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The Lactate-Dependent Enhancement of Hydroxyl Radical Generation by the Fenton Reaction

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The effect of lactic acid (lactate) on Fenton based hydroxyl radical (•OH) production was studied by spin trapping, ESR, and fluorescence methods using DMPO and coumarin-3-carboxylic acid (3-CCA) as the •OH traps respectively. The •OH adduct formation was inhibited by lactate up to 0.4 mM (lactate/iron stoichiometry = 2) in both experiments, but markedly enhanced with increasing concentrations of lactate above this critical concentration. When the H_2O_2 dependence was examined, the DMPO-OH signal was increased linearly with H_2O_2 concentration up to 1 mM and then saturated in the absence of lactate. In the presence of lactate, however, the DMPO-OH signal was increased further with higher H_2O_2 concentration than 1 mM, and the saturation level was also increased dependent on lactate concentration. Spectroscopic studies revealed that lactate forms a stable colored complex with Fe^{3+} at lactate/Fe^{3+} stoichiometry of 2, and the complex formation was strictly related to the DMPO-OH formation. The complex formation did not promote the H_2O_2 mediated Fe^{3+} reduction. When the Fe^{3+}–lactate (1 : 2) complex was reacted with H_2O_2, the initial rate of hydroxylated 3-CCA formation was linearly increased with H_2O_2 concentrations. All the data obtained in the present experiments suggested that the Fe^{3+}–lactate (1 : 2) complex formed in the Fenton reaction system reacts directly with H_2O_2 to produce additional •OH in the Fenton reaction by other mechanisms than lactate or lactate/Fe^{3+} mediated promotion of Fe^{3+}/Fe^{2+} redox cycling.

Keywords: Lactic acid, •OH, Fenton reaction, spin trapping ESR, Fe^{3+}–lactate complex, generation, •OH enhancement

Abbreviations: ESR, electron spin resonance; DMPO, 5,5-dimethyl-1-pyrroline-*N*-oxide; DMPO-OH, 2-hydroxy-5,5-dimethyl-1-pyrroline-*N*-oxide; 3-CCA, coumarin-3-carboxylic acid; 7-OHCCA, 7-hydroxycoumarin-3-carboxylic acid

INTRODUCTION

The Fenton based generation of hydroxyl radical (•OH) has been extensively discussed in oxidative damage in physiological systems[1–11] because •OH is implicated as the major active species

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involved in oxidative degradation of many biological constituents such as DNA,[1] protein,[2] lipids[3] and carbohydrates[4] which eventually lead to various pathological disorders such as cancer,[5] rheumatoid arthritis,[6] diabetes,[7] aging,[8] Parkinson's disease,[9] and Alzheimer's disease.[10] Thus the modulation of intracellular $^{\bullet}OH$ generation is quite important to control these diseases or tumor cell necrosis.

Several chemical systems have been reported to enhance $^{\bullet}OH$ generation. They are mainly metal chelator mediated reactions. For example, 2-oxo-4-thiomethylbutyric acid forms an iron complex which accelerates H_2O_2 decomposition to enhance $^{\bullet}OH$ production.[12] Likewise, gallic acid,[13] carnosic acid,[13] catechol,[14] vitamin B and related compounds[15] have been reported to exhibit both antioxidant and prooxidant activity in the presence of redox active metals such as iron. Nappi *et al.* recently reported that well known radical scavengers such as glutathione, cysteine and ascorbic acid enhanced the $^{\bullet}OH$ production in $Fe^{2+}/EDTA/H_2O_2$ system.[14] In these chemical systems, the enhanced production of $^{\bullet}OH$ was explained by the interaction between the metal chelate and these molecules, which promoted redox cycling of bound metal to facilitate Fenton like reaction. However, the precise mechanism is unknown.

Recently, we showed that α-hydroxy acids (typically lactic acid) enhanced $^{\bullet}OH$ generation in the Fenton reaction as assessed by ESR spin trapping.[16,17] Since lactic acid is a common physiological molecule accumulated in tissues especially under anoxic conditions, it is probable that the lactic acid mediated enhancement of $^{\bullet}OH$ production might play a critical role in the progression of ischemia–reperfusion injury.

Therefore, it is important to clarify how lactic acid modifies the Fenton reaction to increase $^{\bullet}OH$ production. Results reveal that lactate interacts with Fe^{3+}, the primary Fenton product, to form a stable Fe^{3+}–lactate complex at the molar ratio of 1 : 2 which then reacts with H_2O_2 to enhance the $^{\bullet}OH$ generation.

