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# Mechanism of action of some inhibitors of endothelium-derived relaxing factor

(superoxide/vasodilatation/vascular endothelium/redox potential)

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ABSTRACT **The mechanism of the inhibitory action of phenidone, 3-amino-1-[*m*-(trifluoromethyl)phenyl]-2-pyrazoline (BW 755C), dithiothreitol, hydroquinone, and pyrogallol on the vascular relaxation induced by endothelium-derived relaxing factor (EDRF) was investigated. EDRF was released from porcine aortic endothelial cells in culture and bioassayed on a cascade of superfused rabbit aortic strips. These compounds inhibited EDRF-induced relaxation of vascular strips, without affecting the relaxation induced by glyceryl trinitrate, and their inhibitory potency was markedly attenuated (by more than 1 order of magnitude) by the addition of superoxide dismutase (5–15 units/ml) or oxidized cytochrome *c* (20–40 μM) but not by catalase (30 units/ml) or heat-inactivated superoxide dismutase. These data indicate that the above five inhibitors inactivate EDRF through the formation of superoxide ions, which have recently been shown to destroy EDRF. The inhibition of EDRF by these compounds is therefore attributable to their redox properties rather than to any specific biological action.**

Vascular relaxation induced by a number of pharmacological agents is mediated by a humoral factor, derived from vascular endothelial cells, known as endothelium-derived relaxing factor (EDRF) (1). Many substances have been identified as inhibitors of EDRF in arterial preparations. These include antioxidants and sulfhydryl reagents, as well as inhibitors of phospholipase, arachidonic acid lipoxygenase, and cytochrome P-450-dependent enzymes (1–4). All these compounds have diverse biological activities. However, when used as tools to identify the chemical nature of EDRF, their inhibitory effect has often been ascribed to only one of their known biological actions. Therefore, EDRF has alternatively been suggested to be a product of lipoxygenation of arachidonic acid (3), or a product of its oxygenation by a cytochrome P-450-dependent enzyme (4), or an unstable aldehyde, ketone, or lactone (2). This work has led to considerable controversy.

We have recently developed a bioassay system in which the component that generates EDRF (porcine aortic endothelial cells) and the detector component (a series of superfused rabbit aortic strips denuded of endothelium, RbAs) are separated (5). This system allows differentiation between an effect of substances on the generation or on the biological action of EDRF. Using this system, we have demonstrated that superoxide dismutase, but not catalase, increases the stability of EDRF and that $Fe^{2+}$ ions inactivate EDRF. Furthermore, we have observed that this effect of $Fe^{2+}$ ions is markedly reduced by superoxide dismutase. These results led us to suggest that superoxide ions ($O_2^{\cdot-}$) play a role in the inactivation of EDRF (6). A similar conclusion has been reached by others (7).

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We have now used our bioassay system to study the mechanism of action of some previously identified EDRF inhibitors—namely, phenidone, 3-amino-1-[*m*-(trifluoromethyl)phenyl]-2-pyrazoline (BW 755C), hydroquinone, and dithiothreitol.

## METHODS

Porcine aortic endothelial cells were cultured on microcarriers. The cell column and the bioassay cascade were assembled as described (5). In brief, a column containing 2–6 × $10^7$ endothelial cells on microcarrier beads was perfused (5 ml/min) with Krebs' buffer gassed with 95% $O_2$/5% $CO_2$ at 37°C. The column effluent superfused a cascade (8) of up to four RbAs (spirally cut strips of rabbit thoracic aorta denuded of endothelium). The delay between the endothelial cells in the column and consecutive RbAs was 1, 4, 7, and 10 sec, respectively. The bioassay tissues were contracted with a continuous infusion over the tissues (o.t.) of either the 11α,9α-epoxymethano analogue of prostaglandin $H_2$ (U-46619; 30–60 nM) or with phenylephrine hydrochloride (50 nM) (5). The sensitivity of the RbAs was adjusted so that they were relaxed to a similar extent by a standard dose of glyceryl trinitrate (nitroglycerin, $n_3$Gro; 50 nM o.t.).

