Oxygen-derived free radicals, endothelium, and responsiveness of vascular smooth muscle

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RUBANYI, G. M., AND P. M. VANHOUTTE. *Oxygen-derived free radicals, endothelium, and responsiveness of vascular smooth muscle.* Am. J. Physiol. 250 (Heart Circ. Physiol. 19): H815–H821, 1986.—Experiments were designed to determine the role of oxygen-derived free radicals in modulating contractions of vascular smooth muscle and endothelium-mediated relaxations to acetylcholine. The effects of generating or scavenging these radicals were studied in rings of canine coronary arteries suspended for isometric tension recording. Xanthine oxidase plus xanthine caused relaxations, which were greater in rings with endothelium than in rings without endothelium; the relaxations were not affected by superoxide dismutase or mannitol, but could be prevented by catalase. Xanthine oxidase plus xanthine depressed endothelium-mediated relaxations to acetylcholine; this effect was prevented by superoxide dismutase, but was not affected by catalase or mannitol. Exogenous hydrogen peroxide induced catalase-sensitive relaxations, which were depressed by the removal of the endothelium. Superoxide dismutase evoked catalase-sensitive relaxations only in rings with endothelium. Endothelium-mediated relaxations to acetylcholine were slightly depressed by superoxide dismutase or catalase alone; the combination of the two enzymes or mannitol caused a major shift to the right of the concentration-response curve to acetylcholine. In rings without endothelium, relaxations caused by sodium nitroprusside were not affected by the scavengers (alone or in combination) but were augmented by xanthine oxidase plus xanthine. These data suggest that *1*) the endothelium-derived relaxing factor released by acetylcholine is not likely to be an oxygen-derived free radical; *2*) hydrogen peroxide has a direct inhibitory action on coronary arterial smooth muscle and triggers endothelium-dependent relaxations; and *3*) superoxide anions depress and hydroxyl radicals facilitate endothelium-dependent relaxations caused by activation of muscarinic receptors.

acetylcholine; canine coronary artery; catalase; endothelium-dependent relaxations; hydrogen peroxide; hydroxyl radical; mannitol; sodium nitroprusside; superoxide anion; superoxide dismutase; xanthine oxidase

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THE COMPLETE REDUCTION of molecular oxygen to water involves the addition of four electrons that can occur univalently, resulting in a series of intermediates (oxygen-derived free radicals), or quadrivalently by the mitochondrial cytochrome oxidase system, which avoids these reactive intermediates (4, 8). The presence and ubiquity of enzymes (superoxide dismutase, catalase, and peroxidases) that scavenge the byproducts of the univalent pathway in aerobic cells suggest that superoxide anions and hydrogen peroxide are important byproducts of the oxidative metabolism. Actually, there are several pathways in aerobic cells leading to the production of oxygen-derived free radicals (4, 8). Important sources are enzymes associated with the metabolism of arachidonic acid, such as cyclooxygenase, lipoxygenase, and cytochrome P-450 (2, 4, 8). Superoxide anion radicals generated intracellularly or extracellularly by photolysis of water (8) or by xanthine oxidase in the presence of xanthine (1, 15), produce hydrogen peroxide by the dismutation reaction. This reaction can proceed spontaneously or can be catalyzed by superoxide dismutase (20). The hydrogen peroxide formed can be scavenged with catalase (1, 15). The superoxide anion radical and hydrogen peroxide can interact to generate the hydroxyl radical (1), which can be scavenged by mannitol (21) (Fig. 1).

Cultured endothelial cells produce both oxygen-derived free radicals and lipid free radicals (23). Vascular endothelial cells contain the enzymes cyclooxygenase, lipoxygenase (5, 14, 18), and cytochrome P-450 monooxygenases (31), and they produce prostaglandins, lipoxygenase, and epoxigenase intermediates from arachidonic acid (5, 14, 18). Anoxia, inhibitors of lipoxygenase, and cytochrome P-450, and inhibitors of the release of arachidonic acid from certain phosphatides, reduce the endothelium-mediated relaxation to acetylcholine of the thoracic aorta of the rabbit (11, 12, 30) and of the canine coronary (26) and femoral artery (5). This suggests that acetylcholine somehow activates a reaction sequence in vascular endothelial cells, in which arachidonic acid is liberated and then oxidized by lipoxygenases and/or cytochrome P-450 to a product that is the relaxing factor (5, 7, 12, 30). Furchgott and colleagues (12) speculated that the factor might be a labile hydroperoxide or even an oxygen-derived free radical. Inhibition of acetylcholine-induced relaxation with the potential free radical scavenger hydroquinone (11, 12), and by several antioxidant substances (7, 13, 25, 26), is consistent with these speculations. Indirect evidence in favor of the hypothesis that oxygen-derived free radicals contribute to endothelium-dependent vasodilatations comes from experiments on pial arterioles of the cat (16, 17, 24). These dilate in response to superoxide anion radical and other oxygen radicals derived from it (e.g., hydrogen peroxide or free hydroxyl radicals) (16, 17). The vasodilatation of cerebral arterioles induced by arachidonic acid and bradykinin

