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Catechol Adrenergic Agents Enhance Hydroxyl Radical Generation in Xanthine Oxidase Systems Containing Ferritin: Implications for Ischemia/Reperfusion

D. R. Allen,*,†,[1] G. L. Wallis,* and P. B. McCay*

**Free Radical Biology and Aging Research Program, Oklahoma Medical Research Foundation, Oklahoma City, Oklahoma 73104; and †Pulmonary Medicine and Critical Care Section, Department of Medicine, University of Oklahoma, Oklahoma City, Oklahoma 73109*

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Iron chelators have been reported to protect tissues against reperfusion injury. This implies that iron is being released into the plasma or is made accessible in tissues for oxidation–reduction reactions. It has been postulated that ferritin is a likely source for this iron. This report demonstrates that adrenergic agents with the catechol structure, which includes the endogenous catecholamines norepinephrine and epinephrine, are capable of releasing iron from ferritin. It is shown that the net release of iron from ferritin by epinephrine is significantly enhanced under anaerobic conditions. The findings suggest that catecholamines can mediate iron release from ferritin under conditions that can occur during ischemia/reperfusion. Catecholamines are also shown to interact with the released iron and xanthine oxidase to produce highly reactive hydroxyl radicals. The implications of this interaction for ischemia/reperfusion are discussed.

Key Words: **ferritin; iron; catecholamines; ischemia; free radicals; xanthine oxidase.**

Iron chelators have been shown to be effective in ameliorating the damage produced by reperfusion after ischemia (1–9). Normally there is no detectable ionized iron free in the plasma. This is clearly a necessary condition due to iron's electron configuration which renders it capable of readily participating in redox reactions under biological conditions (10). The physiological systems which transport and store iron carry out these functions with the iron remaining bound to proteins which suppress the iron's redox activity (11).

[1] To whom correspondence should be addressed at 825 NE 13th Street, Mail Stop 21, Oklahoma City, Oklahoma 73104. Fax (405) 271-1795.

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In order for iron chelators to exhibit protection against tissue injury caused by ischemia/reperfusion, it is presumed that iron had been released or made accessible for redox reactions by a metabolic process that was perturbed by ischemia/reperfusion. However, the mechanism of iron release by ischemia/reperfusion has not been clearly defined. The sources from which the iron appears to be released during ischemia/reperfusion have not been identified, but ferritin has been considered to be one likely candidate (12).

Ferritin contains the largest pool of readily available iron in the body, storing, on average, 2500 mol of iron per mole of apoprotein. It is ubiquitous, being found in both the extracellular and intracellular compartments (13). Additionally, it is an acute phase reactant whose concentration in plasma increases in response to physiologic stress (14).

Within the ferritin polymer, iron is bound as Fe^{+3} by a ferrioxidase activity of the protein moiety and can be released only by reduction again to Fe^{+2}. Under biological conditions, only a small fraction of the total iron that is stored by ferritin can be released. Winterbourne, Aust, and others have shown that various drugs with *ortho* hydroxyl groups can release iron from ferritin (15–21), which, in the presence of microsomes, can initiate lipid peroxidation.

Epinephrine and norepinephrine are endogenously occurring adrenergic agents that contain the catechol function consisting of *ortho* hydroxyl groups on a benzene ring. Under conditions of stress, catecholamines are secreted by the adrenal glands, leading to increased levels in the plasma. In addition, catecholamines are administered clinically to support the blood pressure and cardiac function of patients in shock.

In the case of ischemic insults to the myocardium, Wollenberger demonstrated that anoxia and ischemia are

two very potent stimuli for the release of norepinephrine from adrenergic neurons associated with the myocardium (22–24). It has been estimated that there is a 2000-fold increase in the catecholamine concentration in ischemic zones of the myocardium (25). During the ischemic period, xanthine dehydrogenase is converted to xanthine oxidase, and oxidative phosphorylation is restricted, leading to ATP depletion. The latter results in an accumulation of xanthine and other purine degradation products that are substrates for xanthine oxidase (26). Superoxide anion, a product of the enzymatic activity of xanthine oxidase, has been shown previously to reductively release iron from ferritin under aerobic conditions (27). Xanthine oxidase has also been demonstrated to have a superoxide-*independent* mechanism that is also capable of reducing and releasing iron from ferritin, presumably via electron transfer from the molybdenum center to ferritin by a process that does not involve intermediate transfer of electrons to oxygen. Such a mechanism could produce iron release from ferritin under anaerobic conditions (27–31).

During reperfusion of ischemic tissue, as oxygen becomes available, the elevated level of xanthine oxidase activity and the increased degradation of ATP to hypoxanthine result in the potential for a significant production of superoxide and H_2O_2. Under conditions where ferrous iron is being released, H_2O_2 would react via Fenton chemistry, resulting in the formation of hydroxyl radicals and other highly reactive oxidizing species (32, 33). These reactive species have been implicated in the pathogenesis of ischemia/reperfusion injury by virtue of the protective effects of prior treatment with antioxidants, being specifically identified by spin trapping with ESR spectroscopy, or implicated by their ability to hydroxylate phenylalanine and other compounds *in vivo* (32–35).

