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Catecholic Iron Complexes as Cytoprotective Superoxide Scavengers against Hypoxia:Reoxygenation Injury in Isolated Hepatocytes

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ABSTRACT. Reactive oxygen species including superoxide radicals ($O_2^{-\bullet}$) have been implicated in the pathogenesis of radiotherapy, ischemia–reperfusion injury, aging, and inflammatory diseases. In the present work, 2:1 catecholic iron complexes were found to be more effective than uncomplexed catechols at protecting hepatocytes against hypoxia:reoxygenation cell injury. They also decreased markedly the level of reactive oxygen species formed before cytotoxicity ensued. Furthermore, these catecholic iron complexes were also more effective than uncomplexed catechols at scavenging superoxide radicals generated both enzymatically and non-enzymatically. The superoxide radical scavenging activity of catecholic iron complexes seemed to correlate with the redox potential of catechols. These results suggest that cytoprotection by catechols may involve an initial chelation with iron to form a complex that is a much more effective superoxide radical scavenger than the catechol itself. BIOCHEM PHARMACOL **56**;7:825–830, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. superoxide dismutase mimics; iron; ischemia:reperfusion injury; catechols; antioxidants; flavonoids

The pharmacological administration of SOD† or cell permeable SOD mimics has been proposed as a therapy to alleviate the deleterious action of superoxide radicals in the pathogenesis of ischemia–reperfusion injury, aging, cancer, and other metabolic, degenerative, and inflammatory diseases [1]. A manganic porphyrin complex or manganic desferrioxamine complex has been shown recently to prevent cell growth inhibition caused by superoxide radical-generating agents [2, 3]. Various low-molecular-weight SOD-like complexes of manganese, copper, and iron have also been reported to have SOD mimic activity [4–11].

At a physiological pH, catechol (i.e. *o*-dihydroxybenzene) readily forms a thermodynamically stable bis complex with ferric iron as a bidentate ligand. The complex formed in alkaline solution and crystallized from organic solvents has been shown by x-ray analysis to be an octahedral chelate tris(catecholato)ferrate(III) [12]. Catecholic compounds are also synthesized and utilized by microorganisms as siderophores, e.g. enterobactin, to sequester and transport iron from the external environment in response to an iron deficiency [13, 14]. These catechols have large stability constants for ferric iron ($\log K_f \geq 40$) and low reduction potentials [15, 16].

Previously, we showed that hepatocyte cytotoxicity induced when hepatocytes were maintained at low oxygen concentrations or treated with respiratory inhibitors could be attributed to ATP depletion and reductive stress, which resulted in intracellular iron release and a marked increase in oxygen activation. The cytotoxicity and oxygen activation were prevented by the catechol caffeic acid or quercetin, a catecholic flavonoid, presumably as a result of their superoxide radical scavenging activity [17, 18]. In the present work, it was found that the catecholic iron chelates were much more potent as superoxide scavengers than uncomplexed catechols, and were markedly effective at preventing hypoxia:reoxygenation hepatocyte injury caused by ROS.

MATERIALS AND METHODS

Chemicals

4-Methylcatechol, protocatechuic acid, quercetin, catechin, XO, hypoxanthine, nitro blue tetrazolium, and SOD (from bovine erythrocytes) were purchased from the Sigma Chemical Co. Collagenase (from *Clostridium histolyticum*), NADH, HEPES, and BSA were obtained from Boehringer Mannheim. Other chemicals were purchased from the Aldrich Chemical Co. Stock solutions of iron chelates were prepared freshly before use, by mixing 5 mM ferric chloride solution with 10 or 15 mM solutions of catechols.

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† *Abbreviations:* NBT, nitro blue tetrazolium; ROS, reactive oxygen species; SOD, superoxide dismutase; and XO, xanthine oxidase.