MATERIALS AND METHODS

Materials

5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) was purchased from LABOTEC Co. Ltd. (Japan) and was used without further purification. Coumarin-3-carboxylic acid (3-CCA), 7-hydroxycoumarin-3-carboxylic acid (7-OHCCA) and *ortho*-phenanthroline were purchased from Wako Chemical Co. Ltd. (Japan). Hydrogen peroxide (H_2O_2 31% w/v) was from Mitsubishi Gas Co. Ltd. (Japan). Lactic acid (98%), $FeSO_4$, and $FeCl_3$, were purchased from Sigma Chemical Co. Ltd. (USA). All other chemicals used were commercially available products of the highest reagent grade.

ESR Spin Trapping Assay

For the ESR spin trapping study, Fenton reaction was carried out in 300 µl aqueous solution in a micro test tube, containing 15 mM of H_2O_2, 0.2 mM of $FeSO_4$ and 20 mM of DMPO. Metal free re-distilled water was used as the solvent for all reagents. Aliquots of lactic acid stock solution (10 mM) were added to the above reaction mixture to make the final concentrations as indicated. Metal free water was added instead of lactate sample for the control reaction. In the experiment carried out in 15 mM Na-phosphate buffer (pH 7.5), the concentration of Fenton reagents was 10 mM H_2O_2, 0.1 mM $FeSO_4$ and 20 mM DMPO. The Fenton reaction was initiated by adding an aliquot of $FeSO_4$ stock solution finally, then 30 µl of the reaction mixture was taken into a disposable micro pipette (Drummond) and the ESR signal was measured at 1 min after the $FeSO_4$ addition. For examining H_2O_2-dependent formation of DMPO-OH, H_2O_2 concentration was varied under the condition where lactic acid and iron concentrations were kept constant at 1.5 and 0.2 mM, respectively.

ESR spectra were recorded by JEOL JES-TE 200 ESR spectrometer (X-Band Microwave Unit). Spectrometer setting was as follows: microwave power,

8 mW; microwave frequency, 9.20 GHz; modulation amplitude, 0.1 mT; time constant, 0.03 s; sweep time, 1 min; center fields, 332.6/322.6 mT.

Coumarin-3-carboxylic Acid Hydroxylation Assay

Hydroxyl radical mediated 7-OHCCA formation was determined by the fluorescence increase at 450 nm (excited at 380 nm)[18] using a Hitachi F-4500 Fluorescence Spectrophotometer. The reaction was carried out at room temperature (22°C) in a 3 ml quartz cuvette containing 10 mM of H_2O_2, 0.2 mM of $FeSO_4$, 20 μM of CCA and various concentrations of lactic acid. Additional reaction conditions are described in the figure legends. Metal free re-distilled water was used for all experiments.

Spectroscopic Determination of Fe^{3+}–Lactate Complex Formation

Various concentrations of lactic acid were reacted with 0.5 mM $FeCl_3$ in a cuvette for 2 min at room temperature, then the formation of Fe^{3+}–lactate complex and decomposition of aqua complex of Fe^{3+} were determined by the absorbance changes at 350 and 300 nm, respectively, using a Hitachi U-3000 UV–V spectrophotometer.

Determination of Fe^{3+} Reduction by Fe^{2+}–*ortho*-Phenanthroline Complex Formation

Kinetic reduction of Fe^{3+} was determined by the increase of absorbance at 512 nm due to the formation of Fe^{2+}–*ortho*-phenanthroline complex in a reaction mixture containing 0.1 mM of $FeCl_3$, 3 mM of H_2O_2, 0.3 mM of lactic acid and 0.3 mM of *ortho*-phenanthroline. The reaction was initiated by H_2O_2 addition.

RESULTS

The effect of lactic acid on the Fenton reaction was examined by ESR using DMPO as a spin trap in aqueous medium (Figure 1(a)). Result showed

