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Quinolinic Acid – Iron(II) Complexes: Slow Autoxidation, but Enhanced Hydroxyl Radical Production in the Fenton Reaction

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Quinolinate (pyridine-2,3-dicarboxylic acid, Quin) is a neurotoxic tryptophan metabolite produced mainly by immune-activated macrophages. It is implicated in the pathogenesis of several brain disorders including HIV-associated dementia. Previous evidence suggests that Quin may exert its neurotoxic effects not only as an agonist on the NMDA subtype of glutamate receptor, but also by a receptor-independent mechanism. In this study we address ability of ferrous quinolinate chelates to generate reactive oxygen species. Autoxidation of Quin-Fe(II) complexes, followed in Hepes buffer at pH 7.4 using ferrozine as the Fe(II) detector, was found to be markedly slower in comparison with iron unchelated or complexed to citrate or ADP. The rate of Quin-Fe(II) autoxidation depends on pH (squared hydroxide anion concentration), is catalyzed by inorganic phosphate, and in both Hepes and phosphate buffers inversely depends on Quin concentration. These observations can be explained in terms of anion catalysis of hexaaquairon(II) autoxidation, acting mainly on the unchelated or partially chelated pool of iron. In order to follow hydroxyl radical generation in the Fenton chemistry, electron paramagnetic resonance (EPR) spin trapping with 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) was employed. In the mixture consisting of 100 mM DMPO, 0.1 mM Fe(II), and 8.8 mM hydrogen peroxide in phosphate buffer pH 7.4, 0.5 mM Quin approximately doubled the yield of DMPO-OH adduct, and higher Quin concentration increased the spin adduct signal even more. When DMPO-OH was pre-formed using Ti^{3+}/hydrogen peroxide followed by peroxide removal with catalase, only addition of Quin-Fe(II), but not Fe(II), Fe(III), or Quin-Fe(III), significantly promoted decomposition of pre-formed DMPO-OH. Furthermore, reaction of Quin-Fe(II) with hydrogen peroxide leads to initial iron oxidation followed by appearance of iron redox cycling, detected as slow accumulation of ferrous ferrozine complex. This phenomenon cannot be abolished by subsequent addition of catalase. Thus, we propose that redox cycling of iron by a Quin derivative, formed by initial attack of hydroxyl radicals on Quin, rather than effects of iron complexes on DMPO-OH stability or redox cycling by hydrogen peroxide, is responsible for enhanced DMPO-OH signal in the presence of Quin. The present observations suggest that Quin-Fe(II) complexes display significant pro-oxidant characteristics that could have implications for Quin neurotoxicity.

Keywords: Iron chelator, reactive oxygen species, excitotoxicity, macrophage

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INTRODUCTION

Quinolinic acid (pyridine-2,3-dicarboxylic acid, Quin) is a direct precursor of nicotinamide cofactors in the kynurenine metabolic pathway, and also an agonist on the *N*-methyl-D-aspartate (NMDA) subtype of brain glutamate receptors[1]. Over-activation of the NMDA receptors leads to neurodegeneration and ultimately cell death by phenomenon known as excitotoxicity[2,3]; accordingly, Quin has attracted considerable research interest as a possible neurotoxic agent[1]. By far the most important source of Quin *in vivo* appears to be immune-activated macrophages[4–7], or microglia[8,9], and Quin neurotoxicity has been implicated specifically in the pathogenesis of brain diseases with a pronounced inflammatory component[10], among them the dementia associated with HIV infection[11,12].

Direct activation of the NMDA receptors apparently cannot account for all neurotoxic effects of quinolinic acid[1,13]; and experimental evidence pointing to the role of oxidative stress in Quin-induced neuronal damage has appeared in the literature[13–15]. For instance, Rios and Santamaria[14] reported that Quin stimulates lipid peroxidation in rat brain homogenates. We performed similar experiments and found that Quin can, depending on concentration used, stimulate as well as inhibit the lipid peroxidation in brain homogenate, and the stimulatory effect critically requires the presence of iron[16]. Our further investigation of complex equilibria between ferrous ion and Quin in solution by means of UV-VIS spectroscopy[17] revealed that quinolinic acid chelates Fe(II) in a manner analogous to picolinic (pyridine-2-carboxylic) acid, another structurally related tryptophan metabolite and a well-known iron chelator[18].

Redox active transition metals, *in vivo* mainly iron and copper, play a crucial role in mediating tissue damage caused by reactive oxygen species[19]. The classical concept of the Fenton reaction[19–22] is commonly presented as an oxidation of ferrous ion by hydrogen peroxide:

$$\begin{aligned} Fe^{2+} + H_2O_2 &\dashrightarrow Fe(H_2O_2)^{2+} \\ &\dashrightarrow Fe^{3+} + \cdot OH + OH^- \end{aligned} \quad (1)$$

The reaction proceeds via an "inner sphere" mechanism and the resulting hydroxyl radical as well as an iron-oxo intermediate (ferryl) may be the highly reactive species ultimately exerting tissue damage[21,22]. A special case, when iron is oxidized by hydrogen peroxide and simultaneously reduced back to Fe(II) by superoxide, is referred to as the Haber-Weiss reaction. Reaction of ferrous ion with molecular oxygen, i.e. autoxidation, can also, by sequential reduction of oxygen, lead to superoxide, hydrogen peroxide and, finally, to the hydroxyl radical or ferryl via the Fenton reaction. This process is perhaps more general in comparison to the Haber-Weiss or Fenton reaction as such, since it does not require prior formation of partially reduced oxygen species[23].

Iron can never be "free" under any physiologically relevant conditions and chelating agents may substantially affect the reactivity of iron towards superoxide and hydrogen peroxide[19,21]. The effect of chelation is not easily predictable; the main factors involved are solubilization of the metal, its redox potential and availability of free coordination sites[21,24]. Ferrous complexes of EDTA, ADP or citrate, for instance, are good Fenton reagents, while chelation by desferrioxamine, phenanthroline or phytate renders iron unreactive. In the body, the most active form of iron in terms of Fenton chemistry, the "low-molecular-weight iron", remains poorly defined. This form of iron is believed to consist of iron bound to polyphosphates, nucleotides and polycarboxylic acids like citrate. All such complexes catalyse the Haber-Weiss reaction[19]. Restriction of the pool of this labile, redox-active iron to a minimum appears to be an important antioxidant defence strategy in the body[20].