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TABLE 1. Prevention of hypoxia:reoxygenation injury cytotoxicity in hepatocytes by iron chelates

Treatment	Cytotoxicity (% of trypan blue uptake)		
	90 min	145 min	180 min
Hypoxia:reoxygenation	42 ± 5	73 ± 6	95 ± 5
+ Caffeic acid (150 μM)	30 ± 4	57 ± 6	62 ± 5*
+ Caffeic acid (20 μM)	43 ± 4	68 ± 5	92 ± 9
+ Caffeic acid–Fe^{3+} (20 μM:10 μM)	30 ± 3	54 ± 5	57 ± 3*
+ Protocatechuic acid (20 μM)	42 ± 4	75 ± 7	95 ± 6
+ Protocatechuic acid–Fe^{3+} (20 μM:10 μM)	31 ± 4	38 ± 4	62 ± 3*
+ 4-*t*-Butylcatechol (20 μM)	41 ± 4	74 ± 7	96 ± 7
+ 4-*t*-Butylcatechol–Fe^{3+} (20 μM:10 μM)	32 ± 4	48 ± 4	68 ± 4*
+ Catechin (20 μM)	38 ± 4	66 ± 6	95 ± 6
+ Catechin–Fe^{3+} (20 μM:10 μM)	31 ± 3	53 ± 6	66 ± 4*
+ Quercetin (20 μM)	40 ± 5	69 ± 7	88 ± 8
+ Quercetin–Fe^{3+} (20 μM:10 μM)	32 ± 6	48 ± 5	58 ± 5*
+ Tiron (30 μM)	42 ± 4	63 ± 7	94 ± 6
+ Tiron–Fe^{3+} (30 μM:10 μM)	43 ± 2	67 ± 4	96 ± 6
+ Fe^{3+} (10 μM)	41 ± 6	71 ± 7	95 ± 5
+ SOD (100 U/mL)	31 ± 3	48 ± 4	52 ± 4*
+ SOD/catalase (100 U/mL)	26 ± 3	31 ± 4	38 ± 3

Rat hepatocytes (10^6 cells/mL) were incubated in Krebs–Henseleit buffer (pH 7.4) under an atmosphere of 95% N_2 and 5% CO_2 and reoxygenated at 90 min with 1% O_2, 94% N_2 and 5% CO_2. Samples were taken at times of interest for viability assessment, which was determined as the percentage of cells taking up trypan blue. Results are the means of three separate experiments (± SEM).

*Significantly different from hypoxia:reoxygenated cells, $P < 0.05$.

Hepatocyte Preparation and Determination of Cytotoxicity and H_2O_2 Formation

Hepatocytes were isolated from male Sprague–Dawley rats (275–300 g) by collagenase perfusion of the liver according to Moldéus *et al.* [19]. Isolated hepatocytes (10^6 cells/mL) were suspended in Krebs–Henseleit buffer (pH 7.4) containing 12.5 mM HEPES in continuously rotating round-bottomed 50-mL flasks, under an atmosphere of 95% O_2 and 5% CO_2 in a water bath of 37° for 30 min. Then the hepatocytes were incubated under an atmosphere of 95% N_2, and 5% CO_2 for 90 min before reoxygenation with 1% O_2, 94% N_2 and 5% CO_2. Reoxygenation with 1% O_2 but not 20% O_2 was found previously to increase markedly hypoxia-induced hepatocyte injury, whereas reoxygenation with 20% O_2 prevented further hypoxic injury by more rapidly restoring ATP levels and redox homeostasis [18]. Cell viability was assessed by determining trypan blue uptake by hepatocytes [19]. H_2O_2 formation was determined using 2′,7′-dichlorofluorescin diacetate, which is hydrolyzed by intracellular esterases and oxidized by H_2O_2/Fe to highly fluorescent dichlorofluorescein [20]. The fluorescence was measured at λ_{ex} = 500 nm and λ_{em} = 520 nm, using a Shimadzu RF-5000 spectrofluorophotometer.

Determination of Superoxide Scavenging Activity

NBT was reduced by superoxide generated by a phenazine methosulfate/NADH system as described by Robak and Gryglewski [21], or by a hypoxanthine/XO system according to Younes and Weser [22]. The superoxide scavenging activities for catechols and catecholic ferric complexes were expressed as IC_{50} values that were obtained by determining the concentration of the catechol or the catecholic iron complex required to inhibit 50% of the initial NBT reduction rate of control. The IC_{50} values were calculated from regression lines, where x was the log of the concentration of the catechol tested, and y was the percentage of the initial rate of NBT reduction in the presence of the catecholic inhibitors over that of the control [21].

Statistical Analysis

Statistical significance of the difference between control and experimental groups was determined by Student's *t*-test. The acceptable level of significance was $P \leq 0.05$.