[both substances cause endothelium-dependent relaxation in several blood vessels (10)] were blocked by superoxide dismutase and catalase (16, 17).

The primary goal of the present experiments was to determine whether oxygen-derived free radicals themselves are the mediator(s) of endothelium-dependent relaxations to acetylcholine in canine coronary arteries. To do so, the effects of various scavengers of oxygen-derived free radicals were determined on contractions of isolated rings of coronary arteries with and without endothelium. In designing the experiments, the following assumptions were made (Fig. 1): *1*) the effect(s) of superoxide dismutase can be attributed to the elimination of superoxide anions and/or the increased generation of hydrogen peroxide; *2*) the effect(s) of catalase can be attributed to elimination of hydrogen peroxide; *3*) if combined treatment with superoxide dismutase and catalase has more pronounced effect(s) than treatment with either enzyme alone, both superoxide anions and hydrogen peroxide, or the production of free hydroxyl radicals may play a role; and *4*) the effects of mannitol can be attributed to scavenging of free hydroxyl radicals (Fig. 1).

METHODS

Experiments were performed on left circumflex coronary arteries taken from mongrel dogs of either sex (18–28 kg), anesthetized with pentobarbital sodium (30 mg/kg iv). The blood vessels were studied in modified Krebs-Ringer bicarbonate solution (control solution) of the following composition (mM): NaCl 118.3, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$, 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25.0, calcium disodium EDTA, 0.026; and glucose 11.1.

Rings (4 mm long) of coronary arteries were immersed in cold control solution. They were cleaned of fat and of loose connective tissue, with special care taken not to touch the luminal surface. In some preparations, the endothelium was removed mechanically (5, 11, 26).

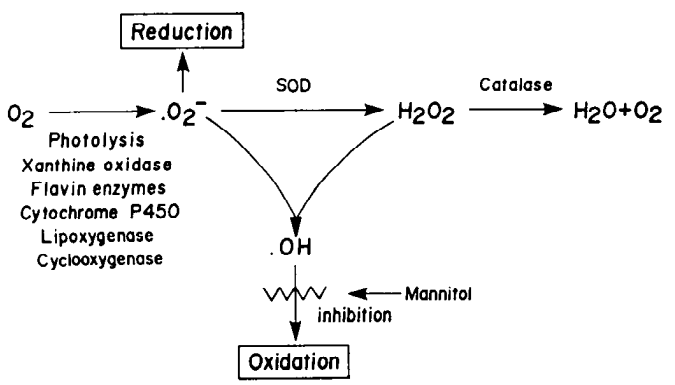

FIG. 1. Formation of O_2-derived free radicals. Superoxide anion ($\cdot O_2^-$) can be generated from molecular oxygen (O_2) by photolysis or by action of xanthine oxidase or of various cellular enzymes. Superoxide anion potentially can act as a reducing agent. It also spontaneously binds to protons to form H_2O_2; this dismutation reaction can be catalyzed by ubiquitous enzyme superoxide dismutase (SOD). Formed hydrogen peroxide can react with superoxide anions to yield highly reactive oxidizing hydroxyl radical ($\cdot OH$). H_2O_2 can be scavenged by another ubiquitous enzyme, catalase. $OH\cdot$ can be scavenged by mannitol (1, 2, 3, 7, 12, 17, 19).

The rings were mounted horizontally in organ chambers filled with 25 ml of control solution (37°C, gassed with 95% O_2-5% CO_2; pH 7.4). The preparations were attached to a strain gauge (Gould UTC 2), and isometric tension was recorded. The rings were progressively stretched to optimal tension (~10 g), determined by repeated stimulation with KCl (2×10^{-2} M). They were allowed to equilibrate for 30 min before experimentation.