A 1-min infusion of bradykinin (20 nM) through the column (t.c.) was used as the standard stimulus for EDRF release, although the calcium ionophore A23187 (0.2–5 μM t.c.) was used occasionally. The inhibitors studied were dissolved in 0.9% NaCl and infused either o.t. or t.c. with a peristaltic micropump. Their inhibitory potency was calculated from the concentration–response curves (Fig. 1). These were constructed by measuring the EDRF-induced relaxation of the uppermost RbA in the presence of the inhibitor and expressed as a percentage of the mean of two bracketing control responses.

## MATERIALS

The following materials were used: Cytodex 3 microcarriers, K16 chromatographic columns, and flow adaptors (Pharmacia); $n_3$Gro, trypsin/EDTA, hydroquinone, phenidone, and BW 755C (Wellcome); bradykinin, phenylephrine hydrochloride, superoxide dismutase, cytochrome *c*, catalase (catalogue no. C-100), pyrogallol, dithiothreitol, and type II collagenase (Sigma); Hepes-buffered Dulbecco's modified Eagle's medium and lyophilized antibacterial-antimycotic solution (Gibco); indomethacin (Merck Sharp & Dohme); U-46619 (Upjohn); fetal bovine serum (Flow Laboratories);

Abbreviations: EDRF, endothelium-derived relaxing factor; RbA, strip of rabbit aorta denuded of endothelium; $n_3$Gro, glyceryl trinitrate; BW 755C, 3-amino-1-[*m*-(trifluoromethyl)phenyl]-2-pyrazoline; U-46619, 11α,9α-epoxymethano analogue of prostaglandin $H_2$; o.t., over the tissues; t.c., through the column.

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and a microcarrier-stirrer system (Techne, Cambridge, U.K.).

## RESULTS

Bradykinin (20 nM t.c.) or ionophore A23187 (0.2–5 μM t.c.) induced release of a RbA-relaxing substance from the column of endothelial cells. This substance was identified as EDRF, since it had a half-life of less than 7 sec, its release was not inhibited by indomethacin, and its action was inhibited by hemoglobin and methylene blue (10 and 50 μM o.t., respectively; data not shown).

When administered t.c., phenidone, BW 755C, hydroquinone, and dithiothreitol caused a concentration-dependent inhibition of EDRF-induced relaxation of the uppermost RbA (Fig. 1). All the compounds were less active o.t. than t.c., but the difference between activity o.t. and t.c. was greater for BW 755C and phenidone than for dithiothreitol and hydroquinone (Fig. 1). The inhibitory effect of all the compounds, whether administered o.t. or t.c., appeared rapidly (within 10 sec), was promptly reversible, and did not increase when the infusion was prolonged. This could be demonstrated during the protracted release of EDRF induced by A23187 (data from an experiment in which hydroquinone was used are shown in Fig. 2).

Infusions of superoxide dismutase (5–15 units/ml, t.c.) caused a relaxation of the assay tissues that was greatest in the uppermost tissue and decreased progressively down the cascade. In addition, it stabilized the EDRF released by bradykinin, as shown by the relaxation of the tissues further down the cascade. The inhibitory effect of all the compounds investigated, whether administered o.t. or t.c., was attenuated by approximately 1 order of magnitude in the presence of superoxide dismutase (15 units/ml, t.c.). Heat-inactivated superoxide dismutase (boiled 1 min) and catalase (30 units/ml) did not affect the potency of these inhibitors. The effect of superoxide dismutase on the inhibitory action of BW 755C is shown in Fig. 3.