RESULTS

As shown in Table 1, iron complexes of the catecholic compounds caffeic acid, protocatechuic acid, 4-*t*-butylcatechol, catechin, and quercetin were much more effective than the uncomplexed catechols at preventing ROS-mediated hypoxia:reoxygenation injury. Much higher concentrations of uncomplexed caffeic acid were required to give the same degree of protection as the caffeic acid–Fe complex. Furthermore, as shown in Fig. 1, the iron complexes of caffeic acid and 4-*t*-butylcatechol were also much more effective than the uncomplexed catechols at inhibiting hepatocyte H_2O_2 formation. By contrast, the tiron–Fe complex was not cytoprotective at the 10 μM Fe^{3+} concentrations used and did not inhibit H_2O_2 formation

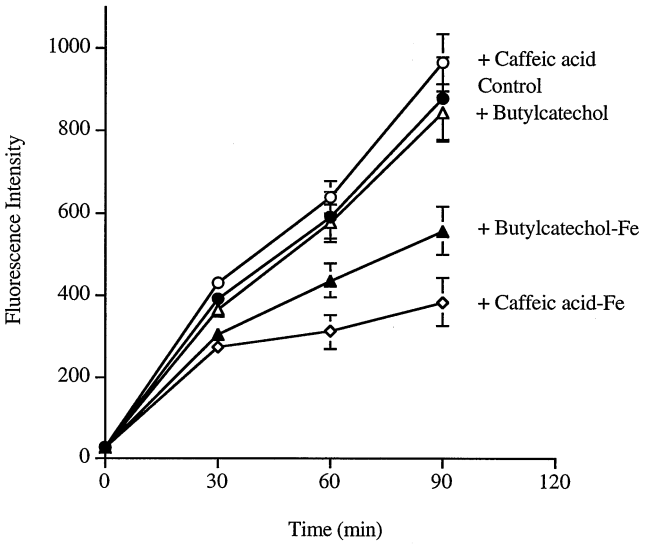


FIG. 1. Inhibition of ROS-mediated dichlorofluorescin fluorescence by catecholic iron complexes. Rat hepatocytes (10^6 cells/mL) were incubated in Krebs–Henseleit buffer (pH 7.4) with 2′,7′-dichlorofluorescin diacetate (8 μM) and catecholic iron complexes (20 μM catechols:10 μM Fe^{3+}) under an atmosphere of 95% N_2, 0.1% O_2, and 5% CO_2. Hepatocytes incubated with 2′,7′-dichlorofluorescin diacetate only were used as the control. Samples were taken at times of interest for fluorescent intensity measurement (λ_{ex} = 500 nm, λ_{em} = 520 nm). Results are means (±SEM) of three separate experiments.

(Table 1). SOD at 100 U/mL also prevented cytotoxicity, particularly when added with catalase (Table 1).

As shown in Table 2, the catecholic ferric complexes were far better inhibitors of superoxide-mediated NBT reduction than their corresponding uncomplexed catechols in the phenazine methosulfate/NADH-mediated superoxide generation system. The catechol–Fe complex had an IC_{50} value 23-fold lower than that for catechol, and the caffeic acid–Fe complex had an IC_{50} value 118-fold lower than that for caffeic acid. Iron chelation also decreased the IC_{50} values of the flavonoids epicatechin, catechin, and quercetin by 25-, 10-, and 14-fold, respectively. The order of superoxide scavenging activity for the catecholic iron complexes was caffeic acid > epicatechin > 3-methoxycatechol, 4-*t*-butylcatechol, 4-methylcatechol, protocatechuic acid, catechin > catechol > quercetin. Free ferric ion at the concentrations used above (1–10 μM) had no detectable superoxide scavenging activity. The order of superoxide scavenging activity for uncomplexed catechols was catechin, epicatechin > 3-methoxycatechol, 4-methylcatechol, 4-*t*-butylcatechol, caffeic acid > quercetin > catechol ≫ protocatechuic acid. A 50% inhibition of NBT reduction was also obtained using 1.35 U/mL of Cu,Zn-SOD. The maximum inhibition of NBT reduction by SOD (100 U/mL) was 82%, which was not further increased by the catecholic iron complexes, indicating that NBT radical intermediates were not re-oxidized by the complexes. The cytochrome *c* assay method for determining SOD activity [3] could not be used, as cytochrome *c* was reduced by the catecholic iron complexes (data not shown).