In view of these findings, we hypothesized that during myocardial ischemia followed by reperfusion, the increased levels of endogenous catecholamines, especially the norepinephrine released in the ischemic zone, along with the elevated activity of xanthine oxidase might produce a significant enhancement of iron release from ferritin, and subsequently result in the generation of a significant flux of reactive oxygen radicals. To examine this hypothesis spin trapping studies were performed to investigate free radical generation resulting from interactions among adrenergic agents, ferritin, and a xanthine oxidase system. Quantitative measurements of iron were also carried out using bathophenanthrolene to determine the extent of iron release from ferritin in these systems under both aerobic and anaerobic conditions.

METHODS

All glassware was acid-washed before use to remove any traces of contaminating iron. Phosphate buffer, NaCl solution, and deionized water were treated with Chelex resin (Bio-Rad, St. Louis, MO) to remove adventitious iron. Phenylephrine, metaproterenol, norepinephrine, isoproterenol, epinephrine, 6-hydroxydopamine, Type I ferritin from horse spleen, hypoxanthine, xanthine oxidase, 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO)[2] and superoxide dismutase were obtained from Sigma Chemical (St. Louis, MO). The adrenergic agents were dissolved in double-distilled deionized water in 10-ml volumetric flasks after the initial addition of 0.1 ml of 2 N HCl to solubilize and prevent spontaneous oxidation of the compounds by lowering the pH. DMPO was charcoal-filtered to remove any trace contaminants that produce an ESR signal and prepared as a 100 mM solution. Type I ferritin was diluted in phosphate/NaCl buffer. Hypoxanthine was dissolved in double-distilled deionized water in a 10-ml volummetric flask after the initial addition of 0.1 ml of 10 N NaOH to solubilize the compound. Desferoximine (Ciba-Geigy, Summit, NJ) and catalase (Worthington Diagnostic Systems, Freehold, NJ) were diluted in buffer. Ethanol (Quantum Chemical Corporation, Tuscola, IL) was diluted in double-distilled deionized water.

Spin Trapping Studies

The basic system consisted of 0.1 M phosphate buffer and 0.09 M NaCl at pH 7.4, hypoxanthine (0.4 mM), xanthine oxidase (0.1 unit/ml solution), and ferritin equivalent to 1 mM total iron content. The final concentration of the other components added to the basic system were as follows: adrenergic agents, 0.4 mM; catalase, 500 units/ml; SOD, 100 units/ml; desferoximine, 0.1 mM; and ethanol, 100 mM. The spin trapping agent, DMPO, was added to these systems at a final concentration of 40 mM. All reaction systems had a final volume of 1.0 ml and were placed in a shaking incubator at 37°C, in room air for 10 min, and then transferred to a quartz ESR flat cell and scanned in a Bruker 300e ESR spectrometer with the following settings: microwave frequency, 9.78 GHz; microwave power, 2×10^1 mW; modulation frequency, 100 Khz; modulation amplitude, 1.053 gauss; time constant, 164.34; sweep time, 83.89 s; center field, 3480 gauss; sweep width, 100 gauss. Each sample was scanned four times with accumulation to decrease noise. The gain ranged from 2.5×10^5 to 1×10^6. Student's *t* test was used to determine the statistical significance of the differences in values between conditions.

Iron Measurements

Ferrous iron release from ferritin was determined in reaction systems under both aerobic and anaerobic conditions. The aerobic iron release studies were done using 2.0-ml systems with the same components as those described above except that DMPO, which had no effect upon iron release under the experimental conditions, was omitted and 1.5 ml of 0.04% disulfonic bathophenanthrolene was added as an indicator for ferrous iron. These systems were incubated in an oscillating waterbath at 37°C for 30 min followed by determination of the absorbance of the bathophenanthrolene-Fe^{+2} complex at 536 nm. The absorbance was measured against a duplicate system with no bathophenanthrolene to correct for adrenochrome formation. The concentration of ferrous iron released was determined by comparing the absorbance of each sample to a standard curve produced by reacting known Fe^{+2} concentrations with bathophenanthrolene.

Anaerobic studies were performed in sealed 50-ml flasks with a side arm connected to the main chamber. The xanthine oxidase and/or the adrenergic agents were placed into the side arm portion of the flask to keep those components separate from the ferritin and the hypoxanthine in the main chamber during the time the systems were being made anaerobic. The contents of the main chamber of the flasks were continuously stirred with a Teflon-coated magnet during deoxygenation. Oxygen was depleted to a very low level by purging the flasks with argon for 4 min, followed by imposing a vacuum for 2 min with a subsequent 15-s purging with argon. This sequence was repeated two more times. At the end of this deoxygenating procedure, the reaction was started by tipping the contents of the side arm into the main chamber of the flask.

[2] Abbreviations used: DMPO, 5,5-dimethyl-1-pyrroline-*N*-oxide; SOD, superoxide dismutase.