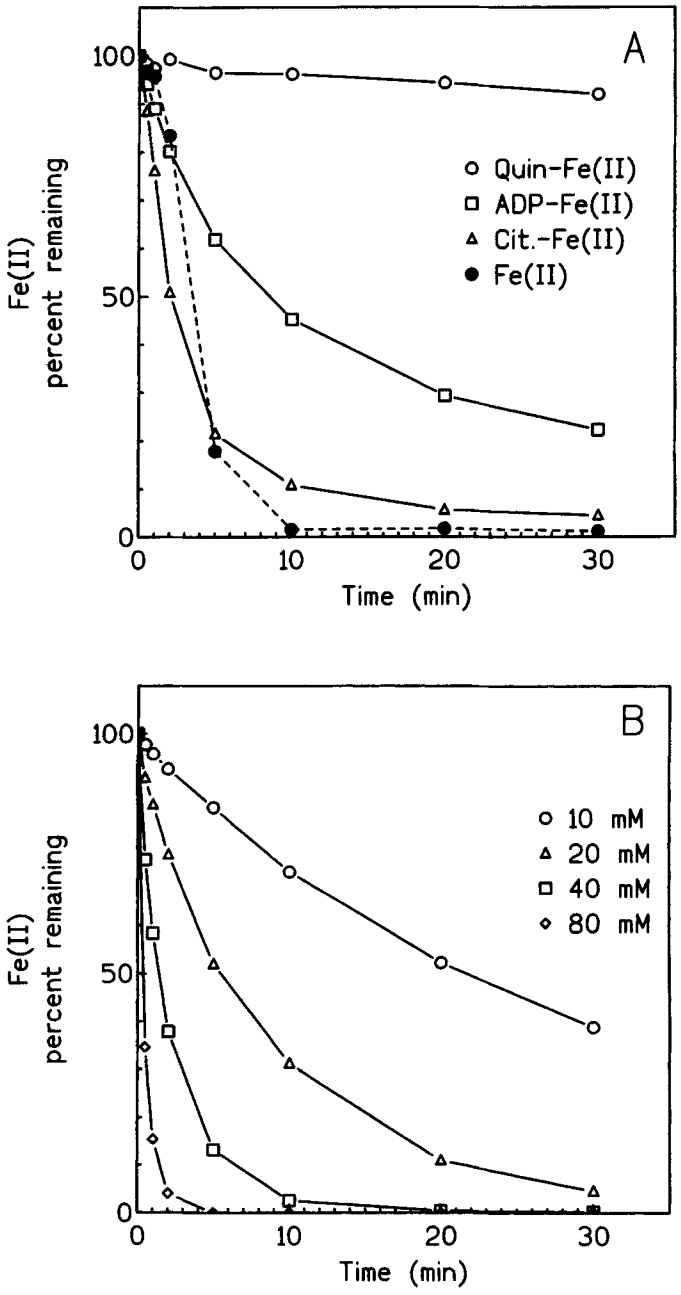

FIGURE 1 **A**. Autoxidation of Fe(II) alone or in presence of Quin, ADP or citrate in buffer Hepes/NaOH 20 mM, pH 7.4. Starting concentration of Fe(II) in the incubation mixture was 0.2 mM, chelator 1 mM. Fe(II) assayed with ferrozine during 30 min. incubation at 25 °C. **B**. Autoxidation of Quin-Fe(II) in sodium phosphate buffer pH 7.4. Concentration of phosphate varied as indicated in the graph; other conditions the same as for A. Both panels show representative results from at least three independent experiments performed for each condition. Note the biphasic course of unchelated Fe(II) autoxidation in Hepes, in accordance with data in the literature[28,33], while oxidation of ferrous iron complexed to Quin, ADP, citrate (or phosphate, not shown) generally followed first- or second-order kinetics

Thus, it is conceivable that iron chelation by Quin might contribute to mechanism of Quin toxicity. However, in the majority of experimental work on Quin neurotoxicity this substance has not been considered as an iron chelator and reactivity of ferrous quinolinate complexes with oxygen radicals has never been investigated in detail. In the present study, we have investigated susceptibility of Quin-Fe(II) complexes to autoxidation, which supposedly determines whether this form of chelated iron is likely to occur in the active, reduced form under the conditions prevailing *in vivo*. Next, we have studied the ability of these complexes to generate hydroxyl radicals in the Fenton reaction with hydrogen peroxide.

MATERIAL AND METHODS

Quinolinic acid, ADP (adenosine diphosphate potassium salt, 98%), ferrozine, EDTA (ethylenediaminetetraacetic acid) disodium salt, DMPO (5,5-dimethyl-1- pyrroline *N*-oxide), and catalase (from bovine liver, thymol free, 10,000 to 25,000 IU/mg) were purchased from Sigma. Hepes [4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid], sodium citrate, sodium ascorbate, activated charcoal, hydrogen peroxide, ferrous chloride tetrahydrate, and ferric chloride hexahydrate came from Fluka; and titanium(III) chloride (15% anaerobic solution) was purchased from Riedel de Haen. All other chemicals were of at least analytical grade. Solutions were generally prepared fresh before each experiment using deionized water (Seralpur PRO90 CN, Seral Ransbach-Baumbach, Germany, final resistance 18 MΩ/cm). Quinolinic acid was dissolved in diluted NaOH and titrated with sodium hydroxide to final pH 6.5–7.0. Ferrous chloride solutions in millimolar range had pH under 5 and no loss of Fe(II) by autoxidation was detectable when used within 6–8 hours from preparation. For spin trapping experiments, however, the Fe(II) and titanium(III) solutions were prepared in water pre-treated with nitrogen gas, and after addition of the metal further purged with nitrogen gas. The solutions were then kept at 4 °C under nitrogen in dark until use. Fe(III) solutions were prepared by dissolving ferric chloride immediately (at most 15 minutes) before use. Buffers were checked for metal contamination using the "ascorbate test" according to Buettner[25]. For spin trapping experiments, the phosphate solution was treated with Chelex 100 resin (Bio-Rad, Hercules, CA, USA) in a batch procedure, before adjustment of pH. We noted that simple passing of prepared phosphate buffer through a Chelex column could dramatically alter the pH of buffer.