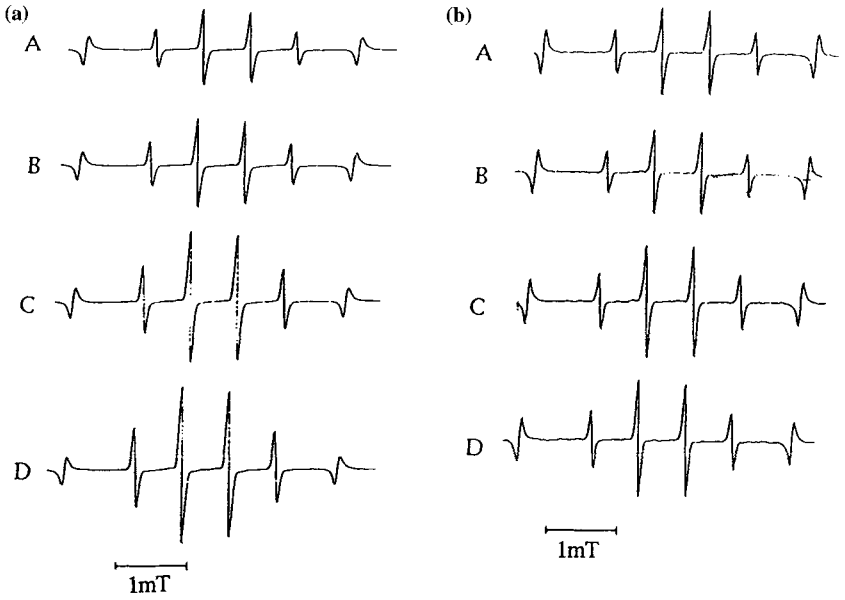

FIGURE 1 Lactate mediated enhancement of DMPO-OH formation in Fenton reaction. Reaction conditions and ESR measurement are described in the Methods section. (a) In aqueous medium; (b) in Na-phosphate buffer (pH 7.5). In (a), (A) without lactic acid, (B) with 0.5 mM lactic acid, (C) with 0.8 mM lactic acid, (D) with 1 mM lactic acid. In (b), (A) without lactic acid, (B) with 0.2 mM lactic acid, (C) with 0.5 mM lactic acid, (D) with 1 mM lactic acid.

that lactic acid markedly enhanced the DMPO-OH signals. When lactic acid was substituted for Na-lactate, the same enhancement of the DMPO-OH signal was observed (data not shown) indicating that lactic acid is taking part in the enhancing reaction as lactate. The enhancing effect by lactic acid was also determined in the presence of Na-phosphate buffer (pH 7.5) even though the extent was smaller than that observed in aqueous medium, possibly due to the $^{\bullet}OH$ scavenging effect of buffer (Figure 1(b)). Thus, the acidification of the reaction mixture was not the cause of the DMPO-OH enhancement. To simplify the reaction system, the following experiments were carried out in an aqueous medium.

When the concentration dependence of lactic acid on the Fenton reaction was precisely examined, the DMPO-OH formation was rather inhibited up to 0.4 mM where the lactic acid/iron stoichiometry was calculated to be 2. However, above this concentration, the DMPO-OH formation was markedly enhanced as lactic acid concentration was increased (Figure 2(a)). Identical results were obtained when the $^{\bullet}OH$ generation was fluorometrically determined using 3-CCA as an $^{\bullet}OH$ trap (Figure 2(b)). 3-CCA reacts with $^{\bullet}OH$ to form a stable fluorescence product, 7-OHCCA.[18] Thus it was confirmed that lactate enhances $^{\bullet}OH$ generation in the Fenton reaction.

To determine whether the lactate itself interacts directly with DMPO-OH, the effect of lactic acid was studied on the DMPO-OH produced by UV (305 nm) irradiation of DMPO/H_2O_2 for 2 min. The DMPO-OH signals obtained at 1 min after the irradiation were the same with and without lactic acid addition indicating that the lactate (or lactic acid) itself does not affect the DMPO-OH spin adducts (data not shown). Lactic acid/DMPO mixture without H_2O_2 does not show any ESR signal by the UV irradiation.

To see the effect of lactate on the decay of DMPO-OH generated in the Fenton reaction, lactic acid was added to Fe^{2+}/H_2O_2 system containing DMPO at various time periods after onset of the Fenton reaction (Figure 3). The DMPO-OH signal formed rapidly decreased in the control Fenton reaction (without lactic acid) with the half decay time approximately 20 min, consistent with the result reported previously.[19] When lactic acid was added shortly after onset of the reaction,