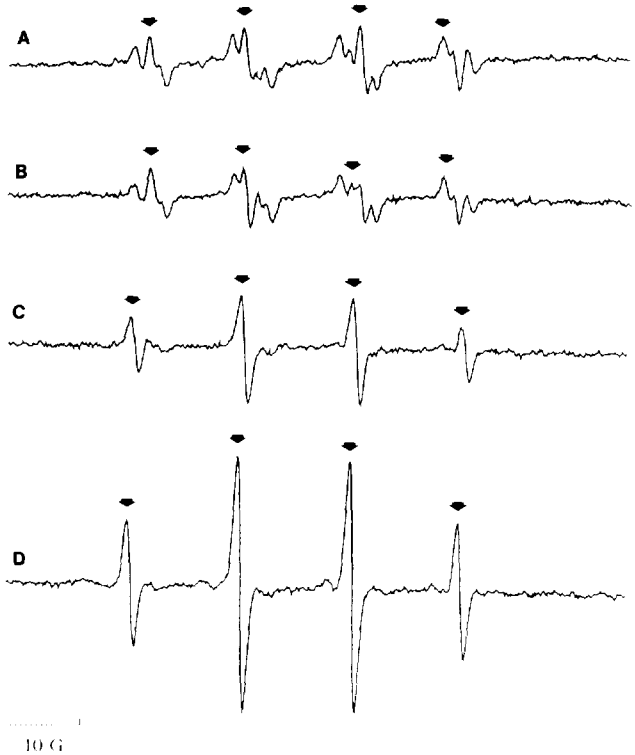


FIG. 1. ESR spectra generated by xanthine oxidase systems under aerobic conditions with the addition of ferritin and epinephrine. ESR scans were done on systems composed of xanthine oxidase (0.1 units/ml) and hypoxanthine (0.4 mM) and the indicated additions. Reactions were carried out at pH 7.4 in 0.1 M phosphate buffer containing 0.09 M NaCl. All systems contained DMPO (40 mM) and were incubated for 10 min at 37°C and then scanned in an ESR spectrometer at room temperature. All spectra are standardized to the same gain. The spectrum produced in the basic system containing hypoxanthine and xanthine oxidase (A) is compared to that produced by the basic system with the addition of (B) ferritin (1 mM total iron content), (C) epinephrine (0.4 mM), or (D) both ferritin and epinephrine. The arrows designate the peaks of the spectrum of the hydroxyl–DMPO adduct. In A and B, the spectrum of the hydroxyl–DMPO adduct is superimposed upon the spectrum of the superoxide–DMPO adduct.

These systems were incubated anaerobically for 30 min at 37°C in an oscillating water bath, and then their absorbance was determined as described for the aerobic systems. Results obtained were compared using Student's *t* test to determine the statistical significance of differences in values. Comparisons were made between groups of results using Duncan's multiple comparison test.

RESULTS

Free Radical Generation Produced by the Addition of Adrenergic Agents and Ferritin to the Xanthine Oxidase System under Aerobic Conditions

Figure 1A shows that under aerobic conditions the basic system consisting of xanthine oxidase, its substrate, hypoxanthine, and DMPO produced an ESR signal that could be assigned primarily to the superoxide anion adduct of DMPO. The spectrum also contained a smaller component of the DMPO adduct of the hydroxyl radical. Addition of ferritin to the basic xanthine oxidase system (Fig. 1B) had no influence on the EPR signal that was observed. The addition of epinephrine (Fig. 1C) to the basic system resulted in the enhancement of the hydroxyl adduct by approximately twofold and loss of the superoxide radical signal. However, the combined addition of both epinephrine and ferritin resulted in hydroxyl radical generation that was twice that observed upon the addition of epinephrine alone (Fig. 1D). The addition of 100 mM ethanol to each of these systems (Figs. 2A–2D) resulted in production of the 1-hydroxyethyl adduct and a corresponding decrease in the hydroxyl adduct in each of the systems consistent with the scavenging of hydroxyl rad-