In order to follow Fe(II) autoxidation under various conditions, a discontinuous colorimetric assay with a potent Fe(II) chelator ferrozine[26,27] was employed, using an experimental protocol of Lambeth *et al.*[28]. A volume of 5 ml of Fe(II) or Quin-Fe(II) complex was added to 20 ml of buffer preincubated in 25 °C water bath. Then, samples were taken from the incubation mixture at specified time points and mixed with 3 volumes of 1 mM ferrozine in 0.25 M acetate buffer pH 5.3. After 11 minutes, absorbance at 562 nm was measured. Quin-Fe(II) chelates were mostly pre-mixed while ADP or citrate, when used, were added to the incubation mixture before starting the reaction with Fe(II). In other experiments, a direct kinetic spectroscopy was used: following decrease in absorbance at 430 nm and/or increase at 316 nm with time provided a good indication of Quin-Fe(II) oxidation unless interfering formation of brown dimeric Quin-Fe(III) species occurred. All spectroscopic and spectrophotometric measurements were performed on diode-array spectrophotometer Hewlett-Packard 8452A interfaced to a computer.

The commercial spin trap was purified as described by Green and Hill[29]: 1 part of DMPO was mixed with 8 parts of deionised water and 1 part of activated charcoal, the headspace above mixture was filled with nitrogen gas, and the test tube was shaken for one hour. After centrifuga-

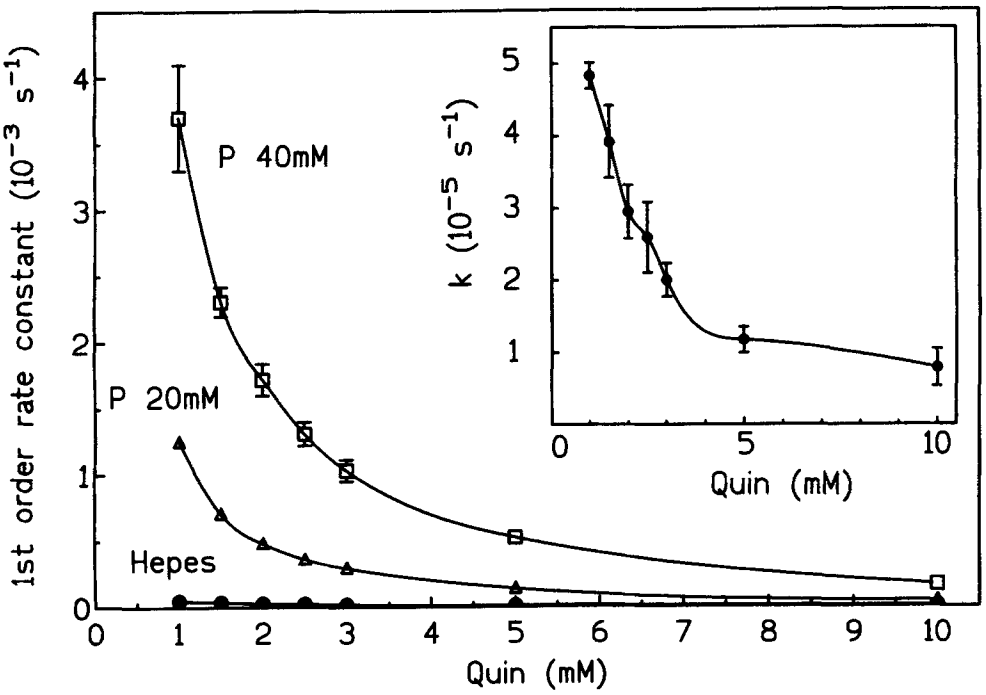

FIGURE 2 Effect of quinolinate concentration on Quin-Fe(II) autoxidation rates. Determined in buffer Hepes/NaOH 20 mM pH 7.4 and in phosphate buffer pH 7.4, conc. 20 mM and 40 mM. Starting concentration of Fe(II) was 0.2 mM, Quin 1–10 mM. The autoxidation was followed directly as change in absorbance at 430 nm for 15–20 minutes, at 25 °C. Data from two separate experiments, each in duplicate (N=4), mean +/-SD. Inset: The data set for Hepes buffer, plotted separately in an adequate scale

tion, the supernatant was divided into aliquots and stored under nitrogen at –18 °C in dark until use.

In a typical electron paramagnetic resonance (EPR) spin trapping experiment, 350 µl of 30 mM phosphate buffer pH 7.4 was mixed with 50 µl of 1 M DMPO and 50 µl of 0.3% hydrogen peroxide; the Fenton reaction was started by adding 50 µl of 1 mM Fe(II) or pre-mixed Quin-Fe(II) solution (to get final concentrations as indicated in legend to Fig. 3), and the sample was transferred to an EPR flat quartz cuvette. The EPR spectrum was recorded 3 minutes after the addition of Fe(II). Effect of various forms of iron on the stability of DMPO-OH radical adduct was investigated according to Burkitt[30]. First, the DMPO-OH was generated by combining 370 µl of 27.027 mM phosphate buffer pH 7.4 with 10 µl of 1 M DMPO, 10 µl of 125 mM titanium(III) chloride and 50 µl of 30 mM hydrogen peroxide (start). After 60 seconds, the excess peroxide was removed by 10 µl of catalase (1000–2500 IU). Following another 60 seconds, 50 µl of Fe(II) or Fe(III) solution, uncomplexed or chelated by Quin or EDTA, was added, and after 4 minutes the spectrum was recorded.

All EPR spectra were obtained at room temperature on X-band operating resonator ESR 220 (Academy of Sciences, Berlin, Germany) with following instrumental settings: microwave frequency 100 kHz, modulation amplitude 2 G (1 Gauss = 10 mT), response time 0.2 or 0.5 s, magnetic field 3380 G, sweep width 200 G, sweep time 3.5 or 4 min, microwave power 10 mW, and attenuation (gain) 12 dB. The amount of spins (radicals) was calculated from signal height and width according to Schoessler *et al.*[31] using Mn^{2+} in ZnS as standard.

