. & Bailey, D.M., pp. 163–195, Orlando: Academic Press.

BACH, M.K., BRASHLER, J.R., SMITH, H.W., FITZPATRICK, F.A., SUN, F.F. & McGUIRE, J.C. (1982). 6,9-Deepoxy-6,9-(phenylimino)-$\Delta^{6,8}$-prostaglandin I$_1$, (U-60,257), a new inhibitor of leukotriene C and D synthesis: in vitro studies. $Prostaglandins$, 23, 759–771.

BAST, A., SAVENIJE-CHAPEL, E.M. & KROES, B.H. (1984). Inhibition of mono-oxygenase and oxidase activity of rat hepatic cytochrome P-450 by H$_2$-receptor blockers. $Xenobiotica$, 14, 399–408.

BLACKWELL, C.J. & FLOWER, R.J. (1978). 1-Phenyl-3-pyrazolidone: an inhibitor of cyclo-oxygenase and lipoxygenase pathways in lung and platelets. $Prostaglandins$, 16, 417–425.

BREEN, K.J., BURY, R., DESMOND, P.V., MASHFORD, M.L., MORPHETT, B., WESTWOOD, B. & SHAW, R.G. (1982). Effects of cimetidine and ranitidine on hepatic drug metabolism. $Clin.$ $Pharmacol.$ $Ther.$, 31, 297–300.

CLARK, D.L. & LINDEN, J. (1986). Modulation of guanylate cyclase by lipoxygenase inhibitors. $Hypertension$, 8, 947–950.

COCKS, T.M., ANGUS, J.A., CAMPBELL, J.H. & CAMPBELL, G.R. (1985). Release and properties of endothelium-derived relaxing factor (EDRF) from cells in culture. $J.$ $Cell.$ $Physiol.$, 123, 310–320.

DIAMOND, L. & GELBOIN, H.V. (1969). Alpha-naphtoflavone: an inhibitor of hydrocarbon cytotoxicity and microsomal hydroxylase. $Science$, 166, 1023–1025.

FÖRSTERMANN, U. & NEUFANG, B. (1984). The endothelium-dependent vasodilator effect of acetylcholine: characterization of the endothelial relaxing factor with inhibitors of arachidonic acid metabolism. $Eur.$ $J.$ $Pharmacol.$, 103, 65–70.

FÖRSTERMANN, U., TROGISCH, G. & BUSSE, R. (1984). Species dependent differences in the nature of endothelium-derived vascular relaxing factor. $Eur.$ $J.$ $Pharmacol.$, 106, 639–643.

FÖRSTERMANN, U. & NEUFANG, B. (1985). Endothelium-dependent vasodilation by melittin: are lipoxygenase products involved? $Am.$ $J.$ $Physiol.$, 249, H14–H19.

FÖRSTERMANN, U., GOPPELT-STRÜBE, M., FRÖLICH, J.C. & BUSSE, R. (1986a). Inhibitors of acyl-coenzyme A:

lysolecithin acyltransferase activate the production of endothelium-derived vascular relaxing factor. *J. Pharmacol. Exp. Ther.*, **238**, 352–359.

FÖRSTERMANN, U., MÜLSCH, A., BÖHME, E. & BUSSE, R. (1986b). Stimulation of soluble guanylate cyclase by an acetylcholine-induced endothelium-derived factor from rabbit and canine arteries. *Circ. Res.*, **58**, 531–538.

FURCHGOTT, R.F. & ZAWADZKI, J.V. (1980). The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature (Lond.)*, **288**, 373–376.

FURCHGOTT, R.F. (1984). The role of endothelium in the responses of vascular smooth muscle to drugs. *Ann. Rev. Pharmacol. Toxicol.*, **24**, 175–197.

GERZER, R., HOFMAN, F. & SCHULTZ, G. (1981). Purification of a soluble, sodium-nitroprusside-stimulated guanylate cyclase from bovine lung. *Eur. J. Biochem.*, **116**, 479–486.

GRIFFITH, T.M., EDWARDS, D.H., LEWIS, M.J., NEWBY, A.C. & HENDERSON, A.H. (1984). The nature of endothelium derived vascular relaxing factor. *Nature (Lond.)*, **308**, 645–647.

HAMASAKI, Y. & TAI, H.-H. (1985). Gossypol, a potent inhibitor of arachidonate 5- and 12-lipoxygenases. *Biochim. Biophys. Acta*, **834**, 37–41.

HIGGS, G.A., FLOWER, R.J. & VANE, J.R. (1979). A new approach to anti-inflammatory drugs. *Biochem. Pharmacol.*, **28**, 959–1961.