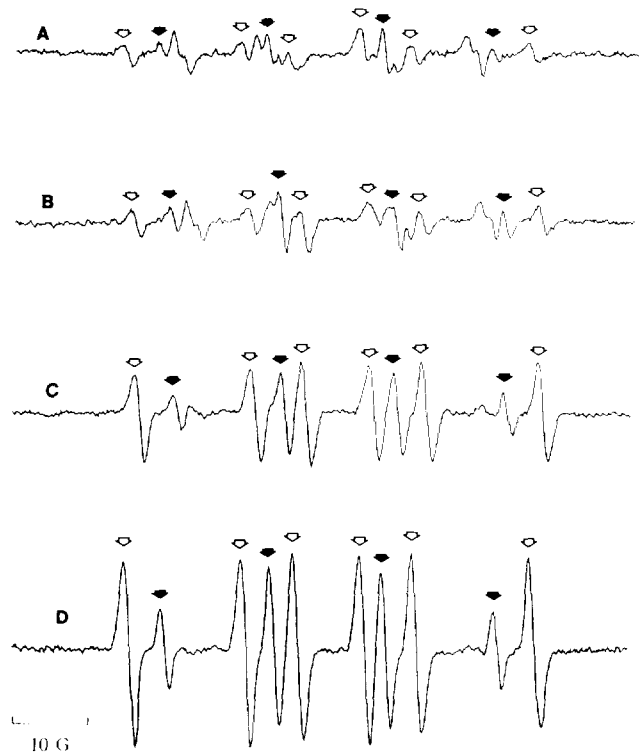


FIG. 2. ESR spectra generated by xanthine oxidase systems containing ethanol under aerobic conditions with the addition of ferritin and epinephrine. ESR scans were done on systems composed of xanthine oxidase (0.1 units/ml), hypoxanthine (0.4 mM), and ethanol (100 mM) along with the indicated additions. Reactions were carried out at pH 7.4 in 0.1 M phosphate buffer containing 0.09 M NaCl. All systems contained DMPO (40 mM) and were incubated for 10 min at 37°C and then scanned in an ESR spectrometer at room temperature. All spectra are standardized to the same gain. The spectrum produced in the basic system (consisting of hypoxanthine and xanthine oxidase) to which 100 mM ethanol was added (A) is compared to that produced by the same system with the further addition of (B) ferritin (1 mM total iron content), (C) epinephrine (0.4 mM), or (D) both ferritin and epinephrine. The filled arrows designate the peaks of the spectrum of the hydroxyl–DMPO adduct; the open arrows designate the spectrum of the 1-hydroxy-ethyl–DMPO adduct. In A and B, both the spectra of the hydroxyl–DMPO adduct and the 1-hydroxy-ethyl–DMPO adduct are superimposed upon the spectrum of the superoxide–DMPO adduct.

icals by ethanol and demonstrating that the spectra in Fig. 1 are due to the trapping by DMPO of authentic hydroxyl radical. Ethanol was added in a similar fashion to every system in which hydroxyl radical was detected in order to confirm authentic hydroxyl radical production. The spectra for the latter experiments are not included in the data presentation.

Figure 3 displays the spectra produced in a set of experiments performed under identical conditions excepting that a noncatechol adrenergic agent, phenylephrine, was added instead of epinephrine. In contrast to epinephrine, phenylephrine had no effect on the ESR signal when added alone or together with ferritin. Another noncatechol adrenergic agent, metaproterenol, also had no significant influence on the ESR spectrum of the basic system (data not shown).

Figure 4 illustrates the relative intensities of hydroxyl radical generation following the addition each of different adrenergic agents or the adrenergic neurotoxin, 6-hydroxydopamine, to the xanthine oxidase system with and without ferritin, all under aerobic conditions. Note that only the agents with the catechol structure caused enhancement of hydroxyl radical production above that of the basic system alone. The inclusion of ferritin along with any one of the catechol adrenergic agents to the basic system resulted in further enhancement of radical production.

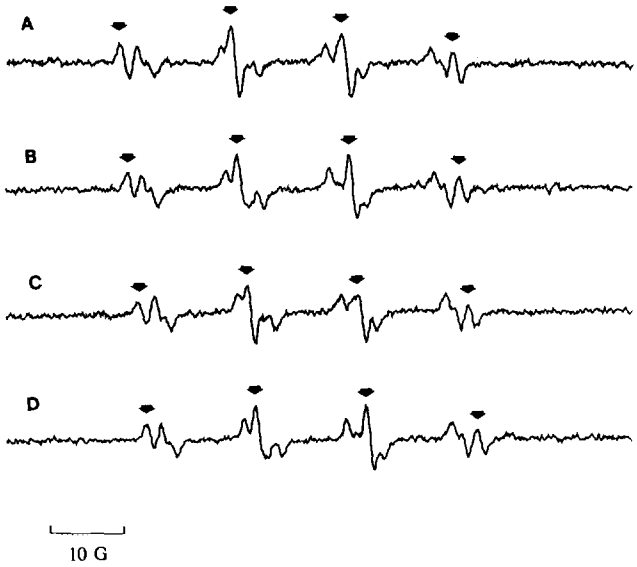


FIG. 3. ESR spectra generated by xanthine oxidase systems under aerobic conditions with the addition of ferritin and phenylephrine. ESR scans were done on the basic system composed of xanthine oxidase (0.1 units/ml) and hypoxanthine (0.4 mM) and with the additions described below. Reactions were carried out at pH 7.4 in 0.1 M phosphate buffer containing 0.09 M NaCl. All incubations contained DMPO (40 mM). Each sample was incubated for 10 min at 37°C, followed by scanning in an ESR spectrometer was done at room temperature. All spectra are standardized to the same gain. The spectrum produced in the basic system (A) is compared to that produced by the basic system with the addition of (B) ferritin, (C) phenylephrine, or (D) both ferritin and phenylephrine. The arrows designate the peaks of the spectrum of the hydroxyl–DMPO adduct. In A through D, the spectrum of the hydroxyl–DMPO adduct is superimposed upon the spectrum of the superoxide–DMPO adduct.