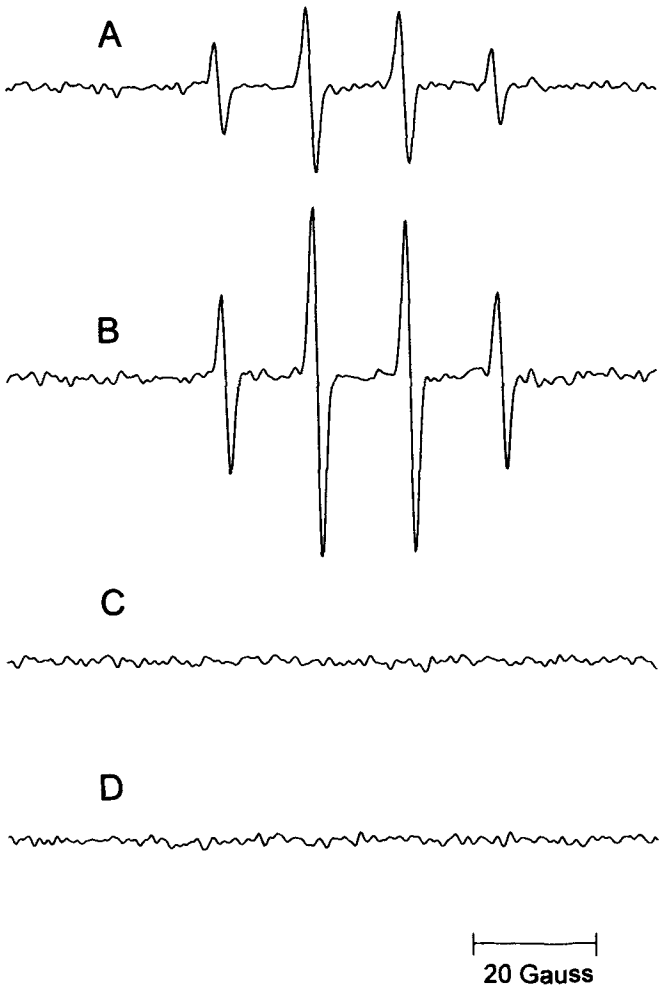

FIGURE 3 Representative results of EPR spin trapping of hydroxyl radicals formed in the Fe(II)- or Quin-Fe(II)-catalysed Fenton reaction. **A**: 8.8 mM H_2O_2, 100 mM DMPO in 21 mM phosphate buffer pH 7.4, and Fe(II) 0.1 mM. **B**: 8.8 mM H_2O_2, 100 mM DMPO in phosphate buffer, and Fe(II) 0.1 mM with Quin 0.5 mM. **C**: 8.8 mM H_2O_2, 100 mM DMPO in phosphate (Quin as well as Fe omitted). **D**: 100 mM DMPO in phosphate buffer, and 0.1 mM Fe(II) with 0.5 mM Quin (H_2O_2 omitted)

RESULTS

Fe(II) Autoxidation

The reaction of unchelated ferrous ion, i.e. hexaaquairon(II), with dioxygen in aqueous solution of pH cca 6.5–7.5, is known to follow first-order terms in iron and dioxygen, and is strongly affected by pH: the rate shows second-order dependence on hydroxide anion concentration[32]. We measured the autoxidation rates of Quin-Fe(II) complex as well as Fe(II) alone in buffer Hepes/NaOH at pH 6.6–7.8. The general finding is that chelation by Quin slows down the autoxidation, i.e. quinolinate stabilizes the iron in ferrous state (Fig. 1

A). There is still pH dependence: when, under conditions given in Fig. 1A, at pH 7.4 in the presence of Quin about 7 % of Fe(II) was lost after 30 minutes, at pH 7.8 it was 38.5 %, while at pH 7.0 only 2 % and at pH 6.6 no Fe(II) loss due to oxidation was detectable within the given period. Plotting the pseudo-first order rate constants of Quin-Fe(II) autoxidation against squared hydroxide anion concentration resulted in linear relationship (not shown) suggesting that the effect of pH is essentially analogous to that described for hexaaquairon(II)[32].

The Hepes buffer should not bind transition metals[28,33], and Fe(II) in this buffer is believed to exist as hexaaquairon(II)[28,33]. We observed similar rates of Quin-Fe(II) or unchelated Fe(II) autoxidation also in solutions buffered with borate or bicarbonate; and the rate only moderately increased when the Hepes concentration (pH 7.8) was gradually raised from 10 to 80 mM (not shown). In phosphate buffer, however, the Quin-Fe(II) autoxidation was much faster, although always slower than oxidation of Fe(II) alone, and the rate was dramatically affected by concentration of phosphate (Fig. 1 B) and also by pH (range of pH 6.6–7.8 tested, data not shown). Inorganic phosphate is known to bind ferrous iron and catalyse its autoxidation[28], so we hypothesized that phosphate might pull Fe(II) from its complex with Quin and then promote its oxidation. However, this is not the case because when phosphate buffer as well as Quin-Fe(II) solutions were purged with nitrogen gas and mixed anaerobically, the characteristic orange colour of Fe(II)-Quin complex persisted and the absorption spectrum did not substantially differ from those of pure aqueous Quin-Fe(II) solutions (results not shown). We also tested whether traces of other transition metals[27], such as copper, could be responsible for catalysis by phosphate: treatment of the buffer with Chelex 100 had essentially no effect on Quin-Fe(II) autoxidation rate, if pH and phosphate concentration were truly kept constant. The Figure 1 A also shows that at pH 7.4, Quin-Fe(II) autoxidizes slowly also in comparison to ferrous complexes of ADP or citrate. A question may arise whether ferrozine, the Fe(II) indicator used, is really able to extract all unoxidized ferrous iron from the other chelators used. However, it can be demonstrated (Table I) that if Fe(II) decay by oxidation is prevented by addition of ascorbate, the amount of ferrous iron measured as complex with ferrozine is not affected by presence of Quin, ADP or citrate. It is as expected given that affinity of ferrozine for ferrous iron is very high[26]. In other experiments we measured Quin-Fe(II) autoxidation rates at various Quin concentration. Both in Hepes and phosphate buffers the reaction rate was inversely proportional to ligand concentration (Fig. 2), further suggesting that chelation by Quin suppresses reactivity of iron towards dioxygen.

