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A NEW SYNTHETIC FLAVONOID PROTECTS ENDOTHELIUM-DERIVED RELAXING FACTOR-INDUCED RELAXATION IN RABBIT ARTERIES *IN VITRO*: EVIDENCE FOR SUPEROXIDE SCAVENGING

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Abstract—A new synthetic flavone derivative, 6,7-dimethoxy-8-methyl-3′,4′,5-trihydroxyflavone, was studied for its capacity to protect the acetylcholine-induced relaxation of rabbit ear and cerebral arteries from inhibition by superoxide anion. This property was evaluated via two types of *in vitro* experiments, using rabbit ear or basilar arteries mounted in organ baths equipped for isometric tension measurement. When a high level of superoxide anion was generated by adding 3×10^{-4} M pyrogallol to the bath, the relaxation to acetylcholine was substantially inhibited. This inhibition was significantly reversed by both superoxide dismutase (25 and/or 50 U/mL) and the flavonoid (3×10^{-6} M and/or 10^{-5} M) in both types of arteries. In the presence of the basal level of superoxide anion, the responses to acetylcholine were significantly potentiated by the flavonoid (10^{-5} M) in the ear but not the basilar artery. Thus this flavonoid protects endothelium-dependent relaxation from high levels of superoxide anion possibly by scavenging superoxide anion and may have a certain therapeutic value as an agent capable of promoting natural vasodilatation.

Key words: EDRF; flavonoid; free radical scavenging; nitric oxide; superoxide; superoxide dismutase; vasodilatation

The flavonoids are benzo-γ-pyrone derivatives forming a class of natural products, widely distributed in the plant kingdom, whose biological and pharmacological properties have received increasing attention during recent years [1–4]. Besides their well-known anti-allergic and anti-inflammatory properties [5], it has been claimed that they possess vasoprotective [6] or hypotensive properties as well [7, 8]. Flavonoid activities are usually associated with their free radical scavenging properties, especially with respect to the superoxide anion [9, 10]. In preliminary experiments with a natural flavonoid, we also found that it reacted rapidly with the superoxide anion generated by potassium dioxide in tetrahydrofuran, leading to oxygenated derivatives. Similar results are described for 3-hydroxyflavones [11]. The generation in biological systems of free radicals, such as superoxide anion, is considered to be an important event contributing to the oxidative stress phenomenon and one associated with many disease states, e.g. inflammation [12]. In particular, the superoxide anion has been shown to affect vascular endothelial cell function since it destroys the EDRF§ [13]. In the present work, we investigated the capacity of a new synthetic flavonoid, 6,7-dimethoxy-8-methyl-3′,4′,5-trihydroxyflavone, to protect the EDRF from inactivation by this radical.

The model chosen to test the capacity of the flavonoid to scavenge free radicals and hence protect EDRF, whether nitric oxide or a nitroso compound [14, 15], consisted of two types of rabbit muscular arteries of roughly similar diameter, one peripheral, the central EA, the other cerebral, the BA. Both ear [16] and cerebral [17, 18] arteries of the rabbit have been shown to respond to ACh by endothelium-dependent relaxation, and they possess a roughly similar number of smooth muscle layers, although the ear artery has a greater overall diameter. However, there are many important differences between these arteries, not the least being the relatively non-vital nature of EA vasomotricity compared to that of the BA.

Two types of *in vitro* experiments were designed. In the first, we artificially created a large increase in the superoxide concentration by administration of pyrogallol [19]. This means of producing a rapid increase in superoxide concentration has recently been used in *in vitro* experiments, especially as a method of inhibiting relaxation to EDRF or nitric oxide (NO) [e.g. 15, 20, 21]. We then determined whether the presence of the flavonoid, or a recognized superoxide scavenger, SOD, could attenuate the inhibition of ACh-induced relaxation of the BA and the EA. In the second, we determined whether the presence of the flavonoid influenced relaxation to ACh, assuming that spontaneous free

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§ Abbreviations: ACh, acetylcholine; BA, basilar artery; EA, ear artery; EDRF, endothelium-derived relaxing factor; SOD, superoxide dismutase.

radical production could inactivate a fraction of the EDRF produced.

The results are compatible with superoxide scavenging activity of the flavonoid tested, which could confer vasoprotective properties to this molecule under certain conditions.