HILDEBRANDT, A.G. (1972). The binding of metyrapone to cytochrome P-450 and its inhibitory actions on microsomal hepatic mixed function oxidation reactions. *Biochem. Soc. Symp.*, **34**, 79–102.

HOPE, W.C., WELTON, A.F., FIEDLER-NAGY, C., BATULA-BERNARDO, C. & COFFEY, J.W. (1983). In vitro inhibition of the biosynthesis of slow reacting substance of anaphylaxis (SRS-A) and lipoxygenase activity by quercetin. *Biochem. Pharmacol.*, **32**, 367–371.

IGNARRO, L.J. & KADOWITZ, P.J. (1985). The pharmacological and physiological role of cyclic GMP in vascular smooth muscle relaxation. *Ann. Rev. Pharmacol. Toxicol.*, **25**, 171–191.

LÜCKHOFF, A., BUSSE, R., WINTER, I. & BASSENGE, E. (1987). Characterization of vascular relaxant factor released from cultured endothelial cells. *Hypertension*, **9**, 295–303.

MANNERING, G.J. (1971). Microsomal enzyme systems which catalyze drug metabolism. In: *Fundamentals of Drug Metabolism and Disposition*, ed. La Du, B., Mandel, H. & Woy, E. pp. 206–252. Baltimore: Williams and Wilkins.

MARTIN, W., VILLANI, G.M., JOTHIANANDAN, D. & FURCHGOTT, R.F. (1985). Selective blockade of endothelium-dependent and glyceryl trinitrate-induced relaxation by hemoglobin and methylene blue in the rabbit aorta. *J. Pharmacol. Exp. Ther.*, **232**, 708–716.

MONCADA, S., PALMER, R.M.J. & GRYGLEWSKI, R.J. (1986). Mechanisms of action of some inhibitors of endothelium-derived relaxing factor. *Proc. Natl. Acad. Sci. U.S.A.*, **86**, 9164–9168.

MÜLSCH, A., BÖHME, E. & BUSSE, R. (1987). Stimulation of soluble guanylate cyclase by endothelium-derived relaxing factor from cultured endothelial cells. *Eur. J. Pharmacol.*, **135**, 247–250.

MURAD, F. (1986). Cyclic guanosine monophosphate as a mediator of vasodilation. *J. Clin. Invest.*, **78**, 1–5.

PALMER, R.M.J., FERRIGE, A.G. & MONCADA, S. (1987). Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature*, **327**, 524–526.

PELKONEN, O. & PUURUNEN, J. (1980). The effect of cimetidine on vitro and in vivo microsomal drug metabolism in the rat. *Biochem. Pharmacol.*, **29**, 3075–3080.

PINTO, A., ABRAHAM, N.G. & MULLANE, K.M. (1986). Cytochrome P-450-dependent monooxygenase activity and endothelial-dependent relaxations induced by arachidonic acid. *J. Pharmacol. Exp. Ther.*, **236**, 445–451.

SINGER, H.A. & PEACH, M.J. (1983). Endothelium-dependent relaxation of rabbit aorta. II. Inhibition of relaxation stimulated by methacholine and A23187 with antagonists of arachidonic acid metabolism. *J. Pharmacol. Exp. Ther.*, **226**, 796–801.

SINGER, H.A., SAYE, J.A. & PEACH, M.J. (1984). Effects of cytochrome P450 inhibitors on endothelium-dependent relaxation in rabbit aorta. *Blood Vessels*, **21**, 223–230.

YAMAMOTO, S., YOSHIMOTO, T., FURUKAWA, M., HORIE, T. & WATANABE-KOHNO, S. (1984). Arachidonate 5-lipoxygenase and its new inhibitors. *J. Allergy. Clin. Immunol.*, **74**, 349–352.

YOSHIMOTO, T., YOKOYAMA, C., OCHI, K., YAMAMOTO, S., MAKI, Y., ASHIDA, Y., TERAO, S. & SHIRAISHI, M. (1982). 2,3,5-Trimethyl-6(12-hydroxy-5,10-dodecadinyl)-1,4-benzoquinone (AA 861), a selective inhibitor of the 5-lipoxygenase reaction and the biosynthesis of slow-reacting substance of anaphylaxis. *Biochim. Biophys. Acta*, **713**, 470–473.

YOSHIMOTO, T., FURUKAWA, M., YAMAMOTO, S., HORIE, T. & WATANABE-KOHNO, S. (1983). Flavonoids: potent inhibitors of arachidonate 5-lipoxygenase. *Biochim. Biophys. Res. Commun.*, **116**, 612–618.