Paired rings (with and without endothelium) from the same artery were studied in parallel. The rings were contracted with prostaglandin $F_{2\alpha}$ (2×10^{-6} M), and the presence or absence of functional endothelium was confirmed by demonstrating the presence and absence, respectively, of relaxations induced by acetylcholine (5, 11, 26).

Drugs

The following pharmacological agents were used (all from Sigma): acetylcholine hydrochloride, catalase (from bovine liver, 40,000 U/mg protein), indomethacin D-mannitol, prostaglandin $F_{2\alpha}$ superoxide dismutase (from dog blood; 3,000 U/mg protein), xanthine, and xanthine oxidase (from buttermilk; 0.52 U/mg protein). All drugs were prepared in distilled water, except indomethacin, which was solubilized in a Na_2CO_3 (1.5×10^{-3} M) solution with sonication, and added to the 25-ml bath in volumes of 146 μl or less. Concentrations are expressed as final concentrations (M or U/ml) in the bath solution. The concentrations of various drugs used in the study were chosen on the basis of preliminary concentration-response studies (e.g., superoxide dismutase), by measuring the specific scavenging function (e.g., prevention by catalase of the effects of hydrogen peroxide), or from the literature (e.g., xanthine oxidase plus xanthine).

Calculations and Statistics

The shift in concentration-response curves to acetylcholine or sodium-nitroprusside caused by the various scavengers or xanthine oxidase plus xanthine was analyzed by the change in the concentration of the agonist inducing 50% (ED_{50}) of the maximal response in the individual tissues. The ED_{50} values are expressed as the negative logarithm of the molar concentration of the agonist. Unless otherwise noted, each experimental group consisted of at least six blood vessels taken from different dogs. The data are shown as means ± standard error of the mean (SEM). Statistical analysis was performed using Student's *t* test for paired and unpaired observations. Differences were considered to be statistically significant when $P < 0.05$.

RESULTS

All experiments were performed on coronary arteries contracted with prostaglandin $F_{2\alpha}$ (2×10^{-6} M) in the presence of indomethacin (10^{-5} M).

Superoxide dismutase, catalase, and mannitol. In arteries with endothelium, superoxide dismutase (150 U/ml) caused relaxations that were reduced or prevented by catalase (1,200 U/ml). In arteries without endothelium,

superoxide dismutase caused significant increases in tension that were not affected by catalase (Table 1).

Catalase (1,200 U/ml) did not cause significant changes in tension in arteries with or without endothelium (Table 1).

Mannitol (80 mM) induced endothelium-independent transient relaxations (Fig. 2; Table 1).

Xanthine oxidase and xanthine. Xanthine (10^{-4} M) did not cause significant changes in tension. Xanthine oxidase (0.1 U/ml) induced transient relaxations followed by increases in tension above the initial contractile response to prostaglandin $F_{2\alpha}$ (Fig. 2). The addition of xanthine in the presence of xanthine oxidase caused transient relaxations, the nadir of which was statistically significantly larger in rings with endothelium than in those without endothelium (Fig. 3). In rings with endothelium, superoxide dismutase (150 U/ml) and mannitol (80 mM) did not affect the relaxations in response to xanthine oxidase plus xanthine (Fig. 2); similar observations were made in rings without endothelium (data not shown). Catalase (1,200 U/ml) prevented the relaxations induced by xanthine oxidase plus xanthine, both in the absence and in the presence of endothelium (Fig. 2). In the presence of catalase, xanthine oxidase plus xanthine augmented contractions in rings with, but not in those without endothelium (Fig. 3).

Hydrogen peroxide. Hydrogen peroxide (8×10^{-7} to 2.4×10^{-5} M) caused concentration-dependent relaxations that were abolished by catalase (1,200 U/ml; 5 min incubation). Removal of the endothelium caused a significant shift to the right of the concentration-response curve to hydrogen peroxide (Fig. 4).

Acetylcholine. Five rings with endothelium from the same coronary artery were studied in parallel. After they were contracted with prostaglandin $F_{2\alpha}$, they were exposed to increasing concentrations (10^{-9} to 3×10^{-6} M) of acetylcholine in control solution or in the presence of either superoxide dismutase (150 U/ml), catalase (1,200 U/ml), the combination of superoxide dismutase plus catalase, or mannitol (80 mM) (5 min preincubation). Superoxide dismutase and catalase alone caused a moderate but significant shift to the right of the concentration-response curve to acetylcholine. The combination of superoxide dismutase and catalase also induced a significant rightward shift of the concentration-response curve to acetylcholine. A similar shift to the right of the dose-response curve to acetylcholine caused by mannitol was not significantly different from that observed with the combination of superoxide dismutase and catalase (Table 2).