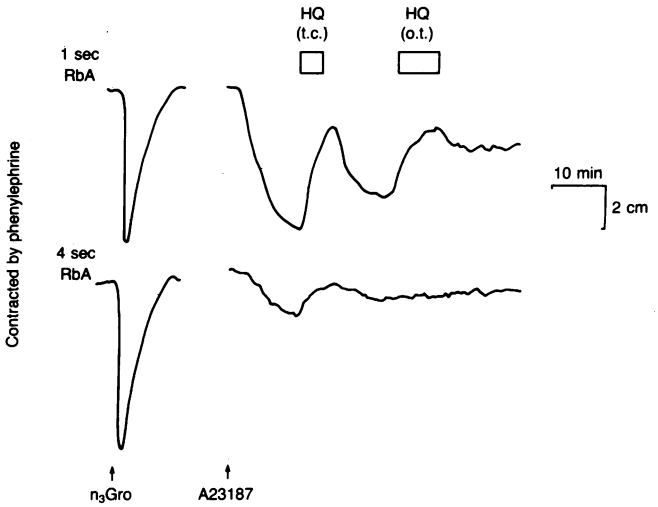


FIG. 2. Hydroquinone (HQ) inhibits the relaxation of RbAs induced by EDRF released in response to A23187. A23187 (5 μM) given o.t. did not have an effect on the assay tissues (data not shown); however, when given t.c. it caused a protracted release of EDRF. The effect of EDRF was reversibly inhibited by short (3–6 min) infusions of HQ (10 μM o.t. or t.c.). The tissues were contracted by an infusion of phenylephrine (50 nM o.t.) and were approximately equisensitive to relaxation induced by $n_3$Gro (50 nM o.t.).

Pyrogallol also inhibited the EDRF-induced relaxation of the bioassay tissues whether administered o.t. or t.c. This compound was more potent t.c. than o.t. and produced complete inhibition at 1 and 30 μM, respectively. An exper-