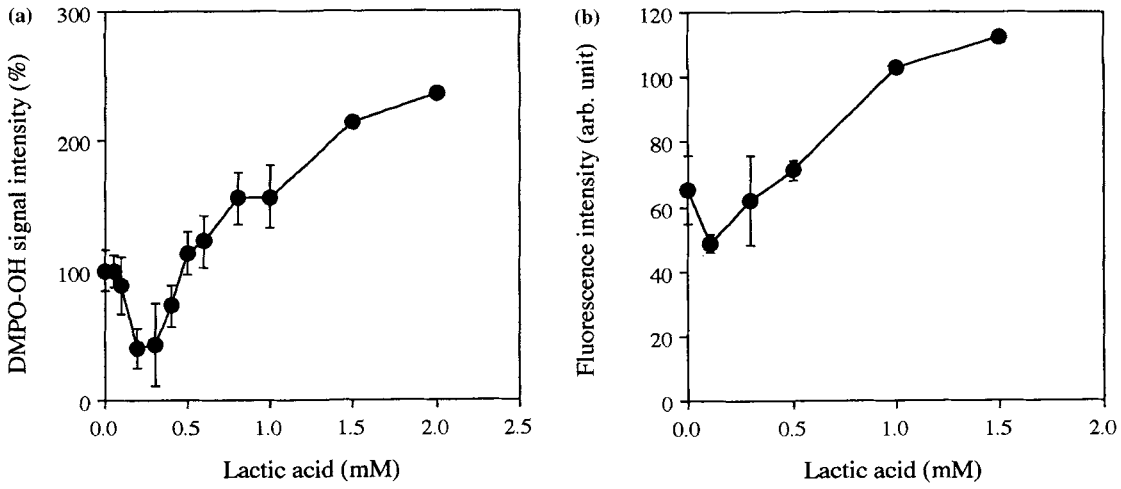

FIGURE 2 Lactate dependence of DMPO-OH and 7-OHCCA formation in Fenton reaction. (a) DMPO-OH formation; (b) 7-OHCCA formation. Reaction conditions for DMPO-OH and 7-OHCCA formation are described in the Methods section. DMPO-OH was determined by ESR spin trapping; 100% indicates DMPO-OH signal intensity obtained for control Fenton reaction (without lactic acid). 7-OHCCA formation was detected by fluorometrically using 380 and 450 nm for excitation and emission wavelengths, respectively. Data points are mean ± standard deviation of six determinations in two independent experiments. No error bars are shown when the deviation is smaller than the symbol.

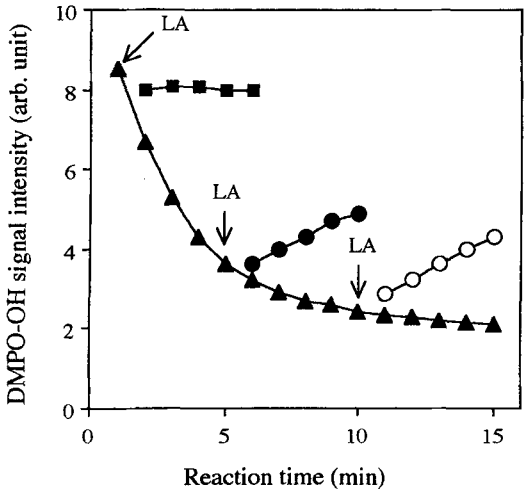

FIGURE 3 Lactate-dependent DMPO-OH formation during Fenton reaction. The condition for the DMPO-OH measurement is given in the Experimental section. The reaction mixture contained 15 mM H_2O_2, 1 mM lactic acid, 150 mM DMPO and 0.2 mM $FeSO_4$. ▲, control Fenton; ■, lactic acid added at 1 min after reaction start; ●, lactic acid added at 5 min after reaction start; ○, lactic acid added at 10 min after reaction start. Data points are the mean of three determinations.

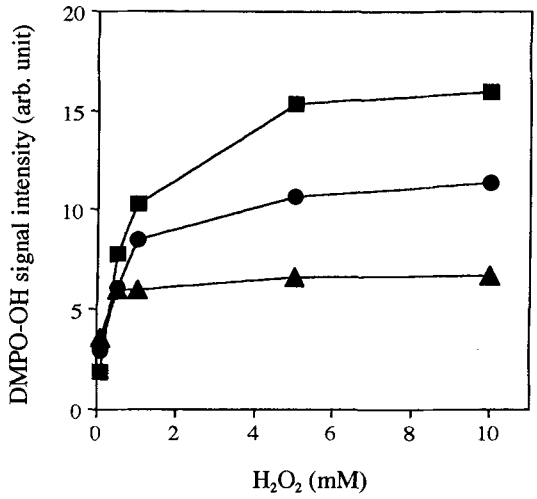

FIGURE 4 H_2O_2 dependence of DMPO-OH formation in Fenton reaction. DMPO-OH spectrum was recorded at 3 min after $FeSO_4$ addition to initiate the reaction. Final concentrations of $FeSO_4$ and DMPO were 0.1 mM and 300 mM, respectively. ▲, without lactic acid; ●, with 1 mM lactic acid; ■, with 10 mM lactic acid. The data points are the mean of three determinations.

the decay of DMPO-OH signal was significantly inhibited, and the signal was maintained at the level observed at the time before the lactic acid addition. When the lactic acid was added at 5 or 10 min after the reaction start, the DMPO-OH signals formed were weak but were found to increase time-dependently. These results above indicated that lactate interacted directly with neither DMPO nor DMPO-OH but reacted with other chemical species present in the reaction mixture to generate additional •OH, thus, enhancing the DMPO-OH signal.

H_2O_2 dependence of the DMPO-OH formation was examined at a fixed Fe^{2+} concentration (0.1 mM) in the presence and absence of lactic acid (Figure 4). The DMPO-OH signal increased linearly with H_2O_2 concentration up to approximately 0.5 mM then saturated in the absence of lactic acid, and no more increase was observed even if H_2O_2 concentration was increased up to 10 mM, consistent with the observation reported by Mizuta *et al.*[20] that the initial •OH generation in the Fenton reaction was primarily determined by the stoichiometric reaction between Fe^{2+} and H_2O_2. On the other hand, when lactic acid was present in the system, the signal was further increased with increased concentrations of H_2O_2 higher than 0.5 mM, however, the initial H_2O_2-dependent growth phase of DMPO-OH signal was the same as in the absence of lactic acid. It was further noted that the H_2O_2-dependent DMPO-OH signal was more markedly enhanced with higher concentrations of lactic acid. These results as well as the observations in Figure 3 indicated that the primary Fenton reaction is not affected by lactate but certain lactate-dependent reactions operated to produce additional •OH in the reaction mixture.