The Fenton Reaction

Although resistant to autoxidation, the ferrous quinolinate complexes are rapidly oxidized by hydrogen peroxide even when Quin is present in excess. For further examination of the ability of Quin-Fe(II) complexes to generate hydroxyl radicals in the Fenton reaction, we employed the electron paramagnetic resonance (EPR) spin trapping with 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO). Addition of Fe(II) (0.1 mM final conc.) alone or together with Quin (0.5–5 mM) into pre-mixed hydrogen peroxide (8.8 mM) and DMPO (100 mM) in 21 mM phosphate buffer pH 7.4 gave rise to the characteristic 1:2:2:1 EPR signal of DMPO-OH adduct (Fig. 3 A, B), with hyperfine splittings $a^N = a^H{}_\beta = 14.96$ G. No signal was observed when either Quin-Fe(II), or hydrogen peroxide were omitted (Fig. 3 C, D). Presence of 0.5 mM Quin approximately doubled the signal intensity in comparison with the same mixture without Quin (Fig. 3 A, B, and Fig. 4); and higher Quin concentrations (1–5mM) increased the yield of the DMPO-OH spin trap adduct even more (Fig. 4), quite as an opposite to the autoxidation rates (Fig. 2).

TABLE I Reliability of the ferrozine autoxidation assay. Ferrous iron (0.2 mM final conc.) was added to Hepes/NaOH buffer 18.75 mM pH 7.4 containing chelator 1 mM as indicated, in the presence or absence of ascorbate (Asc, 5 mM). After exactly 10 minutes standing at room temperature, the reaction mixtures were combined with 3 volumes of the ferrozine reagent (giving final conc. of iron 50 μM, chelator 250 μM and ferrozine 750 μM), and after another 11 minutes the absorbance at 562 nm was read. When ascorbate prevents the loss of Fe(II) due to autoxidation, Quin, ADP or citrate have no effect on the amount of ferous ferrozine complex, indicating that ferrozine is able to bind essentially all ferrous iron present

Chelator	*Absorbance 562 nm (mean ± SD, N=6)*	
	Asc –	*Asc +*
None	0.0204 ± 0.0029	1.4057 ± 0.0176
Quin	1.3597 ± 0.0276	1.4040 ± 0.0079
ADP	0.6131 ± 0.0115	1.4246 ± 0.0381
Citrate	0.1109 ± 0.0035	1.4166 ± 0.0325

This observation suggests that ferrous quinolinate complexes produce more hydroxyl radicals in the Fenton reaction than Fe(II) alone. However, this should be interpreted with caution since the amount of DMPO-OH detected may also reflect other, secondary reactions, such as oxidation of DMPO-OH by Fe(III) or reduction by Fe(II)[30]. In order to test this possibility, we adopted the approach of Burkitt[30]: the DMPO-OH spin trap adduct was first generated by Ti^{3+} and hydrogen peroxide in the presence of DMPO, then catalase was added to remove the residual peroxide, followed by addition of various forms of iron. The results are shown in Fig. 5, surprisingly, only addition of Quin-Fe(II) resulted in significant loss of the spin adduct, while Fe(II), Fe(III), Quin-Fe(III), or even, in contrast to the original report[30], EDTA-Fe(III) were without effect. Thus, chelation by Quin does not appear to increase the DMPO-OH signal by protecting the spin trap adduct from oxidation by Fe(III).

The enhancement of DMPO-OH formation by Quin-Fe(II) can be explained otherwise, if the following simple experiment is taken into consideration. Addition of hydrogen peroxide to an aqueous Quin-Fe(II) solution results in immediate oxidation of Fe(II) as expected. When, however, the Fe(II) detection reagent ferrozine in acetate buffer pH 5.3 is subsequently added, slow, but steady accumulation of the purple ferrozine-Fe(II) complex is observed (Fig. 6 A). No reduced iron appears when the ferrozine reagent is added to Fe(II) alone previously oxidized with hydrogen peroxide (Fig. 6 C); and neither Quin itself nor ferrozine without hydrogen peroxide are able to reduce iron when present from the beginning as Fe(III) (Fig. 6 D). The ferrozine-Fe(II) complex is stable even in the presence of a huge excess of hydrogen peroxide (data not shown). In order to explain this observation, we hypothesise that upon mixing Quin-Fe(II) and hydrogen peroxide, the hydroxyl radicals or another form of the Fenton oxidant in this simple mixture attack quinolinate and convert it into another species, able to redox cycle iron, which in its ferrous state can escape into an inert complex with ferrozine. Fig. 7 shows that the slow accumulation of ferrozine-Fe(II) complex is observable not only in acetate buffer pH 5.3, but also in phosphate pH 7.4 (condition of EPR spin trapping experiments), albeit with much lower rate. Since in these experiments an excess of hydrogen peroxide was used, ongoing oxidation of iron by remaining hydrogen peroxide must be considered. However, the redox cycling of iron in our system in principle proceeds even when the remaining hydrogen peroxide has been removed by catalase (Fig. 7); interestingly, catalase increased the rate of ferrozine-Fe(II) accumulation at pH 7.4, but decreased at pH 5.3 (Fig. 7).

DISCUSSION

We have found that ferrous quinolinate complexes autoxidize slowly in comparison to hexaaquairon(II), or iron chelated by ADP or citrate. Furthermore, the autoxidation rate is inversely

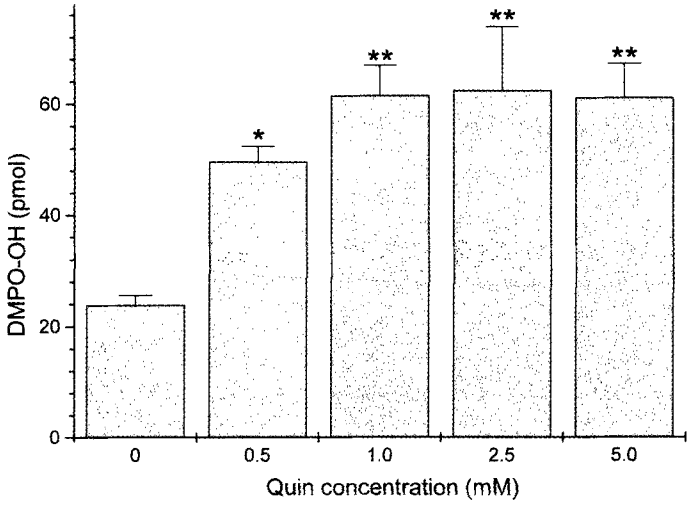

FIGURE 4 Effect of quinolinate concentration on the yield of DMPO-OH spin trap adduct. Mean+/-SEM, N=5–7 (three separate experiments). *... p<0.05, **...p<0.01 vs. control (no Quin), ANOVA, Dunnett's test