MATERIALS AND METHODS

Treatment of arteries

Rabbits were anesthetized with acepromazine (17 mg/kg) and pentobarbital (20 mg/kg), decapitated, and the brain or ears removed. The BA or the intermediate portion of the central EA was carefully dissected out under a HEPES-buffered physiological solution containing oxygen at atmospheric pressure (see Sercombe *et al.* [17] for details). Three millimeter segments were prepared and mounted on a pair of L-shaped stainless steel holders for isometric tension measurement according to the technique of Högestätt *et al.* [22]. The arteries were bathed in a Krebs solution of the following composition (mM): NaCl 126, KCl 5, NaH_2PO_4 1.2, $MgCl_2$ 1.3, $NaHCO_3$ 20, $CaCl_2$ 2.5, glucose 5.5. This solution was gassed with 76% N_2, 20% O_2 and 4% CO_2, and had a pO_2 of 120–150 mm Hg. Its pH was in the range 7.3–7.4.

One millimolar arginine was present throughout the experiments. A 90 min equilibration period was allowed after the arteries were mounted; during this time resting tension was repeatedly adjusted to 600 mg. Preconstrictions of the arteries prior to relaxation with ACh were obtained with noradrenaline for EAs and uridine triphosphate and histamine for BAs (noradrenaline is a poor constrictor of rabbit cerebral arteries [23]). Nitroprusside (10^{-5} and 10^{-4} M) was added at the end of each trial to check the capacity of the vessels to dilate to a non-endothelium-dependent relaxing agent.

Protocols

High-level superoxide experiments. A moderate relaxation to ACh was obtained by the administration of an appropriate intermediate concentration. The ideal response sought was approx. 50 ± 20% relaxation. If the response stabilized, pyrogallol was rapidly administered at a concentration of 3×10^{-4} M, triggering an inhibition of the response to ACh. Such tests were made alternately in the absence or the presence of SOD, the flavonoid or the vehicle, so that each test could be compared with the mean of two controls, immediately before and after. The concentration of ACh was adjusted to obtain an approximately equivalent relaxation at each trial. A typical pair of trials is illustrated in Fig. 1.

Basal-level superoxide experiments. In these experiments the arteries were subjected to cumulatively increasing concentrations of ACh (10^{-7} to 10^{-4} or 3×10^{-4} M). Pilot experiments revealed that the EAs, after reacting very strongly during the first trial, reacted substantially less in the following trial. Thereafter, a further slight decline in the response could be detected with each subsequent trial. We therefore used the first ACh test as a verification of the general state of the vessels, and then alternated controls (no pretreatment) with tests (pretreatment by flavonoid or vehicle); each test was thus preceded and followed by controls. A typical pair of trials is illustrated in Fig. 2. We adopted an identical protocol for the BAs, although they showed much less reduction than EAs in their responses to repeated relaxations by ACh.

Statistical analysis

Each set of results under SOD, the flavonoid or the vehicle was compared with the mean of the preceding and following control values (paired *t*-test). For high-level superoxide experiments, two comparisons were made: firstly, the relaxations to ACh (calculated as the decrease in tension relative to the induced preconstriction) just prior to administration of the pyrogallol, and secondly, the inhibitions induced by pyrogallol. The latter parameter was calculated as the maximal reduction in relaxation caused by pyrogallol, expressed relative to the relaxation (in %). The maximum inhibition was usually obtained between 2 and 3 min following pyrogallol administration. For basal-level superoxide experiments, the values compared were the relaxations to ACh at 10^{-7}, 10^{-6}, 10^{-5}, 10^{-4}, and for BAs only, 3×10^{-4} M.

Probability values were considered significant for $P < 0.05$. Values of all results are expressed as mean ± SEM.

Solutions and drugs

Noradrenaline, uridine 5′-triphosphate, histamine, sodium nitroprusside, bovine liver superoxide dismutase (EC 1.15.1.1) and ACh were obtained from Sigma (U.S.A.), and L-arginine and pyrogallol from Prolabo (France). The flavonoid, 6,7-dimethoxy-8-methyl-3′,4′,5-trihydroxyflavone, was synthesized from phloroacetophenone using a multistep procedure described elsewhere [24].

All compounds except the flavonoid were made up in stock solutions in water saturated with nitrogen and protected from light, and added in small quantities (maximum 150 μL) to the bath to obtain the desired concentration. The flavonoid was dissolved at 10^{-2} M in DMSO, then either used directly (for a final concentration of 10^{-5} M) or after dilution in water to 10^{-3} M (for final concentrations of 10^{-6} and 3×10^{-6} M). Trials with the vehicle used DMSO at a final dilution of × 1000.