Therefore, the interaction between lactate and Fe^{3+} was spectroscopically studied. When lactic acid was added to an aqueous $FeCl_3$ solution, the absorbance at 350 nm was increased linearly due to the formation of Fe^{3+}–lactate complex, and the absorbance at 300 nm decreased due to the loss

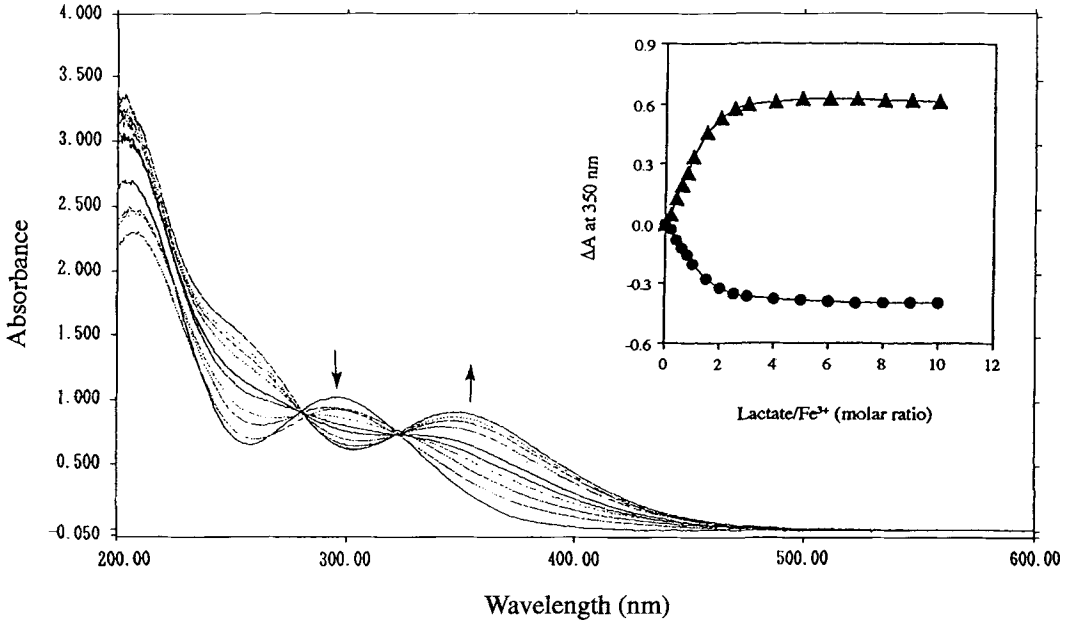

FIGURE 5 Lactate-dependent spectral change of Fe^{3+} aqueous solution. To 0.5 mM Fe^{3+} aqueous solution, lactic acid was added to make the following final concentrations: 0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.75, 1.0, 1.25, 1.5, 2.0, 2.5, 3, 3.5, 4, and 4.5 mM. ↓ indicates the absorbance decrease due to decomposition of aqua complex of Fe^{3+}, and ↑ indicates absorbance increase due to formation of lactate/Fe^{3+} complex. The spectra were recorded at 2 min after mixing Fe^{3+} solution with lactic acid. The data were replotted in the figure insetted.

of the aqua complex of Fe^{3+}. The presence of isosbestic points at 322 and 280 nm indicates that the ligand exchange occurred quantitatively between the aqua and the lactate complexes (Figure 5). From the titration curve (Figure 5, inset), the lactate/Fe^{3+} stoichiometry in the complex was determined as 2.

To evaluate the role of the Fe^{3+}–lactate complex in the •OH enhancing reaction, both DMPO-OH and Fe^{3+}–lactate complex formations were determined at various lactate/Fe^{3+} ratio, then the increased fraction of DMPO-OH (% of control Fenton signal) was plotted against the amount of the Fe^{3+}–lactate complex formed (the net change of absorbance at 350 nm in Figure 6). A good correlation was obtained between the DMPO-OH formation and the Fe^{3+}–lactate complex formation.