As shown in Table 2, the tiron–Fe, 4-nitrocatechol–Fe and tetrachlorocatechol–Fe complexes were much less effective as superoxide scavenger than other catecholic ferric complexes. The IC_{50} concentrations of the 4-nitrocatechol–Fe and tetrachlorocatechol–Fe complexes could not be determined due to solubility problems. The IC_{50} concentration for Cu,Zn-SOD was 1.35 U/mL.

The superoxide scavenging activity of catecholic iron complexes was also determined using the superoxide generating system hypoxanthine/XO. As shown in Table 3, complexation of the catechol with iron increased the superoxide scavenging activity of the catechols several fold. Again, tiron–Fe was the least effective among the catecholic iron chelates tested, having an IC_{50} value more than 9-fold that of caffeic acid–Fe. The catecholic iron com-

TABLE 2. Inhibition of phenazine methosulfate/NADH-mediated NBT reduction

Fe^{3+} chelates	IC_{50} (μM)	Catechols	IC_{50} (μM)
Caffeic acid–Fe	1.15	Caffeic acid	136.3
3-Methoxycatechol–Fe	3.45	3-Methoxycatechol	107.9
4-*t*-Butylcatechol–Fe	3.53	4-*t*-Butylcatechol	129.4
4-Methylcatechol–Fe	3.65	4-Methylcatechol	127.5
Protocatechuic acid–Fe	4.15	Protocatechuic acid	444.4
Catechol–Fe	7.72	Catechol	184.2
Tiron–Fe (3:1)	28.5	Tiron	588.4
Tetrachlorocatechol–Fe (3:1)	>37.5*	Tetrachlorocatechol	>150*
4-Nitrocatechol–Fe (3:1)	>37.5*	4-Nitrocatechol	>150*
Epicatechin–Fe	2.26	Epicatechin	56.4
Catechin–Fe	4.50	Catechin	47.6
Quercetin–Fe	9.94	Quercetin	141.6

The reaction mixture contained 10 μM phenazine methosulfate (PMS), 78 μM NADH, 25 μM NBT, and various concentrations of iron complexes in 0.1 M Tris buffer (pH 7.4). PMS was added last to initiate the reaction. The absorbance at 560 nm was recorded against blank samples containing no PMS. The IC_{50} values were calculated from regression lines, where x was the log of the concentration of the catechol tested, and y was the percentage of the initial rate of NBT reduction in the presence of catecholic inhibitors over that of the control. Seven different concentrations were used for the calculation of the IC_{50} of each compound.

*Precipitation in the reaction systems at higher concentrations of these catechols interfered with the assay.

TABLE 3. Inhibition of hypoxanthine/XO-mediated NBT reduction

Fe^{3+} chelates	IC_{50} (μM)	Catechols	IC_{50} (μM)
Caffeic acid–Fe	26.0	Caffeic acid	80.3
4-Methylcatechol–Fe	28.6	4-Methylcatechol	46.4
Catechol–Fe	36.2	Catechol	119
Tiron–Fe (3:1)	240	Tiron	1850
Tetrachlorocatechol–Fe (3:1)	>225*	Tetrachlorocatechol	>150*
4-Nitrocatechol–Fe (3:1)	>225*	4-Nitrocatechol	>150*

The reaction mixture contained 200 μM NBT, 35 μM hypoxanthine, 25 mU/mL XO, and various concentrations of 2:1 Fe^{3+} complexes in 0.1 M Tris buffer (pH 7.4). XO solution was added last to start the reaction, and the reduction of NBT was measured by following the absorbance at 560 nm. The IC_{50} values for the inhibition of the reduction of NBT by the investigated compounds were calculated as described in Table 2.

*Precipitation in the reaction systems at higher concentrations of these catechols interfered with the assay.

plexes did not affect XO activity even at 100 μM (results not shown). A 50% inhibition of NBT reduction was also obtained using 0.07 U/mL of Cu,Zn-SOD.