Rings with endothelium were exposed to increasing concentrations of acetylcholine in the absence of and in the presence of the combination of xanthine oxidase (0.1 U/ml) plus xanthine (10^{-4} M). The combination shifted the concentration-response curve to acetylcholine to the right and significantly depressed the maximal relaxation induced by the cholinergic transmitter (Figs. 2 and 5; Table 2). Catalase (1,200 U/ml) and mannitol (80 mM) did not significantly affect, but superoxide dismutase (150 U/ml) prevented the inhibitory effect of xanthine oxidase plus xanthine on the response to acetylcholine (Figs. 2 and 5; Table 2).

Sodium nitroprusside. Sodium nitroprusside (10^{-9} to 10^{-7} M) caused concentration-dependent relaxations in arteries without endothelium (ED_{50}: 7.74 ± 0.30). These relaxations were not significantly altered by superoxide dismutase (150 U/ml), catalase (1,200 U/ml), the combination of superoxide dismutase plus catalase, or mannitol (80 mM); they were significantly augmented after 10–15 min incubation with the combination of xanthine oxidase (0.1 U/ml) plus xanthine (10^{-4} M) (ED_{50}: 8.42 ± 0.27; $P < 0.05$). See Fig. 6.

DISCUSSION

The present study demonstrates that oxygen-derived free radicals may have a direct inhibitory action on coronary vascular smooth muscle, and, depending on their chemical identity, either facilitate or inhibit endothelium-mediated responses.

Vascular Smooth Muscle

Xanthine oxidase generates superoxide anions and other oxygen-derived free radicals (9, 15) in the presence of xanthine. This combination inhibits the contractile process in vascular smooth muscle, as evidenced by the relaxations it causes in rings without endothelium. Generation of free radicals by electrical field stimulation also causes relaxation of the canine coronary artery (19). In the present study, hydrogen peroxide, but not superoxide anions, must play the major role in the inhibitory effect induced by xanthine oxidase plus xanthine. This interpretation is based on the demonstration that exogenous hydrogen peroxide relaxes rings without endothelium and that catalase, but not superoxide dismutase, abolishes the relaxations induced by xanthine oxidase plus

TABLE 1. *Effect of oxygen-derived free radical scavengers on canine coronary arteries*

Preparation	*n*	Contraction to Prostaglandin $F_{2\alpha}$, 2×10^{-6} M, g	Superoxide Dismutase, 150 U/ml, %	Catalase, 1,200 U/ml, %	Superoxide Dismutase plus Catalase, %	Mannitol, 80 mM, %
With endothelium	6	5.5±0.5	−25.7±6.4*	−1.7±4.6	−12.8±6.5	−30.2±6.5*†
Without endothelium	3	6.7±0.5	+10.7±1.8*	−4.0±8.3	+10.3±2.2*	−34.2±3.6*†

Values are means ± SEM. Results are expressed in absolute changes in tension (g) for contractions to prostaglandin $F_{2\alpha}$ and in percent changes in tension (%) for effects of scavengers (+, contraction; −, relaxation). * The difference from control (contractions induced by prostaglandin $F_{2\alpha}$) is statistically significant ($P < 0.05$; Student's *t* test for paired observations). † The effect of mannitol was transient. The response shown is the nadir. Within 10 min after the administration of mannitol, tension had returned to a level not significantly different from control.