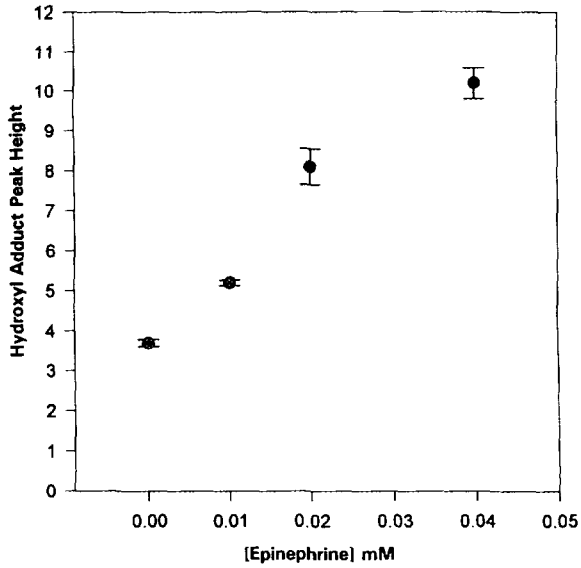


FIG. 4. Effect of varying the epinephrine concentration upon the generation of hydroxyl radicals in xanthine oxidase systems containing ferritin and epinephrine. Hydroxyl radical production was measured as the intensity of the ESR signal of the DMPO–hydroxyl adduct in aerobic systems incubated at 37°C for 10 min. All reactions were carried out at pH 7.4 in 0.1 M phosphate buffer containing NaCl (0.09 M), DMPO (40 mM), xanthine oxidase (0.1 units/ml), hypoxanthine (0.4 mM), and ferritin (1 mM total iron content) and different concentrations of epinephrine. The spectra were standardized to the same gain. The results are the average of three experiments.

Figure 5 shows the relationship between the intensity of hydroxyl production in basic systems containing ferritin at varying concentrations of epinephrine. There is a linear relationship between hydroxyl radical production and epinephrine concentration in the physiologic range from 0.0 to 0.04 mM epinephrine.

Effects of Inhibitors upon Hydroxyl Radical Production by Epinephrine and Ferritin in Xanthine Oxidase Systems under Aerobic Conditions

Desferoximine inhibited the enhancement of hydroxyl radical generation caused by the addition of ferritin and epinephrine to the xanthine oxidase system to the level caused by the addition of epinephrine alone, but did not abolish hydroxyl production completely (Fig. 6). Catalase nearly abolished the signals from all four systems while superoxide dismutase had relatively little effect on the amount of radical generation in any of the systems (Fig. 6).

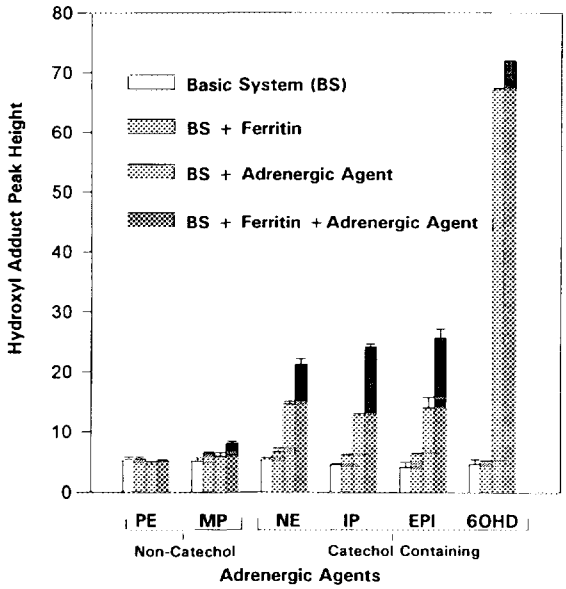


FIG. 5. Effect of adrenergic agents upon the intensity of hydroxyl radical generation in aerobic xanthine oxidase systems. Hydroxyl radical production was measured as the intensity of the ESR signal of the DMPO–hydroxyl adduct in aerobic systems that had been incubated at 37°C for 10 min. Reactions were carried out at pH 7.4 in 0.1 M phosphate buffer containing 0.09 M NaCl. All incubations contained DMPO (40 mM). The spectra were standardized to the same gain. The results are the average of three experiments. The hydroxyl radical production in the basic system (BS), which consisted of xanthine oxidase (0.1 units/ml) and hypoxanthine (0.4 mM), is compared to basic systems with the respective addition of each of the adrenergic agents at a final concentration of 0.4 mM: phenylephrine (PE), metaproterenol (MP), norepinephrine (NE), isoproterenol (IP), epinephrine (EPI), and 6-hydroxydopamine (6OHD) (BS + Adrenergic Agent). Hydroxyl production in basic systems with the addition of ferritin (1 mM total iron content) (BS + Ferritin) are compared to basic systems with addition of ferritin and one of each of the adrenergic agents (BS + Adrenergic Agent + Ferritin).

Iron Release from Ferritin by Adrenergic Compounds under Aerobic Conditions

It was initially considered that the redox potential of the catechol adrenergic agents was related to the intensity of free radical production. However, comparison of the intensity of radical production shown in Fig. 4 with the oxidation–reduction potential of the compounds listed in Fig. 7 demonstrated that the two functions were not closely related (r^2 = 0.60). However, there was a close relationship between the intensity of radical generation shown in Fig. 4 and the quantity of iron released from ferritin by each adrenergic agent shown in Fig. 8 (r^2 = 0.80). The amount of iron released under aerobic conditions was assayed in systems containing an adrenergic agent and ferritin alone, ferritin in the presence of xanthine oxidase alone, or ferritin in the presence of the complete xanthine oxidase system (Fig. 8). In the control systems (i.e., those not containing a catechol adrenergic agent), the addition of xanthine oxidase alone to ferritin increased the measurable iron only by the amount found in the xanthine oxidase preparation itself. The addition of a catechol adrenergic agent to ferritin alone caused a significant release of iron from ferritin compared to the noncatechol agents or the ferritin alone control. In the presence of its substrate, hypoxanthine, xanthine oxidase released iron from ferritin by a superoxide-dependent mechanism. This release was not affected by the addition of *non*catechol adrenergic agents, nor did the addition of any of the catechol-containing adrenergic agents to the xanthine oxidase system produce a significantly greater release of iron from ferritin (Fig. 8).