RESULTS

There was no observable effect of the flavonoid (at 10^{-6}–10^{-5} M) on the basal tone of the arteries.

High-level superoxide experiments

Ear artery. The administration of 3×10^{-4} M pyrogallol to the bath during the relaxation of EAs induced by ACh (in the absence of other agents) caused a rapid inhibition of a mean amplitude of between 90 and 107%, i.e. an approximate return to the baseline preconstriction level (Fig. 3, control columns). In the presence of SOD at 25 or 50 U/mL this inhibition decreased moderately by 16% ($P < 0.05$) and 28% ($P < 0.02$), respectively (Fig. 3A). In the presence of the flavonoid, there was no significant change at 3×10^{-6} M and a decrease in

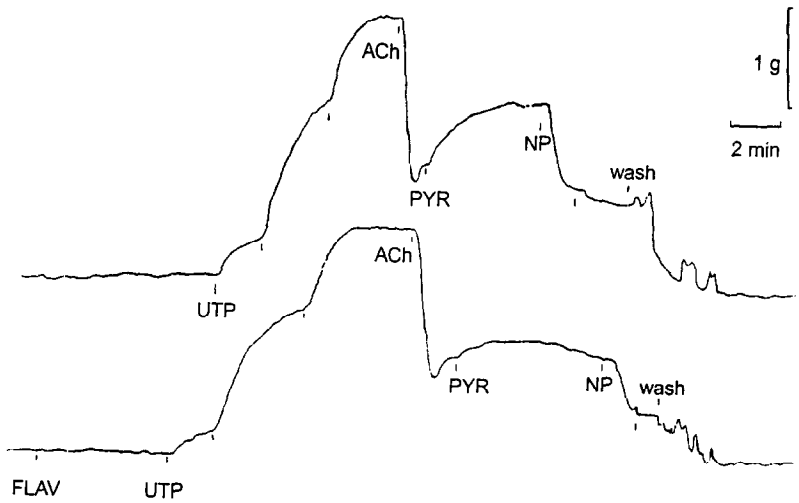

Fig. 1. Original recordings of a BA segment illustrating the protocol used in high-level superoxide experiments. The sequence was identical in both upper (control) and lower (test) panels: after preconstriction with uridine triphosphate (UTP) and histamine (unlabelled signals), a significant, approximately similar, relaxation was obtained in each case with a suitable concentration of acetylcholine (ACh) (not necessarily identical in the two tests). At the moment the relaxation stabilized, 3×10^{-4} M pyrogallol (PYR) was rapidly added to the bath, inducing an inhibition of the relaxation. NP, nitroprusside at 10^{-5} and 10^{-4} M 30 min between the wash and the following test. FLAV: 10^{-5} M flavonoid added.

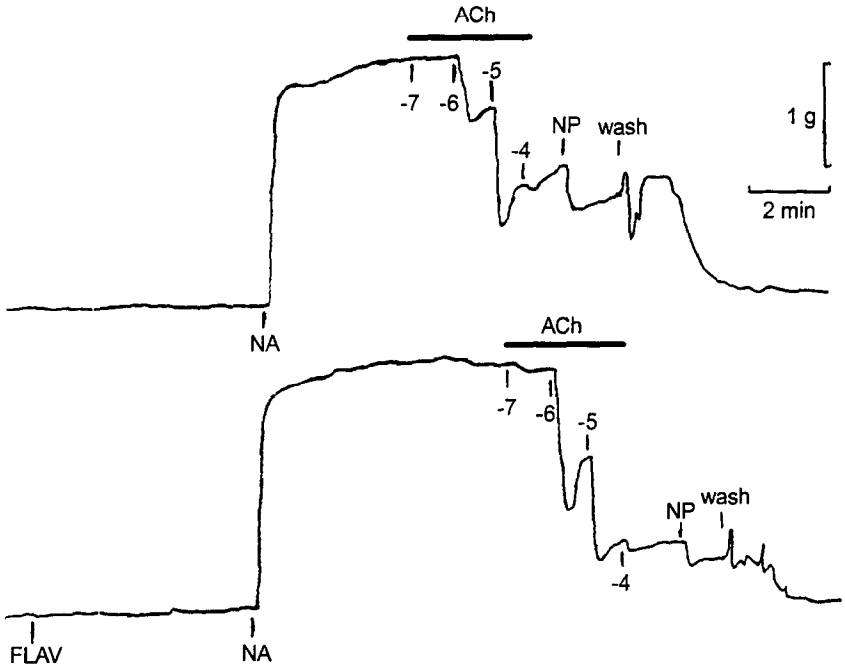

Fig. 2. Original recordings of an EA segment illustrating the protocol used in basal-level superoxide experiments. The sequence was identical in both upper (control) and lower (test) panels: after preconstriction with noradrenaline (NA), relaxation was obtained with increasing concentrations of acetylcholine (ACh) at the log molar concentrations shown. NP, nitroprusside 10^{-5} M 30 min between the wash and the following test. FLAV: 10^{-5} M flavonoid added.

inhibition of approx. 37% ($P < 0.01$) at 10^{-5} M (Fig. 3B).