dependent on the quinolinate concentration. Our spectroscopic investigation of complex equilibria between ferrous ion and Quin in solution[17] indicates that Quin forms successively at least three complex species with Fe(II):

$$\mathrm{Fe(II)} + \mathrm{Quin} \leftrightarrow \mathrm{Fe(II)Quin} \qquad (2)$$
$$\log \beta_1 = 2.95$$

$$\mathrm{Fe(II)Quin} + \mathrm{Quin} \leftrightarrow \mathrm{Fe(II)(Quin)_2} \qquad (3)$$
$$\log \beta_2 = 5.05$$

$$\mathrm{Fe(II)(Quin)_2} + \mathrm{Quin} \leftrightarrow \mathrm{Fe(II)(Quin)_3} \qquad (4)$$
$$\log \beta_3 = 6.36$$

The equilibria extensively overlap and under all conditions employed in this study we essentially deal with a mixture of all three species[17]. When this is considered together with the dependence of autoxidation rate on ligand concentration (Fig. 2), it can be reasonably hypothesized that reactivity towards dioxygen decreases in the folowing order: unchelated Fe(II), 1:1, 1:2 and 1:3 species.

The autoxidation of Quin-Fe(II) complexes still shows dependence on pH (squared hydroxide anion concentration) and is markedly catalysed by phosphate, although phosphate is not able to pull iron from the complex with Quin. It is known that Fe(II) autoxidation in general is catalysed by various anions, e.g. pyrophosphate, phosphate, chloride, sulfate and perchlorate, which bind to Fe(II) with affinity decreasing in the named order and stabilize Fe(III) state in the transitional iron-dioxygen complex[34]. As suggested by Jewett *et al.*[34], the effect of hydroxide may be just a special case of the anion catalysis. The effects of pH or buffers on Quin-Fe(II) autoxidation that we observed, then, can be simply explained in terms of the anion catalysis, acting mainly on the small pools of unchelated and partially chelated iron, which pull the complexation equilibria (reactions 2–4) to the left.

It has been generalized that chelators with oxygen donors, e.g. EDTA, citrate or pyrophosphate, preferentially bind Fe(III) and so promote the autoxidation of Fe(II), while nitrogen donors, like phenanthroline or our Fe(II) detector ferrozine, preferentially bind and stabilize Fe(II)[28]. Our observation that quinolinate slows down iron autoxidation in comparison to citrate, ADP or hexaaquairon, seems to conform well to this

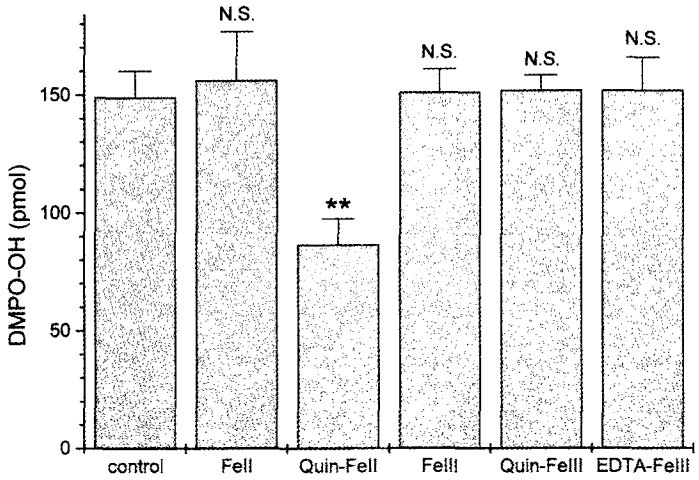

FIGURE 5 Stability of pre-formed DMPO-OH spin trap adduct in the presence of various forms of iron. DMPO-OH was generated with Ti^{3+}/H_2O_2, followed by addition of catalase and chelated or unchelated iron as described in Material and methods, giving final concentrations of Ti 2.5 mM, H_2O_2(calc. initial) 3 mM, DMPO 20 mM, catalase 0.2 mg/ml (1000–2500 IU), Fe 0.1 mM, chelator 5 mM, and phosphate buffer 20 mM, pH 7.4. Mean+/-SEM, N=5–11 (three separate experiments). N.S. ...not significant, **...$p<0.01$ vs. control, ANOVA, Dunnett's test

rule since Quin coordinates by one oxygen and one nitrogen atom and so, accordingly, its effect on iron autoxidation should lie somewhere in the middle between the oxygen and nitrogen donors.

Another important finding is that chelation by Quin substantially increases the yield of hydroxyl radicals in the Fenton reaction, measured as intensity of the EPR signal of DMPO-OH spin trap adduct. During preparation of this manuscript, the same observation has appeared in the report of Iwahashi *et al.*[35]. Using EPR spin trapping with DMPO, Iwahashi *et al.*[35] found that iron chelation by quinolinic and picolinic (pyridine-2-carboxylic) acids enhance the signal of DMPO-OH generated in the Fenton reaction in phosphate, but not carbonate buffer. The ability of iron picolinate complex to catalyse Haber-Weiss reaction has been also briefly demonstrated by Bannister *et al.*[36]. To understand how a metal chelator can enhance the yield of DMPO-OH adduct in the Fenton reaction, a variety of mechanisms can be considered. For instance Floyd[37] reported this for nucleoside di- and triphosphate iron complexes; in his experiments, however, the iron was added before hydrogen peroxide and so it could be mostly lost due to autoxidation before start of the reaction. When the author reversed the order of the additions and started the reaction with Fe(II), i.e., just as we did, the opposite was observed: the signal of DMPO-OH was stronger with Fe(II) alone than with Fe(II)-ADP complex[37]. Floyd himself concluded the influence of the chelator lies mostly in keeping iron longer divalent and so active for the Fenton reaction. Biaglow and Kachur[23], using a fluorescent probe for hydroxyl radical reported that chelation of Fe(II) by tripolyphosphate enhances the yield of hydroxyl radicals in autoxidation as well as in the Fenton reaction; and proposed that chelation by polyphosphate leads to preference of one-electron reduction of hydrogen peroxide over a two-electron, no radicals producing mechanism. In contrast, Burkitt[30]. showed for EDTA and DTPA iron chelates that the observed intensity of DMPO-OH signal does not reflect only the rate and stoichiometry of the Fenton reaction, but also other side reactions like