Under aerobic conditions, in the complete xanthine oxidase system without epinephrine, the addition of superoxide dismutase decreased the iron release by 50% (−SOD, 10.0 ± 0.9, n = 12; +SOD, 5.0 ± 0.3, n = 5). On the other hand, when epinephrine was present in addition to the complete system, superoxide dismutase had no significant effect on iron release (−SOD, 12.1 ± 2.2, n = 8; +SOD, 10.6 ± 2.9, n = 6).

Although all of the systems containing an adrenergic agent and the complete xanthine oxidase system produced some iron release from ferritin, enhancement of hydroxyl

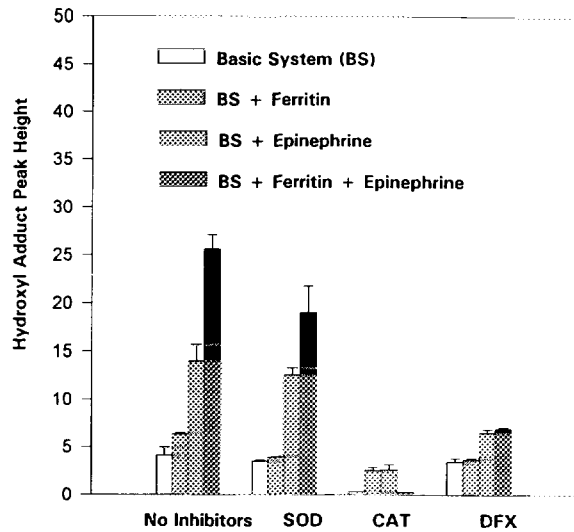


FIG. 6. Effect of inhibitors upon hydroxyl radical production in aerobic xanthine oxidase systems with epinephrine and ferritin. Hydroxyl radical production was measured as the intensity of the ESR signal of the DMPO–hydroxyl adduct in aerobic systems that had been incubated at 37°C for 10 min. All reactions were carried out at pH 7.4 in 0.1 M phosphate buffer containing 0.09 M NaCl. All incubations contained DMPO (40 mM). The spectra were standardized to the same gain. The results are the average of three experiments. The hydroxyl radical production in the basic system composed of xanthine oxidase (0.1 units/ml) and hypoxanthine (0.4 mM) (BS) is compared to basic systems with the following additions: epinephrine (0.4 mM) (BS + Epinephrine), ferritin (1 mM total iron content) (BS + Ferritin), and epinephrine with ferritin (BS + EPI + FER). The effect of superoxide dismutase (100 units/ml) (SOD), catalase (500 units/ml) (CAT), and desferioximine (0.1 mM) (DFX) on hydroxyl radical production in each of the above system variations is also shown.

Adrenergic Agents

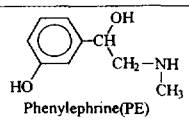
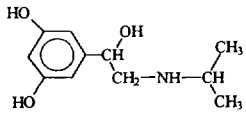
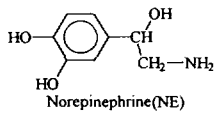
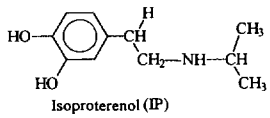
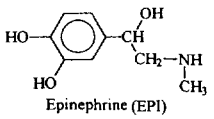
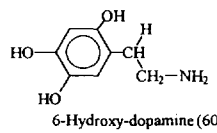
Compound and Structure	Redox Potential (V)	Reference
 Phenylephrine(PE)	?	
 Metaproterenol (MP)	.70	44
 Norepinephrine(NE)	.44	44
 Isoproterenol (IP)	.37	44
 Epinephrine (EPI)	.40	44
 6-Hydroxy-dopamine (6OHD)	< 20	45

FIG. 7. Adrenergic agents: Structures and redox potentials.

radical generation was observed *only* in systems containing the agents possessing the catechol function (Fig. 4). Indeed, 6-hydroxydopamine in the absence of ferritin generated nearly the same intensity of hydroxyl radicals in the absence of ferritin as with ferritin. Even so, iron appears to be essential for most of the enhancement of hydroxyl radical generation caused by the adrenergic agents containing the catechol structure.

Release of Iron from Ferritin by Epinephrine in Xanthine Oxidase Systems under Anaerobic Conditions

Table I shows the iron release from ferritin was significantly greater under anaerobic conditions compared to aerobic ones. Under aerobic conditions, the epinephrine-induced iron release is unaffected by the addition of xanthine oxidase with or without its substrate, hypoxanthine. Under anaerobic conditions, however, epinephrine, in the presence of the complete xanthine oxidase system, releases more than twice the amount of iron as in the absence of the xanthine oxidase system. In addition, under anaerobic conditions, the addition of epinephrine to the complete xanthine oxidase system resulted in twice the net iron release as the same system under aerobic conditions (Table I).