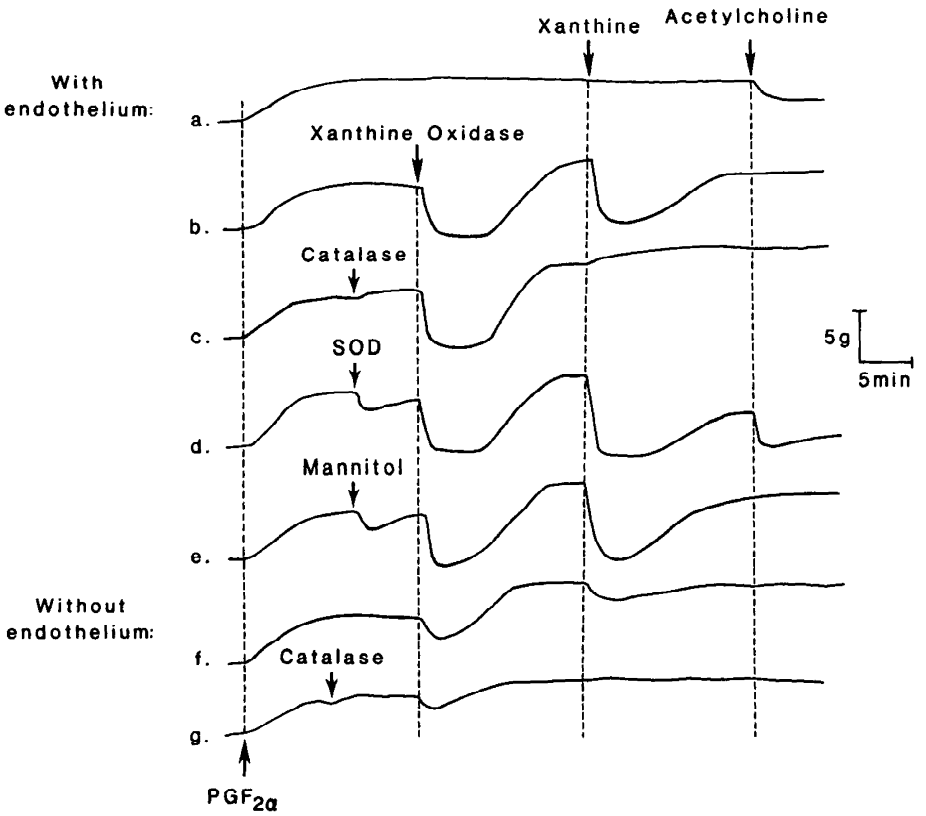

FIG. 2. Effect of xanthine (10^{-4} M) and xanthine oxidase (0.1 U/ml), alone and in combination, on contractions to prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$, 2×10^{-6} M) and relaxation to acetylcholine (10^{-7} M) in canine coronary artery rings with (*a–e*) or without (*f*, *g*) endothelium in absence and presence of various scavengers of O_2-derived free radicals [see also Fig. 1; catalase, 1,200 U/ml; superoxide dismutase (SOD), 150 U/ml; mannitol, 80 mM].

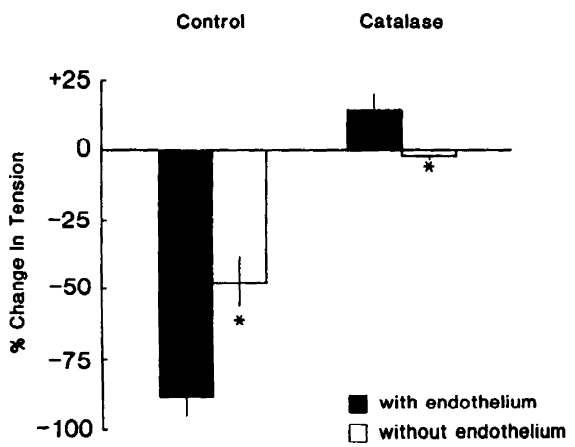

FIG. 3. Effect of catalase (1,200 U/ml, 5 min incubation) on relaxations induced by xanthine oxidase (0.1 U/ml) plus xanthine (10^{-4} M) in canine coronary artery rings with or without endothelium. Rings were contracted with prostaglandin $F_{2\alpha}$ (2×10^{-6} M) in presence of indomethacin (10^{-5} M). Data are expressed as % of initial contraction (100% = 5.1 ± 0.8 and 5.5 ± 0.7 g, respectively, in rings with and without endothelium) and shown as means ± SE, $n = 5$. * Difference between responses observed in rings with and without endothelium is significant ($P < 0.05$).

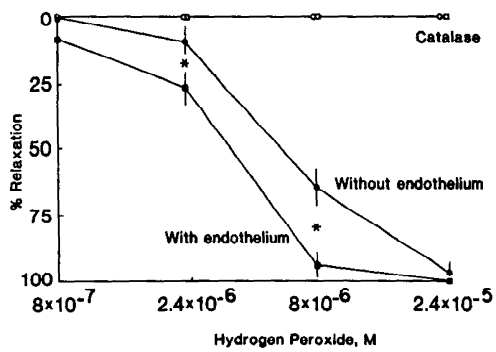

FIG. 4. Concentration-response curves to H_2O_2 in canine coronary arteries contracted with prostaglandin $F_{2\alpha}$ (2×10^{-6} M). Symbols: *filled squares*, rings with endothelium; *filled circles*, rings without endothelium; *open circles*, *open squares*, rings treated with catalase (400 U/ml, 5 min incubation). Data expressed as % of initial contraction to prostaglandin $F_{2\alpha}$ (100% = 5.7 ± 1.2 and 6.2 ± 1.7 g, respectively, in rings with and without endothelium) and shown as means ± SE ($n = 3$). * Difference between rings with and without endothelium is statistically significant ($P < 0.05$).