DISCUSSION

Previously, we showed that quercetin partly prevented hypoxia:reoxygenation injury in isolated hepatocytes or when hepatocytes were incubated with mitochondrial respiratory inhibitors under aerobic conditions. The marked increase in ROS formation measured by luminol chemiluminescence that preceded the cytotoxicity was also inhibited [17, 18]. Flavonoids have also been shown previously to protect mammalian and bacterial cells from ROS-mediated cell injury, which was attributed to the catechol moiety of the B-ring [23, 24]. In the present work, it was discovered that iron complexes of catechols and catecholic flavonoids were much more effective than the uncomplexed catecholic compounds at preventing hypoxic hepatocyte injury as well as inhibiting H_2O_2 generation, as determined by dichlorofluorescin fluorescence. SOD at 100 U/mL also protected the hepatocytes against hypoxic injury, particularly when added with catalase. This suggests that cytotoxicity can be attributed to extracellular ROS. The cytoprotective effectiveness of the ferric complexes was similar even though the catechols used had widely differing partition coefficients, and this suggests that the catecholic iron complexes may protect the hepatocyte cell membrane extracellularly. Recently hypoxia–reoxygenation cytotoxicity was attributed to extracellular superoxide that had been formed in an intracellular compartment and had egressed through plasmalemma anion channels [25].

The complexation of catechols with ferric ion was also found to increase their SOD scavenging activity markedly. As shown in Fig. 2, this superoxide scavenging activity, expressed as the logarithm values of IC_{50} of the phenazine methosulfate/NADH system, seemed to correlate with the redox potentials (V vs NHE) of catechols [26, 27]. A linear correlation with an r^2 value of 0.95 for catechols and 0.98 for catecholic iron complexes was obtained between the reduction potentials and the superoxide scavenging activities. However, the iron complexes of caffeic acid and protocatechuic acid did not fit this linear correlation and were more active than expected, suggesting that the acid side chain may also contribute to iron chelation and, thus, $O_2^{-\bullet}$ scavenging properties.

At a physiological pH, most of the catechols form 2:1 complexes with ferric ions, whereas catechols with low pK_a values and high redox potentials such as tiron, 4-nitrocatechol, and tetrachlorocatechol form 3:1 complexes with ferric ion [13, 28, 29]. The iron complexes of tiron, tetrachlorocatechol, and 4-nitrocatechol, however, were much less effective as superoxide scavengers than those of other catechols, probably because of the stereochemistry of the stable 3:1 catecholic iron complexes formed at a physiological pH. Graf *et al.* [30] suggested that the availability of an iron coordinate site that is open or occupied by a readily displaceable ligand such as water facilitates the reaction of iron chelates with $O_2^{-\bullet}$, although it is not a

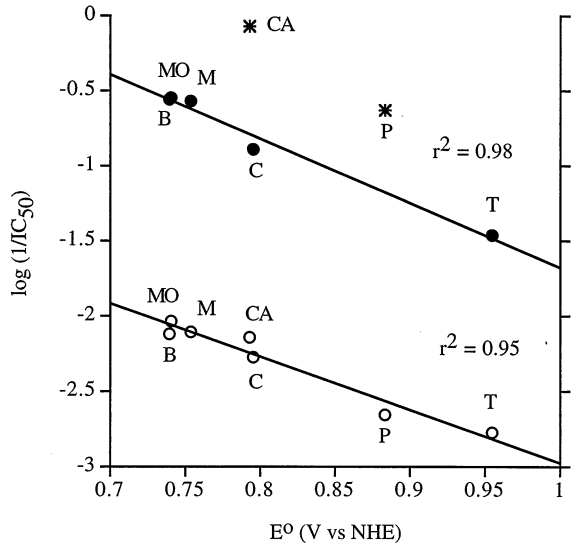
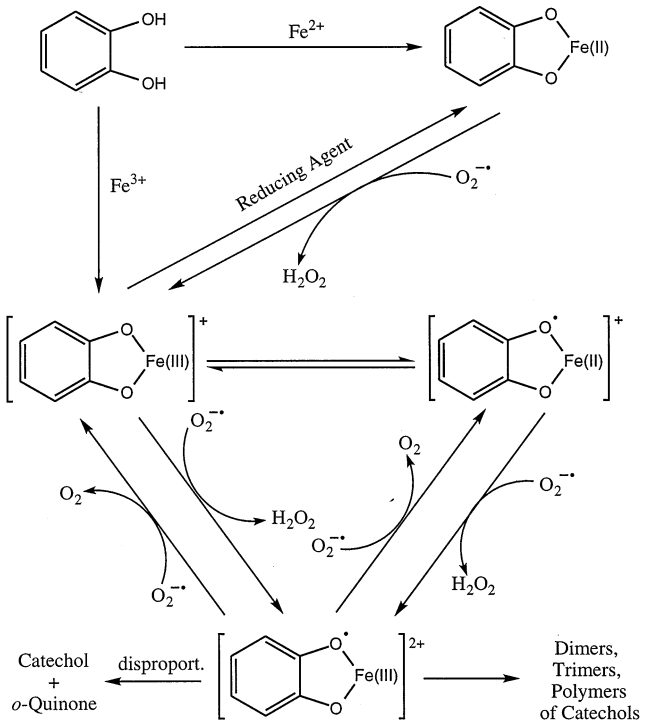