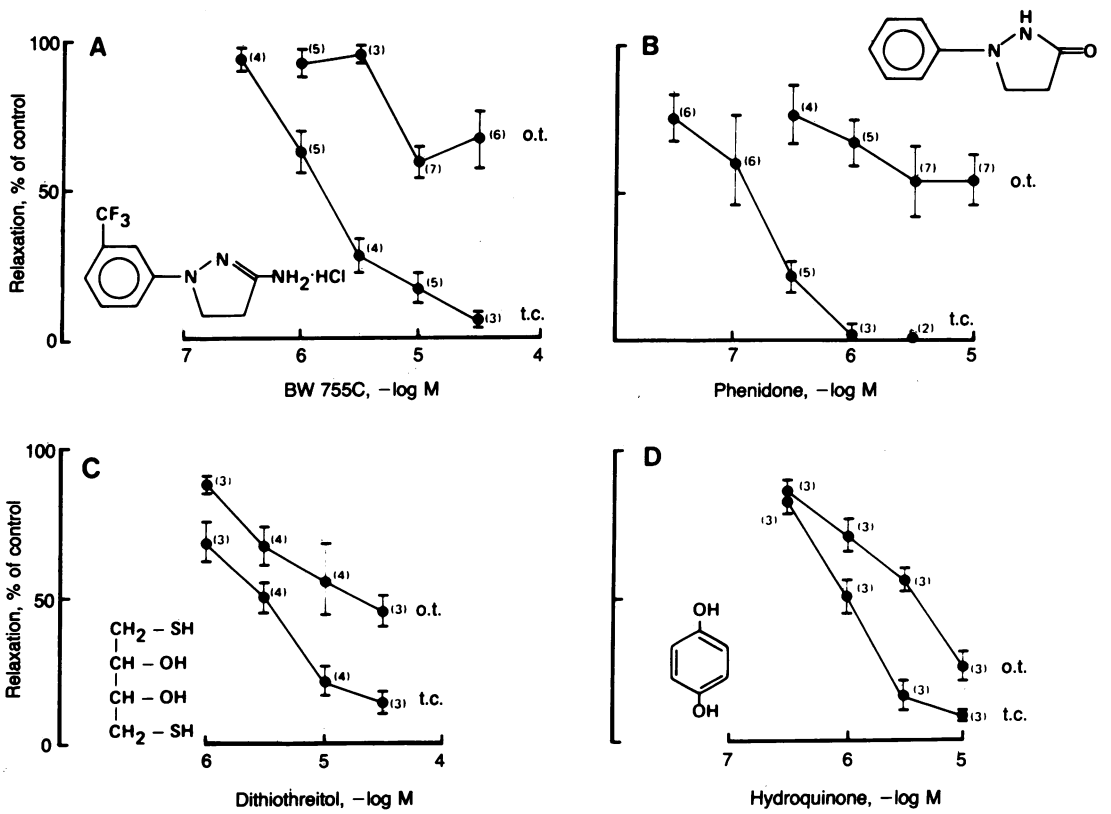


FIG. 1. Concentration–response curves for the inhibition of EDRF-induced relaxation of the uppermost RbA in the cascade by BW 755C (*A*), phenidone (*B*), dithiothreitol (*C*), and hydroquinone (*D*) infused for 5 min either o.t. or t.c. at the concentrations indicated. Each point is the mean ± SEM of (*n*) separate determinations.

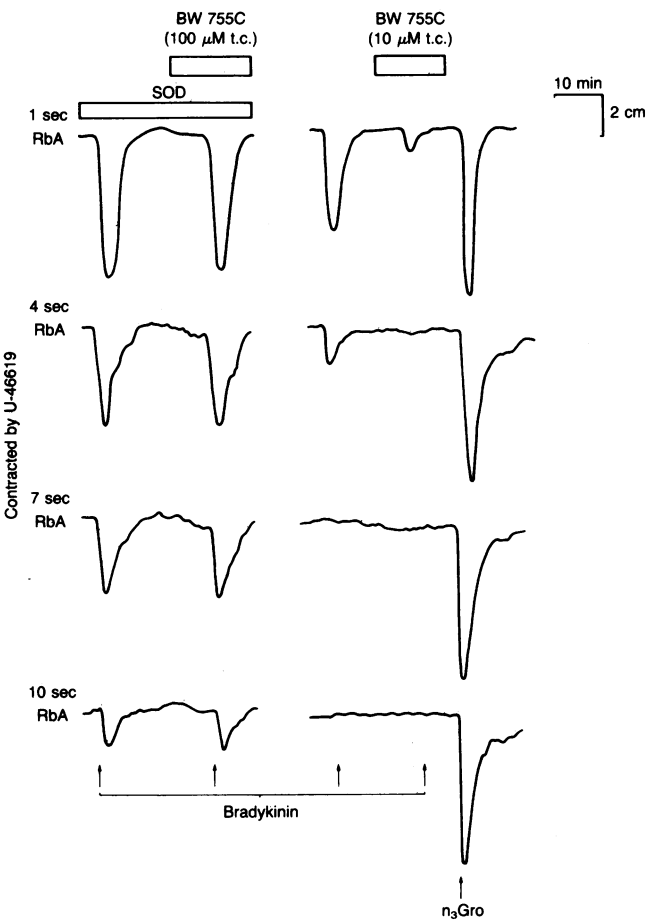


FIG. 3. Superoxide dismutase (SOD) diminishes the inhibition by BW 755C of EDRF-induced relaxation. Superoxide dismutase (15 units/ml, t.c.) induced a relaxation of the assay tissues (not shown). During the infusion of superoxide dismutase, bradykinin (20 nM t.c.) released EDRF, which survived as far as the fourth RbA in the cascade, and infusion of BW 755C (100 μM t.c.) had no effect on the release of EDRF. In the absence of superoxide dismutase, EDRF was less stable (total disappearance by the third RbA) and BW 755C (10 μM t.c.) caused a significant inhibition of its action. The tissues were contracted by an infusion of U-46619 (30 μM o.t.) and were approximately equisensitive to relaxation induced by $n_3$Gro (50 nM o.t.).

iment in which this compound was administered t.c. is shown in Fig. 4. Furthermore, the effect of pyrogallol was decreased by more than 1 order of magnitude by superoxide dismutase (Fig. 4).