Basilar artery. The administration of 3×10^{-4} M pyrogallol to the bath during ACh-induced relaxation of BAs (in the absence of other agents) inhibited the relaxations by between 54 and 107% (Fig. 4, control columns). In the presence of SOD at 25 or 50 U/mL, these inhibitions were decreased

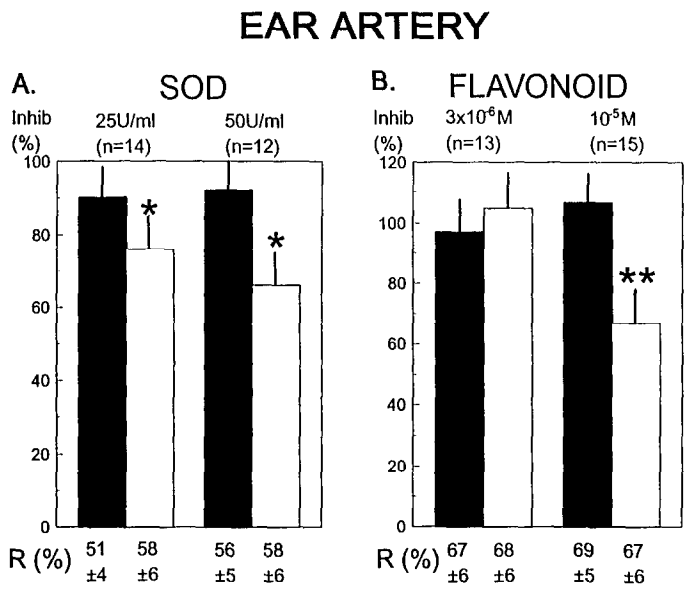

Fig. 3. Influence of SOD (A) or flavonoid (B) on the inhibition of the ACh-induced relaxation of ear arteries triggered by addition of 3×10^{-4} M pyrogallol. *R* (%) represents the initial relaxation to ACh, as percentage of preconstriction amplitude; Inhib (%) = inhibition of relaxation in percent. Control = filled columns; test = empty columns. Significant differences are indicated by * $P < 0.05$ or ** $P < 0.01$, paired *t*-test.

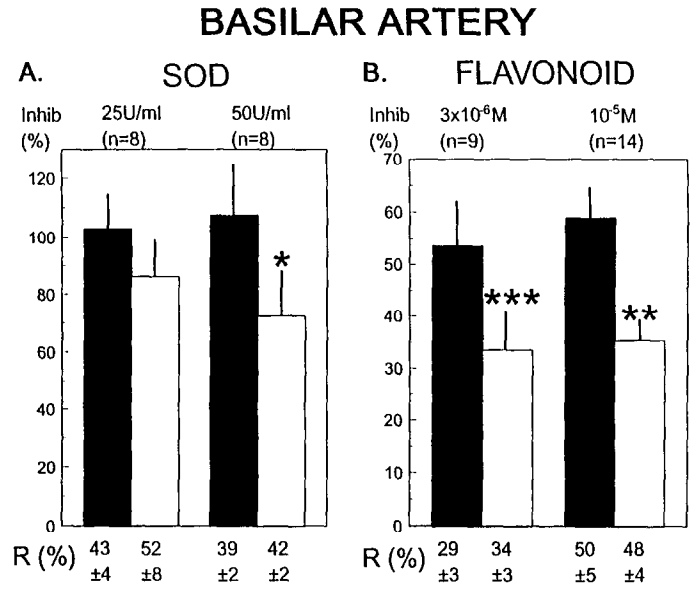

Fig. 4. Influence of SOD (A) or flavonoid (B) on the inhibition of the ACh-induced relaxation of basilar arteries triggered by addition of 3×10^{-4} M pyrogallol. *R* (%) represents the initial relaxation to ACh, as percentage of preconstriction amplitude; Inhib (%) = inhibition of relaxation in percent. Control = filled columns; test = empty columns. Significant differences are indicated by * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$, paired *t*-test.

moderately by 16% (NS) and 38% ($P < 0.02$), respectively (Fig. 4A). In the presence of the flavonoid, at 3×10^{-6} M and 10^{-5} M, the inhibitions were decreased by 37% ($P < 0.001$) and 40% ($P < 0.01$), respectively (Fig. 4B).