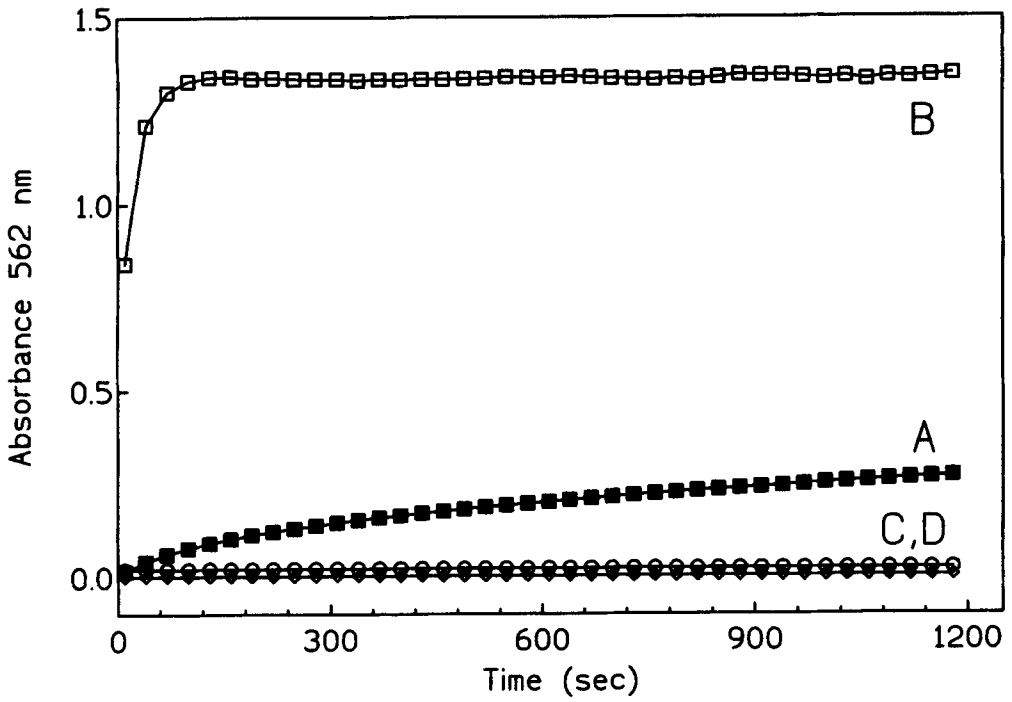

FIGURE 6 Redox cycling of iron following reaction of ferrous quinolinate complex with hydrogen peroxide. In the basic experimental setting, ferrous (0.2 mM final conc.) quinolinate (1 mM) solution in the spectrophotometric cuvette was mixed with hydrogen peroxide (4.4 mM), the resulting solution had pH 6–6.5. After exactly 1 minute, 3 volumes of 1 mM ferrozine in 0.25 M acetate buffer pH 5.3 were added; giving final conc. of Fe 0.05 mM, Quin 0.25 mM, ferrozine 0.75 mM and H_2O_2 (calc. initial) 1.1 mM. During next 20 minutes, the Fe(II) ferrozine complex formation was monitored by recording the absorbance at 562 nm. **A**: Quin-Fe(II) combined with hydrogen peroxide, then ferrozine reagent. **B**: Quin-Fe(II) combined with water instead of hydrogen peroxide, then ferrozine. **C**: Fe(II) alone combined with hydrogen peroxide, then ferrozine. **D**: Quin-Fe(III) combined with water, then ferrozine

decay of DMPO-OH spin adduct due to its oxidation by chelated-Fe(III) or redox cycling of iron by hydrogen peroxide. Although EDTA-Fe(II) reacts faster with hydrogen peroxide than DTPA-Fe(II), the DMPO-OH signal is higher with DTPA just because DMPO-OH is destroyed more efficiently by EDTA-Fe(III) than by DTPA-Fe(III)[30]. Using similar experimental protocol, however, we did not find support for the role of DMPO-OH oxidation by Fe(III) in our system since neither Fe(III), nor Quin-Fe(III) decomposed pre-formed DMPO-OH (Fig. 5). The reason why, in opposition to study of Burkitt[30], in our hands even EDTA-Fe(III) was inefficient is unclear, and perhaps could be ascribed to differences in buffer composition and/or chelator concentration used. In fact, from all forms of iron tested (Fig. 5), only Quin-Fe(II) significantly decomposed the pre-formed spin trap adduct, probably by reduction of DMPO-OH to an EPR-silent hydroxylamine. This might seem puzzling given that Quin-Fe(II) also enhances DMPO-OH generation in the Fenton reaction, however, it can well be assumed that in the presence of hydrogen peroxide all the Quin-Fe(II) is oxidized by peroxide, rather than by DMPO-OH[30].

Further experimental evidence described in the previous section indicates what emerges as the likely candidate mechanism underlying the