When cytochrome *c* (20–40 μM) was given o.t. in the absence of the column effluent, it had no effect on the assay tissues. However, when given either t.c. or o.t. in the presence of effluent from the column, it induced a relaxation of the assay tissues that was greater in the uppermost RbA and decreased the further down the cascade the detector tissue was situated. The effect of 40 μM cytochrome *c* t.c. was equivalent to that observed with 15 units of superoxide dismutase per ml t.c. and, like that of superoxide dismutase, was less pronounced when given o.t. The stability of EDRF released by bradykinin was also increased by infusions of cytochrome *c* (20–40 μM t.c.), as shown by the relaxation of the tissues further down the cascade. Furthermore, cytochrome *c* significantly attenuated the effect of all the inhibitors, including pyrogallol, whether they were administered o.t. or t.c., but did not affect the relaxations induced by $n_3$Gro. The data from an experiment showing the interaction of cytochrome *c* with phenidone are shown in Fig. 5.

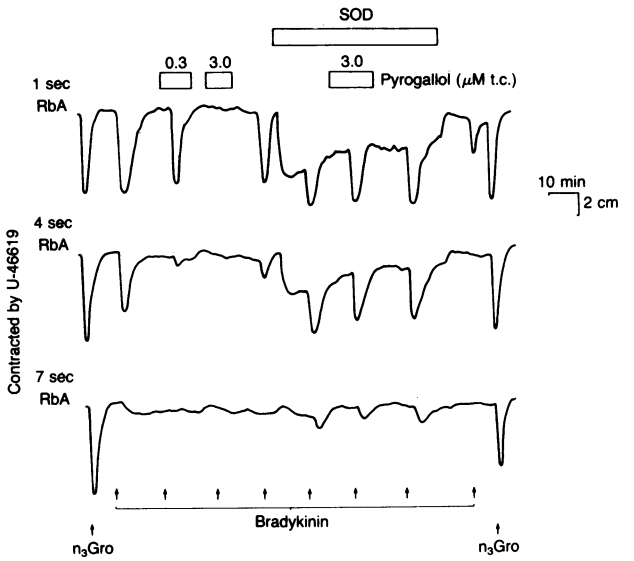


FIG. 4. Superoxide dismutase (SOD) diminishes the inhibition by pyrogallol of EDRF-induced relaxation. Bradykinin given o.t. had no effect on the assay tissues (data not shown); however, when given t.c. it induced the release of EDRF as shown by the relaxation of the upper two RbAs in the cascade. This was inhibited completely by 3 μM but not by 0.3 μM pyrogallol (t.c.). Infusions of superoxide dismutase (15 units/ml, t.c.) produced a relaxation of the assay tissues, which diminished down the cascade, revealing the spontaneous release of EDRF. EDRF released by bradykinin in the presence of superoxide dismutase caused a relaxation of all the tissues in the cascade that was not affected by pyrogallol (3 μM t.c.). The tissues were contracted by an infusion of U-46619 (30 nM o.t.) and were approximately equisensitive to relaxation induced by $n_3$Gro (50 nM o.t.).

## DISCUSSION

We have used our bioassay system to confirm that hydroquinone, dithiothreitol, phenidone, and BW 755C inhibit vascular relaxation induced by EDRF released by bradykinin and A23187 from porcine aortic endothelial cells in culture. Unlike hemoglobin and methylene blue (9), these four compounds do not affect the response to $n_3$Gro, indicating that they do not inhibit EDRF-mediated relaxation by inhibition of guanylate cyclase. Therefore, these inhibitors selectively affect EDRF action by some other mechanism, such as inhibition of its synthesis or action or by its accelerated destruction.