The possible influence of the vehicle was tested on the most sensitive arteries (the BAs) by performing identical experiments ($N = 8$) in the presence of the highest concentration of DMSO used (1/1000 (v/v), corresponding to 10^{-5} M flavonoid).

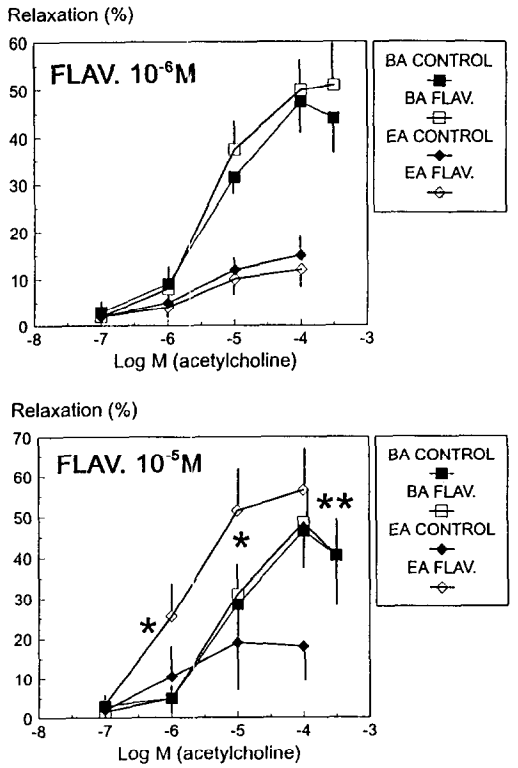

Fig. 5. Influence of flavonoid (FLAV) at 10^{-6} M and 10^{-5} M on the response of basilar arteries (BA) and ear arteries (EA) to ACh in basal-level superoxide experiments. Relaxation is expressed as a percentage of the amplitude of the preconstriction. Significant differences between control (mean of preceding and following trials) and flavonoid values shown by * $P < 0.05$ and ** $P < 0.01$, paired *t*-tests.

Following relaxations of 54 ± 4% (controls) and 51 ± 5% (DMSO) (difference NS), we found inhibitions of 73 ± 4% and 74 ± 9% (difference NS).

The capacity of 3×10^{-4} M pyrogallol to constrict arteries (and hence to simulate inhibition of relaxation) was tested by administering it to non-preconstricted BAs: effects were zero (N = 14) or extremely small (up to 126 mg, N = 3), and could in no case explain the increases in tension induced during relaxation to ACh. Finally, we found that both arteries still showed substantial relaxation to nitroprusside after pyrogallol treatment.

Basal-level superoxide experiments

The flavonoid was tested at three concentrations: 10^{-6} and 10^{-5} M on both types of arteries, and 3×10^{-6} M on BAs. As illustrated in Fig. 5, there was no significant change in the relaxations to ACh for either type of artery at 10^{-6} M flavonoid, whereas at 10^{-5} M flavonoid the relaxations of the EAs, but not the BAs, were substantially increased to nearly 3-fold at the highest concentration of ACh. At the intermediate concentration of 3×10^{-6} M (not shown), the flavonoid still had no significant influence on the relaxations of the BAs: the greatest mean increase in relaxation induced by the presence of flavonoid attained only 5.3% at 10^{-5} M ACh.

The possible influence of the vehicle was checked by performing identical tests on the EAs in the absence and the presence of the highest concentration of DMSO used (1/1000 (v/v), corresponding to 10^{-5} M flavonoid). Values of control responses and responses under DMSO to 10^{-7}, 10^{-6}, 10^{-5} and 10^{-4} M ACh, respectively, were 2 ± 1% and 2 ± 1% (NS); 9 ± 3% and 12 ± 3% ($P < 0.01$); 19 ± 4% and 21 ± 6% (NS); 21 ± 4% and 21 ± 5% (NS).