FIG. 2. Correlation between the redox potentials [26, 27] and the superoxide scavenging activities (for Table 2). The open circles represent catechols, and the filled circles represent catecholic iron complexes. Key: (B) 4-*t*-butylcatechol; (C) catechol; (CA) caffeic acid; (M) 4-methylcatechol; (MO) 3-methoxycatechol; (P) protocatechuic acid; and (T) tiron. NHE = normal hydrogen electrode.



SCHEME 1. Mechanisms of superoxide scavenging by catecholic iron complexes.

stringent requirement. In the 3:1 complexes, all the ligand sites are occupied by the catechols, which consequently hinders a free electron transfer between the complex and a superoxide radical.

The dismutation of superoxide radicals by catecholic iron complexes could be explained by the redox cycling of the metal and the ligand as shown in Scheme 1, in which a 1:1 catechol–Fe complex is used as an example for simplicity. Catechols bind tightly with ferric ion to form a complex [12], which could be oxidized to semiquinone ferric complexes by superoxide radicals. The catechol–Fe complex has also been hypothesized to undergo an internal redox reaction to generate a semiquinone radical intermediate of catechol chelated to the ferrous iron [31, 32], a process that is favored at low pH or by low redox potential catechols. The semiquinone ferrous complex is then oxidized by $O_2^{-\bullet}$ to form a semiquinone ferric complex. The resulting semiquinone ferric complexes are readily reduced to catecholic ferric complexes, while the superoxide radicals are converted to oxygen. They may also form catecholic dimers or trimers, or undergo disproportionation to give the parent catechol and the *o*-quinone, which could also be reduced by $O_2^{-\bullet}$. Alternatively, the initial catecholic ferric complexes could also be reduced by $O_2^{-\bullet}$ or intracellular reducing agents to form catecholic ferrous complexes. The superoxide scavenging activity arises because the catecholic ferrous complexes have much lower redox potentials compared with that of Fe^{3+}/Fe^{2+}.

The potentiated superoxide scavenging activity of catechols by forming iron complexes could result from the increased tendency of catechols to be oxidized to semiquinones in the iron complexes. Semiquinone radicals of tiron chelated to Mg^{2+} or Zn^{2+} have been detected by EPR, and the increased yield of the semiquinone of tiron in the presence of metal ions has been attributed to the spin stabilization of the radicals [29]. Complexation with iron may also decrease the reduction potential of the semiquinone/catechol redox pair. As a consequence, catechols in the complexes are relatively more readily oxidized by superoxide radicals than uncomplexed catechols. Part of the cytoprotective activity of the catechols could also involve removal of extracellular H_2O_2 by the catecholic ferrous complexes.

Caffeic acid has been reported to be genotoxic in bacterial and mammalian *in vitro* genotoxicity tests, which was ascribed to H_2O_2 formed during autoxidation [33, 34]. However, millimolar concentrations of caffeic acid were used and stored for days. The caffeic acid–Fe complex was not cytotoxic even when the complex at 1 mM was incubated with hepatocytes under an atmosphere of 95% O_2 (data not shown).

Our studies suggest that iron complexes of catecholic compounds scavenged superoxide radicals generated extracellularly. In the absence of iron, much higher concentrations of catecholic compounds were required to reach the same level of cytoprotection against hypoxic injury and could involve chelation with intracellular iron to form complexes with more powerful superoxide scavenging activity.

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