Various biochemical properties are usually associated with these compounds. For example, hydroquinone is an aromatic antioxidant that prevents lipid peroxidation (10), dithiothreitol is a sulfhydryl reagent that reactivates a number of sulfhydryl group-containing enzymes (11), and phenidone (12) and BW 755C (13) are best known as mixed-function lipoxygenase/cyclooxygenase inhibitors. Could such a diversity of biochemical and chemical actions underlie a single effect of these compounds as inhibitors of EDRF?

We have recently shown that superoxide dismutase protects EDRF against inactivation (6). Since superoxide dismutase is a specific enzymic inactivator of $O_2^-$ ions, its effect is generally accepted as evidence for the involvement of these ions in a particular system. As a result we examined the effect of superoxide dismutase on the action of these compounds and found that this enzyme significantly attenuates their inhibitory potency. The potency of the compounds was not affected either by heat-inactivated superoxide dismutase or by catalase, indicating that this effect of superoxide dismutase is dependent on its enzymic activity and that $O_2^-$ ions and not other oxygen-derived radicals are the active species. The mechanism of this attenuation is probably not due to

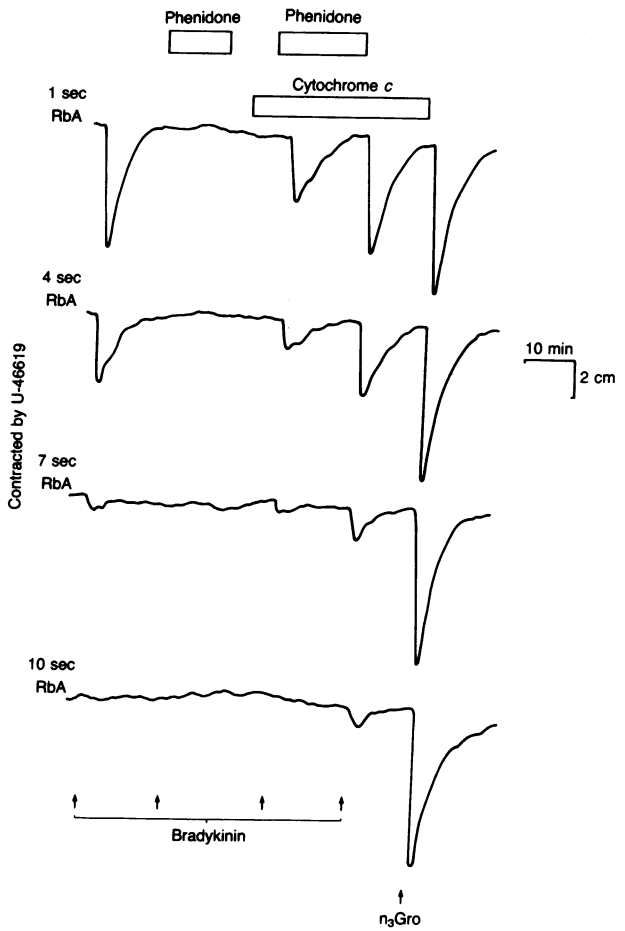


FIG. 5. Cytochrome *c* attenuates the inhibition by phenidone of EDRF-induced relaxation. Bradykinin (20 nM t.c.) induced the release of EDRF, which was completely inhibited by phenidone (1 $\mu$M t.c.). In the presence of cytochrome *c* (20 $\mu$M t.c.), this concentration of phenidone produced only ≈50% inhibition of the relaxation induced by EDRF. EDRF was stabilized by cytochrome *c*, as shown by the relaxation of the third and fourth RbAs in the cascade. The tissues were contracted by an infusion of U-46619 (30 nM o.t.) and were approximately equisensitive to relaxation induced by $n_3$Gro (50 nM o.t.).

increased amounts of EDRF overcoming the action of the inhibitors, as one would still expect to see an effect of the inhibitors on relaxations smaller than the control. This is not the case. As seen in Fig. 3, the control relaxation of the top tissue, which is 60–70% of that induced by the standard $n_3$Gro (50 nM), is greatly reduced by 10 $\mu$M BW 755C, whereas, in the presence of superoxide dismutase, smaller responses in the lower tissues are totally unaffected by a 10-fold greater dose of BW 755C (100 $\mu$M). This clearly indicates that the effect of superoxide dismutase is not due to a shift of the dose–response curve of EDRF but to an abrogation of the mechanism by which EDRF is destroyed.