Since the acceleration of redox cycling of iron is implicated in many other chemical systems

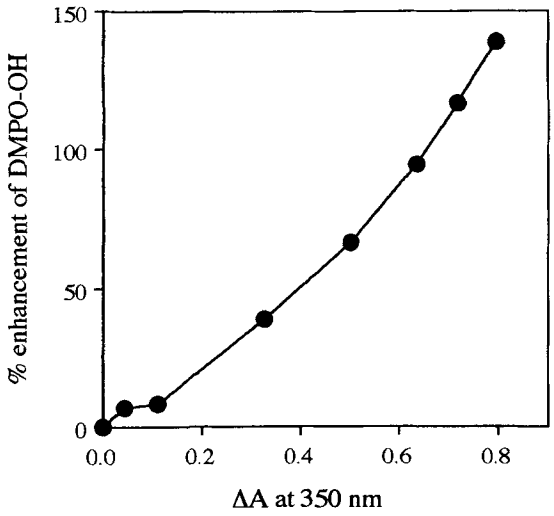

FIGURE 6 Relationship between Fe^{3+}–lactate complex formation and enhanced fraction of DMPO-OH. Reaction conditions were described in the Methods section. The increased fraction of DMPO-OH (% increase of control Fenton signal) was plotted against the net change of absorbance at 350 nm (ΔA 350 nm).

that enhance •OH generation,[12–15,21–25] Fe^{3+} reduction was kinetically determined in the presence and absence of lactic acid by Fe^{2+}–*ortho*-phenanthroline complex formation assay.

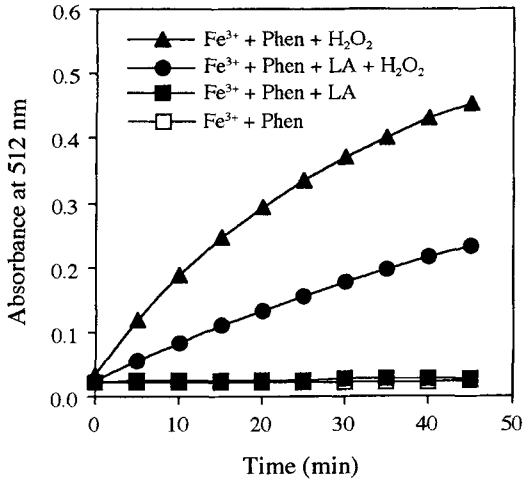

FIGURE 7 Inhibition of H_2O_2 mediated Fe^{3+} reduction by lactic acid. Fe^{3+} reduction was followed by absorbance change at 512 nm due to the formation of *ortho*-phenanthroline/Fe^{2+} complex at 22°C. The reaction mixture contained 0.3 mM *ortho*-phenanthroline, 0.3 mM lactic acid, 0.1 mM $FeCl_3$ and 3 mM H_2O_2.

ortho-Phenanthroline is known to bind Fe^{2+} to form a specific colored complex with the λ_{max} at 512 nm.[26] Results indicated that lactic acid (or lactate) did not accelerate but inhibited the H_2O_2-dependent Fe^{3+} reduction (Figure 7). The same inhibitory effect of lactic acid was determined when ferrozine was used as Fe^{2+}-chelator instead of *ortho*-phenanthroline (data not shown).

The role of the Fe^{3+}–lactate complex was further studied fluorometrically. When the preformed Fe^{3+}–lactate (1 : 2) complex was reacted with H_2O_2 in the presence of 3-CCA, the 7-OHCCA fluorescence increased with time. Further, the initial rate of the 7-OHCCA formation was linearly increased with H_2O_2 concentrations up to 0.4 mM, thus the reaction between the Fe^{3+}–lactate complex and H_2O_2 followed pseudo-first-order kinetics (Figure 8) although the reaction profile changed at higher H_2O_2 concentrations. On the other hand, no detectable amount of 7-OHCCA fluorescence was formed when Fe^{3+} was reacted with H_2O_2 during the first 5 min observed even with increased H_2O_2 concentrations. The result thus indicated that the Fe^{3+}–lactate (1 : 2) complex formed in the Fenton