DISCUSSION

The main findings of the present work are as follows. First, the synthetic flavonoid substantially attenuated the inhibition of ACh-induced relaxation, at high levels of superoxide, triggered by the administration of pyrogallol in both the BA and the EA, in a way very similar to SOD and with comparable efficiency. Second, at basal levels of superoxide, the flavonoid potentiated ACh-induced relaxation in the EA but not in the BA (at the concentrations tested). We shall discuss these results in terms of our hypothesis that the superoxide scavenging property previously attributed to flavonoids [9] is responsible for this activity, which should thus favour vasodilatation in both vascular territories by decreasing the destruction of the EDRF.

Since the flavonoid was solubilized in a non-aqueous solvent (DMSO) and administered after a minimal dilution of 1/1000 (v/v), it is essential to consider the possible action of the vehicle itself. In high-level superoxide experiments the vehicle had strictly no effect on the inhibition, so could not have contributed to the observed attenuation with the flavonoid. In basal-level superoxide experiments on the EA, responses to ACh were increased 0–41% by DMSO alone, but this was only significant for 10^{-6} M ACh. This is to be compared with increases of 88–212% (149% at 10^{-6} M ACh) (Fig. 5) in the presence of the flavonoid at the corresponding concentration. It is clear that, although DMSO had a minor potentiating effect, this could not explain the observed augmentation in relaxation. The concentration of DMSO tested corresponds to approximately 10^{-2} M. Pitts *et al.* [25] found little or no dilatory action on cat cerebral arteries either *in vitro* or *in vivo* at this concentration of DMSO. However, there is evidence for a hydroxyl radical scavenging activity of DMSO [26, 27], which could account for its minor potentiating action in basal-level superoxide experiments, where the hydroxyl radical may play a significant role, and its ineffectiveness in high-level superoxide experiments.

It is worth emphasizing that the present experiments were performed at a nearly physiological level of pO_2, i.e. 130–150 mm Hg, 20% O_2 in gas mixture, compared to the 400–500 mm Hg, 95% O_2, commonly used. According to Kelm *et al.* [27], this difference will result in a significantly longer half-life of nitric oxide (approx. 4 sec at 500 mm Hg, and 5.6 sec at 150 mm Hg in their experiments), presumably because both free oxygen and superoxide react rapidly with NO.

Both hydroxyl and superoxide radicals contribute to EDRF/NO destruction [13, 27–30], but it has been proposed, on the basis of experiments with the xanthine/xanthine oxidase system, that flavonoids scavenge the superoxide anion in particular [9].

In the present experiments the addition of pyrogallol created a pool of superoxide anion [19] at the moment when the response to ACh was stabilized. The resulting rapid inhibition of the relaxation to ACh was significantly attenuated in both arteries by both SOD and the flavonoid. The slight difference between control values of the inhibition (SOD versus flavonoid) in BAs (Fig. 4), probably due to the fact that two different series of rabbits were used for these experiments, is of no consequence since the controls and corresponding tests are obtained in the same arteries. The most obvious hypothesis that could explain the reduction in inhibition is that the scavenging properties of these two agents reduced the concentration of superoxide anion in the bath, allowing more EDRF/NO to reach and penetrate into the smooth muscle cells. Since the ACh responses were matched in controls and tests (see R values in Figs 3 and 4), up until the creation of the superoxide pool, it seems unlikely that SOD and the flavonoid could have acted upon the production of NO or on its effects in the smooth muscle. Since we did not actually measure superoxide concentrations, we cannot exclude other possible actions, such as some kind of binding of the NO, which is thus protected and released at the smooth muscle. A possible direct contractile effect of superoxide anion [31] seems of small significance since pyrogallol administered to arteries at basal tone had little or no contractile activity.

In the case of experiments without artificially elevated superoxide concentrations, the flavonoid was able to potentiate the responses to ACh in the EA at 10^{-5} M, but not in the BA. If, as the first set of experiments suggests, the flavonoid scavenges superoxide anion, the potentiation observed could be explained by a reduction in superoxide concentration. In this case, the difference between the two types of arteries might be due to greater diffusion distances for EDRF in the EA, more efficient endogenous free radical scavenging within the wall of the BA, or the release of a more resistant form of EDRF (a nitroso compound) in the BA [32, 33].

The flavonoid studied here appears to be a potentially useful tool for investigating phenomena involving free radicals, especially the superoxide anion (see Sies [34] for discussion). The present experiments suggest that it can protect nitric oxide from oxidative attack, especially at high levels of superoxide anion. Further work is required to elucidate definitively the mechanisms involved. Therapeutic applications of this and related flavonoids may be hoped for, especially if the problem of low water-solubility can be solved, while retaining a distinct degree of lipid-solubility useful for applications to the brain.

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