DISCUSSION

The findings demonstrate that catecholamines can release a significant amount of iron from ferritin under both aerobic and anaerobic conditions. Indeed, under aerobic conditions, catecholamines by themselves were able to release essentially as much iron from ferritin as occurred when the catecholamine and the xanthine oxidase system were incubated together with ferritin (Fig. 8). Very little of the release in this complete aerobic system seemed to be superoxide-dependent in view of the fact that the addition of superoxide dismutase produced only a small inhibition of iron release (data given in previous paragraph). Even so, it has been established that the xanthine oxidase system by itself under aerobic conditions is capable of releasing some iron from ferritin (27–29).

Under anaerobic conditions the release of iron from ferritin when both epinephrine and the xanthine oxidase

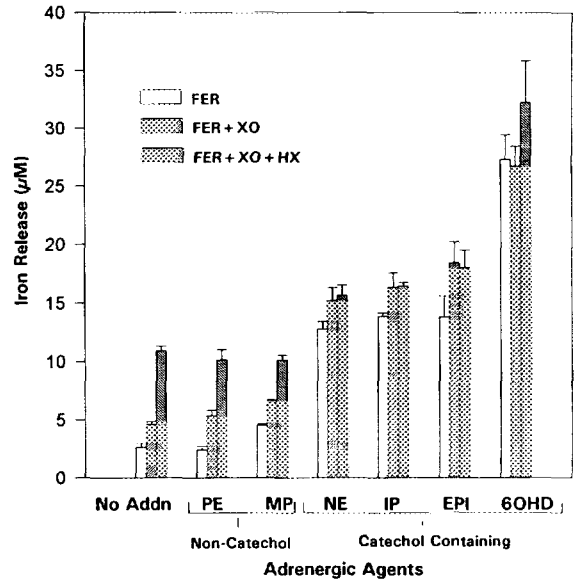


FIG. 8. Effect of adrenergic agents upon iron release from ferritin in aerobic xanthine oxidase systems. Reductive release of iron was measured using bathophenanthrolene (0.01%) as an indicator of ferrous iron in systems that had been incubated at 37°C for 30 min. All reactions were carried out at pH 7.4 in 0.1 M phosphate buffer containing 0.09 M NaCl. The results are the average of three experiments. Fe^{+2} release from ferritin was determined in systems containing ferritin (1 mM total iron content) only (FER), ferritin plus xanthine oxidase (0.1 units/ml) alone (FER + XO), and ferritin plus the complete xanthine oxidase system with hypoxanthine (0.4 mM) (FER + XO + HX). The effect of different catechol and noncatechol adrenergic agents on Fe^{+2} release in these systems was also determined: phenylephrine (PE), metaproterenol (MP), norepinephrine (NE), isoproterenol (IP), epinephrine (EPI), 6-hydroxydopamine (6OHD). All adrenergic agents were at a final concentration of 0.4 mM.

TABLE I

Iron Release from Ferritin

System Components	Aerobic			Anaerobic		
	n	Iron release (μM)	SEM	*n*	Iron release (μM)	SEM
XO	2	2.1	0.65	4	2.3	0.60
FER	8	2.4	0.31	2	2.7	0.06
FER + XO	10	7.5[a]	0.35	12	9.6[b,c]	0.79
FER + EPI	8	12.0	1.65	13	15.0	0.83
FER + XO + EPI	3	11.4	4.61	3	20.3	1.20
FER + XO + HX	12	10.0	0.87	15	14.0[c]	1.23
FER + XO + HX + EPI	8	12.1	2.18	13	28.5[b,c]	2.09

Note. Ferrous iron concentrations were measured in 0.1 M phosphate buffer containing 0.09 M NaCl at pH 7.4 using bathophenanthrolene (0.01%) after 30-min incubations of the indicated components: (XO) xanthine oxidase (0.1 units/ml); (HX) hypoxanthine (0.4 mM); (EPI) epinephrine (0.4 mM); (FER) ferritin (1 mM total iron content). The xanthine oxidase and ferritin preparations contained small amounts of ferrous iron. The values for Fe^{+2} in xanthine oxidase alone and ferritin alone were not included in the Duncan's multiple comparison analysis.

[a] Significantly different from the remainder of the aerobic group by Duncan's multiple comparison test.

[b] Significantly different from the remainder of the anaerobic group by Duncan's multiple comparison test.

[c] Significantly different from the corresponding aerobic incubation by Student's *t* test ($P < 0.05$).

system were present was more than double the amount released by the same system under aerobic conditions (Table I). It is interesting to note in Table I that when the amount of iron released under anaerobic conditions in the FER + EPI system (20.3 μM) is added to the amount released by the FER + XO + HX system (14.0 μM), the sum of the two systems individually closely approximated the amount released by the combination of these two systems (FER + XO + HX + EPI) (28.5 μM), suggesting that iron released by epinephrine might be from a different iron pool associated with the ferritin molecule than that released by the xanthine oxidase. This contrasts with the same systems incubated under aerobic conditions where no enhancement of iron release by the combined incubation of epinephrine and the xanthine oxidase system was observed. The potentiation of xanthine oxidase-mediated iron release from ferritin by epinephrine under anaerobic conditions may have a bearing on the release of iron associated with ischemia, particularly in myocardial tissue in which, as mentioned earlier, there can be as much as a 2000-fold increase in catecholamine concentration in the ischemic myocardium (25) along with conversion of xanthine dehydrogenase to xanthine oxidase and an elevation of hypoxanthine.