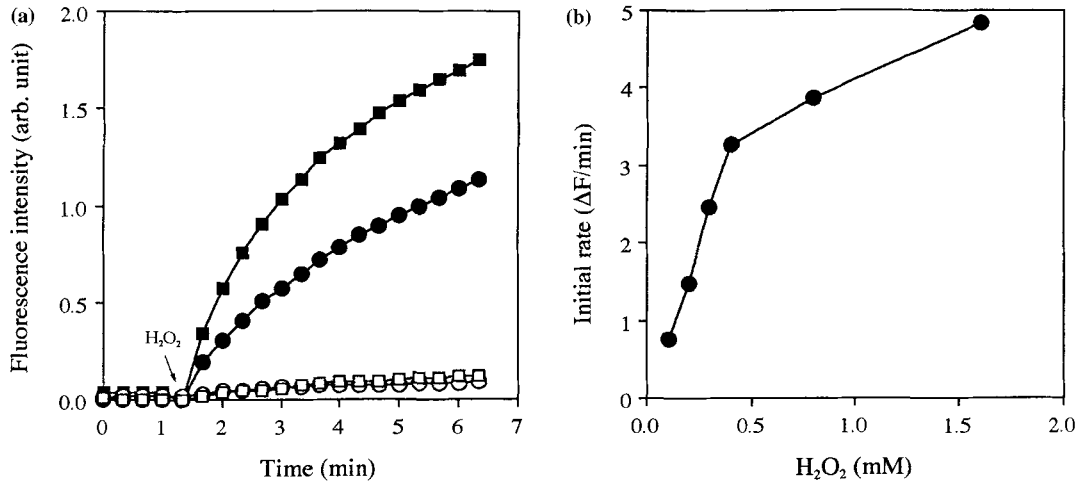

FIGURE 8 Kinetics of 7-OHCCA formation by the reaction of Fe^{3+}–lactate and H_2O_2. The 7-OHCCA formation was kinetically determined by the fluorescence change at 450 nm (excitation at 380 nm) after the addition of a series amounts of H_2O_2 to the reaction mixture containing 3-CCA (20 µl), Fe^{3+}/lactate (0.1 mM/0.2 mM) or Fe^{3+} (0.1 mM). In (a), ■, 0.4 mM H_2O_2 with Fe^{3+}/lactate complex; □, 0.4 mM H_2O_2 with Fe^{3+}; ●, 0.2 mM H_2O_2 with Fe^{3+}/lactate complex; ○, 0.2 mM H_2O_2 with Fe^{3+}. In (b), initial rate of 7-OHCCA formation was plotted against H_2O_2 concentrations.

reaction mixture successively reacted with H_2O_2 to give rise to $^{\bullet}OH$ formation.

DISCUSSION

In the present experiments, the formation of a Fe^{3+}–lactate complex (1 : 2) was found to be a prerequisite for the lactate mediated enhancement of $^{\bullet}OH$ generation in the Fenton reaction. The $^{\bullet}OH$ trapping studies by both DMPO and 3-CCA (Figures 2 and 4) clearly indicated that the $^{\bullet}OH$ generation in the lactate-modified Fenton reaction resulted from a composite of two distinct $^{\bullet}OH$ generating processes, that is, the initial lactic acid-independent Fenton reaction and the following lactic acid-dependent reaction. The latter reaction was also dependent on H_2O_2 concentration. The enhanced $^{\bullet}OH$ generation in the lactate-modified Fenton reaction was mainly attributed to the latter reaction in that a complex of lactate and Fe^{3+} with the stoichiometry of 2 : 1 is involved (Figure 6). Lactate enhanced the $^{\bullet}OH$ generation only when its concentration was higher than that required for the complex formation, and the amount of complex formed was finely correlated with the enhanced fraction of $^{\bullet}OH$ generation. When the reaction of the Fe^{3+}–lactate (1 : 2) complex with H_2O_2 was further studied kinetically by 7-OHCCA formation, the initial rate of 7-OHCCA formation was linear with H_2O_2 concentration up to 0.4 mM (Figure 8) indicating that the complex reacts directly with H_2O_2 to produce $^{\bullet}OH$.

Metal-chelate mediated $^{\bullet}OH$ generations in the Fenton system have been discussed in several reports[12–15,21–25] and the $^{\bullet}OH$ enhancement was rationalized as a result of either superoxide anion radical ($O_2^{\bullet-}$) or H_2O_2 mediated redox cycling of metal ions. For example, Gutteridge *et al.*[21] showed that the Fe^{3+}–EDTA complex directly reacts with H_2O_2 to form EDTA–Fe^{3+}–OOH^- complex, reacting further with H_2O_2 to form a ferryl–EDTA complex (FeO^{2+}–EDTA) and hydroperoxyl radical $HO_2^{\bullet}$ (protonated superoxide radical). The $O_2^{\bullet-}$ thus formed reduces Fe^{3+}–EDTA to Fe^{2+}–EDTA which then acts by Fenton chemistry to produce $^{\bullet}OH$. The $O_2^{\bullet-}$-dependent $^{\bullet}OH$ formation from other ferric-chelates and H_2O_2 was also shown by Gutteridge.[22]

A similar mechanism could be drawn for the lactate-modified Fenton reaction. The Fe^{3+} produced by the primary Fenton reaction is trapped by lactates to form a stable Fe^{3+}–lactate complex, which might react directly with H_2O_2 to form $O_2^{\bullet-}$. The $O_2^{\bullet-}$ produced then could mediate a redox recycle of iron to attenuate the $^{\bullet}OH$ generation. This idea, however, was not supported by the previous experiment[17] that SOD, an enzyme to dismutate $O_2^{\bullet-}$, did not inhibit the enhancing effect of lactate at all, whereas the presence of catalase completely inhibited the $^{\bullet}OH$ generation. Thus the $O_2^{\bullet-}$ mediated reduction of Fe^{3+}–lactate to Fe^{2+}–lactate did not take place in the lactate enhanced $^{\bullet}OH$ production in the Fenton system.