xanthine. If superoxide anions were to exert a direct inhibitory action on coronary vascular smooth muscle, the combination of xanthine oxidase and xanthine should have caused relaxations in the presence of catalase as well, since this enzyme does not decrease the concentration of generated superoxide anions but only that of hydrogen peroxide produced from them via the dismutation reaction (1, 4, 15).

In contrast to the catalase-sensitive inhibitory action of xanthine oxidase plus xanthine, the transient relaxations induced by xanthine oxidase alone were not sensitive to any of the free radical scavengers used in the study. Thus the action of the enzyme alone is not mediated by oxygen-derived free radicals. The augmentation by the combination xanthine oxidase plus xanthine, of the relaxations to sodium nitroprusside (in contrast to the inhibitory effect when given separately), was not influenced by catalase, indicating that a different mech-

anism may be responsible for the two events. The augmentation of the relaxation evoked by sodium nitroprusside may be due to the facilitation of the relaxing action of the nitro-vasodilator, but the possibility cannot be ruled out that nitroprusside and xanthine-xanthine oxidase are causing relaxation by different mechanisms.

Although superoxide dismutase augmented contractile responses in rings without endothelium, this cannot be due to the removal of superoxide anions (since the relaxations evoked by the combination of xanthine oxidase and xanthine were not prevented by superoxide dismutase), or to facilitation of the production of hydrogen peroxide (since the contractile response to superoxide dismutase was not sensitive to catalase). Thus it may reflect a nonspecific direct action of the enzyme on coronary vascular smooth muscle.

TABLE 2. *Effect of oxygen-derived free radical scavengers and xanthine oxidase plus xanthine on ED_{50} values and maximal responses to acetylcholine in canine coronary arteries*

	ED_{50}	Maximal Response
Control I	7.25±0.13	98.8±2.5
Superoxide dismutase, 150 U/ml	7.07±0.10*	95.3±2.6
Catalase, 1,200 U/ml	6.99±0.08*	97.1±2.9
Superoxide dismutase + catalase	6.88±0.10*	88.5±6.7
Mannitol, 80 mM	6.77±0.15*	79.9±10.2
Control II	6.90±0.09	98.3±1.7
Xanthine oxidase, 0.1 U/ml + xanthine, 10^{-4} M	6.03±0.22*	73.0±8.0*
Superoxide dismutase + xanthine oxidase + xanthine	6.82±0.09	100±0
Catalase + xanthine oxidase + xanthine	6.12±0.20*	88.8±7.0
Superoxide dismutase + catalase + xanthine oxidase + xanthine	6.55±0.22	97.7±2.3
Mannitol + xanthine oxidase + xanthine	5.93±0.22*	57.5±9.5*

Values are means ± SEM; $n = 6$ of Control I group, and $n = 4$ of Control II group. ED_{50}, negative logarithm of acetylcholine concentration (M) inducing 50% relaxation of prostaglandin $F_{2\alpha}$-induced contraction. Maximal response expressed as % relaxation of contractions caused by prostaglandin $F_{2\alpha}$ (2×10^{-6} M) in response to highest concentration (3×10^{-6} to 10^{-5} M) of acetylcholine that did not cause further relaxation. * Difference from control is statistically significant ($P < 0.05$).

Endothelium-Dependent Responses

Under our experimental conditions, superoxide anions and other oxygen-derived free radicals must be produced either extracellularly by photolysis (21) or intracellularly during various enzymatic oxidative reactions (2, 4, 8) initiated by stimulation with acetylcholine, as evidenced by the significant effects of various scavengers on endothelium-mediated responses. To ensure that the observed endothelium-dependent relaxations were due to endothelium-derived relaxing factor(s) and not to prostacyclin, the experiments were performed in the presence of an inhibitor of cyclooxygenase. This might have excluded one potential intracellular source of free radical generation (4, 8), but without using a cyclooxygenase inhibitor we would not be able to analyze the effect of free radical scavengers on the production and action on smooth muscle of a nonprostaglandin endothelium-derived relaxing factor. However, since treatment with inhibitors of cyclooxygenase (including indomethacin) has no significant effect on endothelium-dependent relaxations to acetylcholine in a variety of blood vessels (including the canine coronary artery) (see Ref. 12), a major influence of free radicals generated by this metabolic pathway for arachidonic acid on endothelium-mediated relaxations can be excluded.