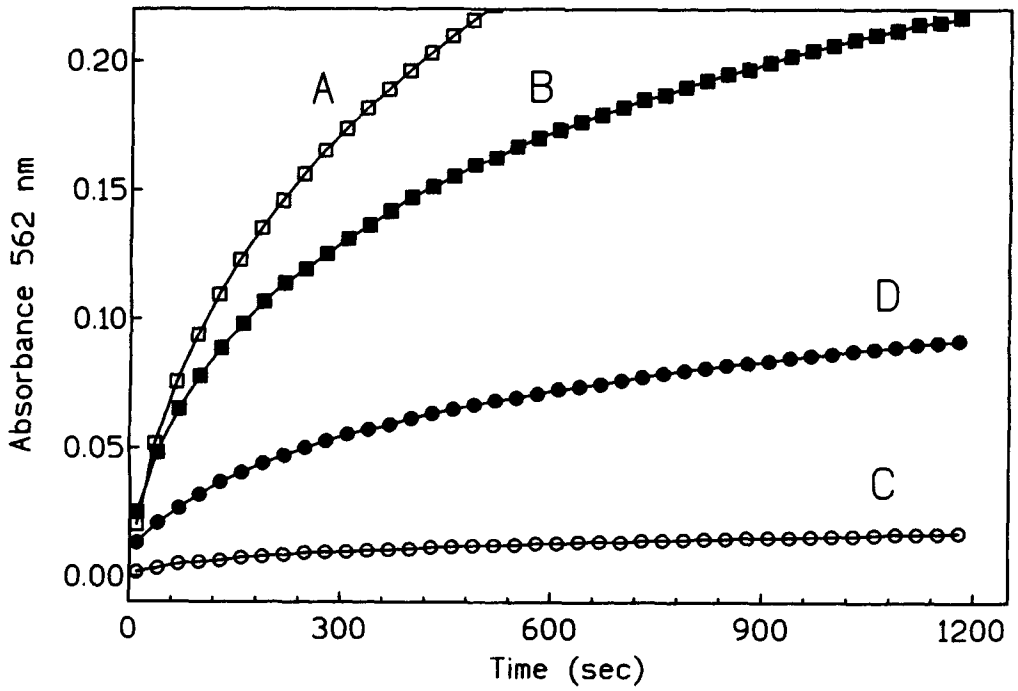

FIGURE 7 Redox cycling of iron proceeds even at pH 7.4 and following addition of catalase. Here, ferrous (0.2 mM) quinolinate (1 mM) solution was combined with hydrogen peroxide (4.4 mM), after 1 min catalase (525 IU) or water (control) was added. Following another 30 sec, the mixture was combined with 3 volumes of the ferrozine reagent either in 0.25 M acetate buffer pH 5.3, or 28 mM phosphate buffer pH 7.4, and recording of the absorbance at 562 nm began. **A**: pH 5.3; **B**: pH 5.3 + catalase; **C**: pH 7.4; **D**: pH 7.4 + catalase

apparent enhancement of the Fenton reaction by Quin: the redox cycling of iron by a quinolinate derivative, arising from the reaction between Quin and the Fenton oxidant. Yet another possibility, redox cycling of chelated Fe(III) by hydrogen peroxide, also demonstrated for EDTA and DTPA chelates[30], has not been directly addressed in our experiments and thus cannot be excluded. However, this pathway is unlikely to be the major mechanism for enhancement of DMPO-OH signal by Quin-Fe(II) since, first, the redox cycling observed upon reaction of Quin-Fe(II) with hydrogen peroxide and measured as accumulation of ferrous ferrozine complex is not abolished by addition of catalase, and, second, in phosphate buffer pH 7.4 (exactly the condition of the EPR measurements), the rate of ferrozine-Fe(II) accumulation is much higher with catalase, suggesting that hydrogen peroxide acts as an oxidant, rather than reductant for iron under this condition.

The exact nature of the redox active Quin derivative(s) remains elusive and requires further investigation. Reactions of radiolytically generated hydroxyl radicals with quinolinic acid and other pyridinecarboxylates have been studied[38–40]: the hydroxyl radicals add to the meta or ortho position with respect to the ring nitrogen atom[38,39], and decarboxylation might also occur due to ipso-addition[40]. We hypothesize that some of these reactions can lead to redox-active compounds, for that a striking, although somewhat peculiar analogy can perhaps be found in the iron chelators secreted by

some wood decay fungi. These are polyhydroxyphenolic compounds not only able to bind Fe(III), but also promote its reduction providing Fe(II) as a reagent for the Fenton reaction that ultimately allows the fungi to degrade cellulose[41]. The chelator simultaneously undergoes complex redox cycling involving semiquinone radicals and molecular oxygen. In fact, many redox active iron chelators are found among antitumor and antibiotic drugs; supposedly this property plays a key role in the therapeutic action and/or toxicity of these drugs[42].

In conclusion, chelation by quinolinate (1) slows down iron autoxidation so that the ferrous state is relatively stabilized at physiological pH, (2) but enhances the generation of hydroxyl radicals with hydrogen peroxide as far as can be assessed by the EPR spin trapping with DMPO, (3) probably because initially hydroxyl radicals attack Quin and convert it into a compound able to redox cycle iron. Altogether, the overall impression is that if such complexes occur *in vivo*, they would be long-lived and rather pro-oxidant. The cumulative stability constant of 1:3 ferrous quinolinate complex ($\log \beta_3 = 6.36$[17]) indicates that Quin is a chelator of much lower affinity for Fe(II) than compounds like EDTA ($\log K \sim 14.3$[43]) or phenanthroline ($\log \beta_3 \sim 21.3$[43]), but, on the other hand, can successfully compete with weak endogenous ligands such as citrate, which has value of log K only about 4.4[44]. It is worth noting that iron-chelating properties of quinolinic acid are essentially comparable to that of structurally related picolinic acid ($\log\beta_3 = 6.45$[17]). Unlike Quin, picolinic acid is a well-known iron chelator that has been even used as an experimental tool, for example in studies demonstrating that iron chelators inhibit growth of tumor cells in culture[45].

The mechanism of quinolinate neurotoxicity is not fully understood and involves features that cannot be explained solely by action on the NMDA type of glutamate receptors, for instance, dependence on glutamatergic afferentation[1], or possibility to prevent Quin-induced damage by co-administration with slight excess of picolinic acid[46,47]. Quinolinic acid was reported to enhance lipid peroxidation *in vitro*[13,14], and *in vivo*[15]; the neurodegeneration following intracerebral injection of Quin can be prevented by receptor-independent actions of melatonin or deprenyl[13]; and even attenuated by treatment with the spin trap α-phenyl-tert-butyl nitrone[48]. These reports suggesting a role for oxidative stress in the quinolinate neurotoxicity, however, largely do not address the question *how* is quinolinate supposed to generate the oxygen radicals. Similarly, the reason why activated macrophages produce quinolinic acid remains enigmatic[6,7]. The scope of this study does not allow for broad speculations about all possible biomedical implications, but we believe if the fact that quinolinic acid forms highly redox-active complexes with ferrous iron is appreciated, it can substantially change our view both on quinolinate neurotoxicity and physiological significance of this compound in the activated macrophage.

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