Under anaerobic conditions, the release of iron from ferritin by xanthine oxidase plus hypoxanthine was significantly more than the same system under aerobic conditions (14.0 ± 1.2 μM versus 10.0 ± 0.9 μM), $P < 0.05$ (Table I). Ulvik and Bolann previously demonstrated the enhancement of iron release from ferritin by the xanthine oxidase system under anaerobic conditions. They attributed this release to a direct xanthine oxidase-mediated transfer of electrons from hypoxanthine to ferritin iron (29). In addition, non-oxygen-dependent electron transfer from various electron donors to ferritin has been shown to be catalyzed by flavin-free xanthine oxidase (36).

In our experiments, reductive release of ferritin iron under anaerobic conditions by the xanthine oxidase system and by epinephrine was always considerably greater than the same systems incubated under aerobic conditions. Some of this difference may be due to continuous rebinding of part of the iron by ferritin under aerobic conditions driven by the ferroxidase activity of ferritin. In addition, when oxygen is present, it would compete efficiently for electrons from xanthine oxidase and epinephrine, and in effect, inhibit iron release.

Ferritin is capable of storing a large amount of iron, but only a small portion of the total can be released under biological conditions (37). Due to the nature of the ferric hydrophosphate micelles that are the storage medium for ferritin iron, newly stored iron is thought to be more easily released since these micelles have a larger surface area to volume ratio than older ones (38). It is possible that during the acute phase, newly synthesized ferritin might provide an increased amount of more easily released iron.

The results of the spin trapping studies also suggest the possibility for an additional role for catecholamines in tissue injury after ischemia/reperfusion. The data in this report demonstrate that catecholamines can interact with xanthine oxidase to produce hydroxyl radicals by a mechanism that is not totally abolished by the addition of desferoximine (Fig. 6), suggesting that epinephrine, or more likely its corresponding *ortho*-semiquinone form, can react with the xanthine oxidase system as an organic Fenton reagent. Kalyranaraman *et al.* demonstrated similar behavior concerning the reaction of anthracycline-derived *o*-semiquinones with xanthine oxidase systems. However, these investigators did not observe any direct

reduction of hydrogen peroxide by simple *ortho*-semiquinones in their systems (38). The spin trapping results also demonstrate that catecholamines can interact with ferritin in the presence of the xanthine oxidase system to produce a very significant enhancement of hydroxyl radical production. This interaction has an absolute requirement for the catechol configuration which consists of *ortho* hydroxyl groups on a benzene ring. The noncatechol adrenergic agents (phenylephrine, with its single hydroxyl group on the benzene ring, and metaproterenol, with hydroxyl groups in the *meta* arrangement), produced no enhancement in hydroxyl radical production when added to the xanthine oxidase system, or to ferritin, or to a combination of these components (Fig. 3). Iwahashi *et al.* demonstrated enhancement of hydroxyl radical production by the plant catechol, caffeic acid, and other catechol-containing compounds by EDTA-chelated Fe^{+3} added directly to hydrogen peroxide (39). Similar chemistry may be involved in the enhancement of hydroxyl radical production by catecholamines in the presence of the xanthine oxidase system described in this report.

In the aerobic systems, the formation of hydroxyl radical correlates with iron release. Interestingly, the iron release studies demonstrated that under aerobic conditions the superoxide produced by the xanthine oxidase system released the same amount of iron from ferritin in the presence or absence of epinephrine (Table I). However, it is only the systems that had both an iron source and a catechol-structured adrenergic agent that were effective producers of the hydroxyl radical (Fig. 5).

The most plausible explanation for the observed enhancement of radical production by catechol compounds is that they may play a dual role. First, they are able to reductively release iron from ferritin both aerobically and anaerobically, and second, the oxidized form of the catecholamine is capable of chelating ferrous iron. Such chelates often are more redox active than either compound alone; for example, chelation of Fe^{2+} by EDTA enhances the reactivity of the iron with hydrogen peroxide (40). It is well-documented that the hydroquinone oxidation products of catecholamines readily bind divalent metal ions (41, 42).

An additional feature that is associated with the catecholamines and other ortho hydroquinones is their capacity for producing superoxide by redox cycling during the course of their oxidation to quinones (43, 44). Superoxide production by this mechanism taking place in the presence of traces of iron could result in hydroxyl radical production by Fenton chemistry.

In summary, catechol adrenergic agents were shown to promote iron release from ferritin under aerobic and anaerobic conditions. It was considered significant that epinephrine released considerably more iron from ferritin under anaerobic conditions and was especially effective in combination with the xanthine oxidase system. Since this augmentation occurred only under anaerobic conditions, it cannot be due to the reductive release of iron by superoxide anion produced by xanthine oxidase activity. It is known, however, that xanthine oxidase activity can release iron from ferritin by a superoxide-independent mechanism that operates under anaerobic conditions. Such conditions might be realized during cardiac ischemia wherein markedly elevated catecholamine levels and the increases in the components of the xanthine oxidase system could release a significant amount of iron from ferritin in the ischemic zone. When reperfusion occurs, production of hydrogen peroxide resulting from xanthine oxidase activity and its interaction with the released iron could then result in oxyradical generation that could contribute to tissue injury. A current aim in this laboratory is to determine the contribution of endogenous and exogenous catecholamines to iron release in the ischemic myocardium.

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