Hydrogen peroxide. The combination of xanthine oxidase and xanthine caused larger relaxation in rings with than in those without endothelium. A similar endothelium-dependent component was observed during relaxa-

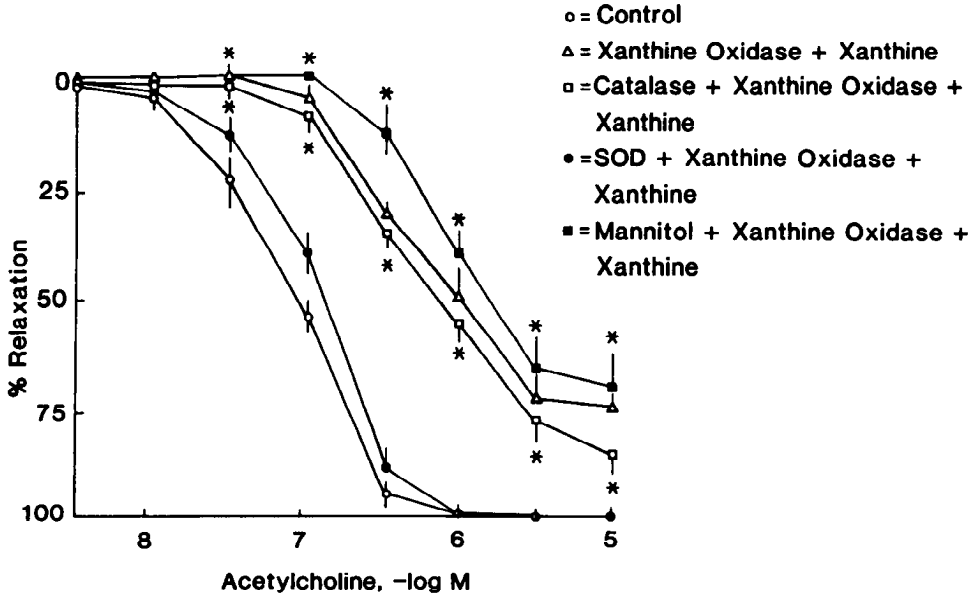

FIG. 5. Effect of combination of xanthine oxidase (0.1 U/ml) plus xanthine (10^{-4} M) (10–15 min incubation) on relaxations of canine coronary arteries with endothelium caused during contractions to prostaglandin $F_{2\alpha}$ (2×10^{-6} M) by increasing concentrations of acetylcholine (the total time required to generate the dose-response curves varied between 20 and 30 min), in the absence and presence of free radical scavengers (5 min incubation). Symbols and initial contractions (g) to prostaglandin $F_{2\alpha}$: *open circles*, control solution (5.3 ± 1.0); *open triangles*, xanthine oxidase plus xanthine (XO + X) (3.1 ± 1.3); *open squares*, XO + X in the presence of catalase (1,200 U/ml) (6.6 ± 2.0); *filled circles*, XO + X in presence of superoxide dismutase (150 U/ml) (5.1 ± 2.4); *filled squares*, XO + X in presence of mannitol (80 mM) (3.9 ± 1.2). Data expressed as % of initial contraction to prostaglandin $F_{2\alpha}$ and shown as means ± SE ($n = 4$). * Difference from control is statistically significant ($P < 0.05$).