This possibility was further examined in experiments with oxidized cytochrome *c*, another scavenger of $O_2^{\cdot-}$ ions. Oxidized cytochrome *c* is readily reduced by $O_2^{\cdot-}$ ions and is therefore widely used in spectrophotometric methods for their detection (14). Indeed, we found that cytochrome *c* has a superoxide dismutase-like effect, since it increases the stability of EDRF, as demonstrated by an increase in the EDRF-induced relaxation of the tissues further down the cascade and the unmasking of its spontaneous release from the endothelial cells. Cytochrome *c* also attenuated the inhibitory potency of all four compounds studied. While superoxide dismutase achieves this effect by dismuting $O_2^{\cdot-}$ ions, cytochrome *c* could be acting either by removing $O_2^{\cdot-}$ ions or by interacting directly with the active species of these compounds to prevent their reduction of $O_2$ to $O_2^{\cdot-}$ ions.

From all these observations we predicted that pyrogallol, a potent generator of $O_2^{\cdot-}$ ions in aqueous solutions (15), should also be an effective inhibitor of EDRF-induced vascular relaxation and that cytochrome *c* should reduce the inhibitory activity of pyrogallol. We confirmed this in additional experiments in which we also showed that superoxide dismutase markedly attenuated the activity of pyrogallol.

Our data indicate that there is a common mechanism for the inhibitory action of the four compounds investigated, which is also shared by pyrogallol: namely, the generation of $O_2^{\cdot-}$ ions which in turn inactivate EDRF. Such an action could also explain the inactivation of EDRF by catecholamines (16), for these compounds can also generate $O_2^{\cdot-}$ ions during their autoxidation (17). Final confirmation of this mechanism of action is dependent on direct measurement of $O_2^{\cdot-}$ ions. Preliminary experiments using electron-spin resonance (ESR) have failed to show the presence of $O_2^{\cdot-}$ ions; however, the quantity of $O_2^{\cdot-}$ ions likely to be present in our system is below the limit of detection of this method.

All the compounds tested are likely to reduce $O_2$ to $O_2^{\cdot-}$ ions either directly or subsequent to their conversion to semiquinones or radical cations (18–20). The generation of free radical products and/or $O_2^{\cdot-}$-derived radicals during autoxidation of high concentrations (20 mM) of these inhibitors has been confirmed by ESR (T. F. Slater, personal communication). A similar activation reaction is reported to underlie the cardiotoxicity of antitumor anthracyclines (21). In this instance, the quinone moiety is reduced in microsomes to the semiquinone, which then generates cytotoxic $O_2^{\cdot-}$ ions. All EDRF inhibitors studied may be activated to varying degrees by endothelial cells, leading to the generation of $O_2^{\cdot-}$ ions by a similar mechanism. Such an effect could account for the greater potency of these compounds t.c. than o.t. Additional support for the proposed mechanism of action comes from the observation that the inhibition of EDRF by these compounds, including pyrogallol, was rapid in onset and promptly reversible, suggesting a direct chemical interaction rather than an enzymic inhibition, which one would expect to be slower in onset and longer-lasting.