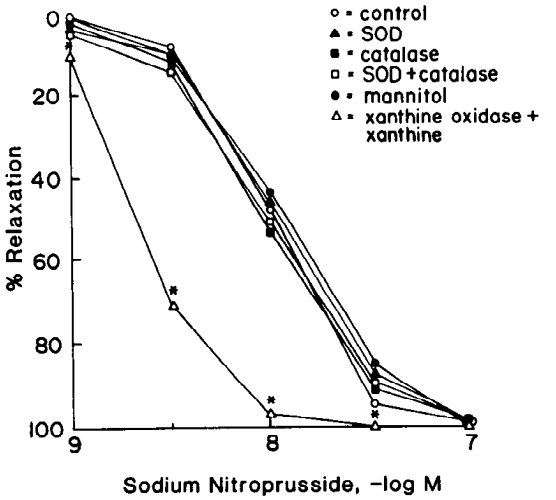

FIG. 6. Relaxations of canine coronary arteries without endothelium caused by increasing concentrations of sodium nitroprusside (total time required to generate dose-response curve varied between 15 and 25 min) during contractions evoked by prostaglandin $F_{2\alpha}$ (2×10^{-6} M). Symbols: *open circles*, control solution ($n = 6$); *filled triangles*, superoxide dismutase (150 U/ml; 5 min incubation; $n = 6$); *filled squares*, catalase (1,200 U/ml; 5 min incubation; $n = 6$); *open squares*, superoxide dismutase plus catalase ($n = 6$); *filled circles*, mannitol (80 mM; 5 min incubation; $n = 6$); *open triangles*, xanthine oxidase (0.1 U/ml) plus xanthine (10^{-4} M; 10–15 min incubation; $n = 4$). Data expressed as % of initial contraction to prostaglandin $F_{2\alpha}$ (100% = 6.6 ± 0.8 g) and shown as means; for sake of clarity SE were omitted. * Difference from control is statistically significant ($P < 0.05$).

tions induced by exogenous hydrogen peroxide. Catalase abolished the endothelium-dependent responses both to xanthine oxidase plus xanthine and to hydrogen peroxide. These observations suggest that hydrogen peroxide, in addition to a direct depression of the contractions of vascular smooth muscle, also initiates the release of endothelium-derived relaxing factor(s). This conclusion is strengthened by the demonstration that superoxide dismutase evokes catalase-sensitive endothelium-dependent relaxation. These observations are explained best if under our experimental conditions there were a continuous generation of small amounts of superoxide anions, which do not trigger the release of endothelium-derived relaxing factor except after accelerated transformation to hydrogen peroxide by superoxide dismutase (20).

Hydroxyl radicals. The lack of protective effect of superoxide dismutase (or mannitol) on relaxations induced by xanthine oxidase plus xanthine in rings with endothelium suggests that, under these conditions, either free hydroxyl radicals are not generated or they do not trigger the production and release of endothelial relaxing factor(s), the latter explanation seems the most likely.

The present study confirms earlier observations (5, 6, 10–13, 25, 26) that acetylcholine causes endothelium-dependent relaxation. It demonstrates that free oxygen radicals generated during univalent reduction of molecular oxygen are not the endothelium-derived relaxing factor(s) released by acetylcholine (29). Indeed, none of the free radical scavengers used in this study prevented acetylcholine-induced relaxations of canine coronary artery rings with endothelium. However, the present experiments suggest that hydroxyl radicals facilitate the relaxation caused by acetylcholine. This conclusion is prompted by the inhibitory effects on acetylcholine-induced relaxations of either superoxide dismutase or catalase and by the more pronounced inhibitory effect of the combination of both enzymes or of mannitol, which scavenges hydroxyl radicals (9, 21). The transient, endothelium-independent decreases in tension caused by mannitol are probably due to an increase in osmolality (22).

Superoxide anions. That superoxide anions depress endothelium-mediated relaxations to acetylcholine is obvious in intact rings only when it is produced in excess, as is the case during exposure to xanthine oxidase plus xanthine. Indeed, the latter combination markedly inhibits relaxations to acetylcholine in the rings with endothelium; in the presence of catalase, it induces endothelium-dependent contractions (probably due to inactivation of the relaxing factor(s) released under basal conditions; 14, 28). That superoxide anions are responsible for the inhibition is suggested by the observation that superoxide dismutase (but not catalase or mannitol) prevents the depression of the relaxations to acetylcholine by xanthine oxidase plus xanthine. The inhibitory effect of the superoxide anion is not obvious under control conditions (29; this study), which implies that very small amounts of it are produced under such conditions. Although the present study does not rule out the possibility that superoxide anions depress the relaxation because they break down acetylcholine, bioassay experiments indicate that these oxygen-derived free radicals can inactivate the endothelium-derived relaxing factor after its release from endothelial cells (28).

In addition to their direct depressing action on vascular smooth muscle, oxygen-derived free radicals can have opposing effects on endothelium-dependent responses in canine coronary arteries. Hydrogen peroxide triggers the release of endothelium-derived relaxing factor(s), and free hydroxyl radicals facilitate its liberation by acetylcholine. By contrast, superoxide anions depress endothelium-dependent responses to acetylcholine.

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