The relevance of our findings to the *in vivo* situation is difficult to assess. It is likely that the potency of these compounds is highly dependent on the local concentration of $O_2$. This could explain why they are less potent in other experimental conditions (1–4) than in our cascade superfusion where continuously flowing hyperoxic buffer is used. Nevertheless, $O_2^{\cdot-}$ ions are reported to be responsible for the conversion of the acetylcholine-induced cerebral arteriolar dilatation to vasoconstriction during acute experimental hypertension (22) and to participate in the tissue damage induced by postischemic reperfusion in several organs (23). These effects are prevented by superoxide dismutase (23). It remains to be seen whether EDRF levels are altered in postischemic reperfusion damage and in other types of oxygen-related damage and, if this is so, what is the biological significance of this effect. What is evident from our experiments is that before any chemical can be classified as a specific inhibitor of EDRF, its potential to generate $O_2^{\cdot-}$ ions by a redox mechanism should be examined. In this respect it is likely that compounds may act either as inhibitors or as stabilizers of EDRF, according to their ability to oxidize $O_2^{\cdot-}$ ions or reduce $O_2$.

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1. Furchgott, R. F. (1984) *Annu. Rev. Pharmacol. Toxicol.* **24,** 175–197.
2. Griffith, T. M., Edwards, D. H., Lewis, M. J., Newby, A. C. & Henderson, A. H. (1984) *Nature (London)* **308,** 645–647.
3. Singer, H. A. & Peach, M. J. (1983) *J. Pharmacol. Exp. Ther.* **226,** 796–801.
4. Pinto, A., Abraham, N. G. & Mullane, K. M. (1986) *J. Pharmacol. Exp. Ther.* **236,** 445–451.
5. Gryglewski, R. J., Moncada, S. & Palmer, R. M. J. (1986) *Br. J. Pharmacol.* **87,** 685–694.
6. Gryglewski, R. J., Palmer, R. M. J. & Moncada, S. (1986) *Nature (London)* **320,** 454–456.
7. Rubanyi, G. M. & Vanhoutte, P. M. (1986) *Am. J. Physiol.* **250,** H222–H227.
8. Vane, J. R. (1964) *Br. J. Pharmacol. Chemother.* **23,** 360–373.
9. Martin, W., Villani, G. M., Jothianandan, D. & Furchgott, R. F. (1985) *J. Pharmacol. Exp. Ther.* **232,** 708–716.
10. Geissman, T. A. (1968) *Principles of Organic Chemistry* (Freeman, San Francisco), pp. 667–670.
11. Peach, M. E. (1974) *The Chemistry of the Thiol Group* (Wiley, London), pp. 765–771.
12. Blackwell, G. J. & Flower, R. J. (1978) *Prostaglandins* **16,** 417–425.
13. Higgs, G. A., Flower, R. J. & Vane, J. R. (1979) *Biochem. Pharmacol.* **28,** 1959–1961.
14. Babior, B. M., Kipnes, R. S. & Curnutte, J. T. (1973) *J. Clin. Invest.* **52,** 741–744.
15. Marklund, S. & Marklund, G. (1974) *Eur. J. Biochem.* **47,** 469–474.
16. Rubanyi, G. M., Lorenz, R. R. & Vanhoutte, P. M. (1985) *Am. J. Physiol.* **249,** H95–H101.
17. Misra, H. P. & Fridovich, I. (1972) *J. Biol. Chem.* **247,** 3170–3175.
18. Misra, H. P. (1974) *J. Biol. Chem.* **249,** 2151–2155.
19. Marnett, L. J., Siedlik, P. H. & Fung, L. W. M. (1982) *J. Biol. Chem.* **257,** 6957–6964.
20. Lee-Ruff, E., Lever, A. B. P. & Rigaudy, J. (1976) *Can. J. Chem.* **54,** 1837–1839.
21. Peters, J. H., Gordon, G. R., Kashiwase, D., Lown, J. W., Yen, S. F. & Plambeck, J. A. (1986) *Biochem. Pharmacol.* **35,** 1309–1323.
22. Wei, E. P., Kontos, H. A., Christman, C. W., DeWitt, D. S. & Povlishock, J. T. (1985) *Circ. Res.* **57,** 781–787.
23. Halliwell, B. & Gutteridge, J. M. C. (1985) *Mol. Aspects Med.* **8,** 89–193.