Sandstrom *et al.*[25] reported the stimulatory effect of Fe^{3+}–quin2 complex on $^{\bullet}OH$ production in the Fenton reaction. In the report, H_2O_2 mediated direct reduction of the Fe^{3+}–quin2 to Fe^{2+}–quin2 complex was shown as the cause of the $^{\bullet}OH$ generation. Likewise, Nappi *et al.*[14] reported the prooxidant activity of glutathione and ascorbate which are known radical scavengers. Both Fe^{3+} and O_2 are reduced by these scavenger molecules directly to form Fe^{2+} and $O_2^{\bullet-}$, respectively, which in turn produces $^{\bullet}OH$ by a Haber–Weiss type reaction. Another example of the enhanced production of $^{\bullet}OH$ was obtained by catechols which are oxidized either by molecular oxygen or Fe^{3+} to generate semiquinones, superoxide and H_2O_2.[27,28] Thus in all these reaction systems, the enhanced production of $^{\bullet}OH$ was explained as the result of attenuated iron redox cycling.

Our results, on the contrary, showed that, although the lactate formed a stable Fe^{3+}–lactate complex, the complex did not effect the redox cycling of iron as a cause of enhanced $^{\bullet}OH$ production because lactate did not enhance the H_2O_2

mediated Fe^{3+} reduction but considerably inhibited it (Figure 7). Indeed, no correlation was observed between the lactic acid-dependent $^{\bullet}OH$ formation and the rate of H_2O_2 mediated Fe^{3+} reduction (data not shown). Therefore, the role of Fe^{3+}–lactate complex is different from other chemical systems reported so far to enhance the production of $^{\bullet}OH$ in the Fenton system.

Although further studies are needed to clarify the precise mechanism of $^{\bullet}OH$ production due to direct interaction between the Fe^{3+}–lactate complex and H_2O_2, and also the chemical species playing as an electron donor, the present findings suggest a possible involvement of the Fe^{3+}–lactate complex in the tissue damaging process after ischemia–reperfusion because large amounts of lactate are well known to be formed during the early hypoxic condition, and also free iron is released from the cell during hypoxia/reoxygenation.[29]

Acknowledgments

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References

[1] B. Halliwell and O.I. Aruoma (1993) *DNA and Free Radicals*. Ellis Harwood, pp. 67–93.
[2] T. Reinheckel, B. Nedelev, J. Prause, W. Augustin, H.U. Schulz, H. Lippert and W. Halangk (1998) Occurrence of oxidatively modified proteins: An early event in experimental acute pancreatitis. *Free Radical Biology and Medicine*, **24**, 393–400.
[3] K.H. Cheeseman (1993) Lipid peroxidation and cancer. In: B. Halliwell and O.I. Aruoma (Eds.), *DNA and Free Radicals*, Ellis Harwood, pp. 109–144.
[4] O.I. Aruoma (1993) *Free Radicals in Tropical Diseases*. London Harwood Academic Publishers.
[5] P.A. Cerutti (1994) Oxy-radicals and Cancer. *Lancet*, **344**, 862–863.
[6] B. Halliwell (1990) In: B. Henderson, J.C.W. Edward and E.R. Pettipher (Eds.), *Free Radical and Rheumatoid Disease*, Academic Press, London, pp. 301–316.
[7] S. Roy, C.K. Sen, H.J. Tritschler and L. Packer (1997) Modulation of cellular reducing equivalent homeostasis by alpha-lipoic acid. Mechanism and implications for diabetes and ischemic injury. *Biochemical Pharmacology*, **53**, 393–399.
[8] K.B. Beckman and B.N. Ames (1997) Oxidant, Antioxidant, and Aging. In: J.G. Scandalios (Ed.), *Oxidative Sress and the Molecular Biology of Antioxidant Defenses*, Cold Spring Harbor Laboratory Press, pp. 201–246.
[9] E.C. Hirsch and B.A. Faucheux (1998) Iron metabolism and Parkinson's disease. *Movement Disorders*, **13**, 39–45.
[10] D.A. Loeffler, J.R. Connor, P.L. Juneau, B.S. Snyder, L. Kanaley, A.J. DeMaggio, H. Nguyen, C.M. Brickman and P.A. LeWitt (1995) Transferrin and iron in normal, Alzheimer's disease, and Parkinson's disease brain regions. *Journal of Neurochemistry*, **65**, 710–724.
[11] B. Halliwell, C.E. Cross and J.M.C. Gutteridge (1992) Free radicals, antioxidant and human diseases. Where are we now? *Journal of Laboratory Clinical Medicine*, **119**, 598–620.
[12] G.W. Winston, O.M. Eibchutz, T. Strekas and A.I. Cederbaum (1986) Complex-formation and reduction of ferric iron by 2-oxo-4-thiomethylbutyric acid, and the production of hydroxyl radicals. *Biochemical Journal*, **235**, 521–529.
[13] C. Smith, B. Halliwell and O.I. Aruoma (1992) Protection by albumin against the pro-oxidant actions of phenolic dietary components. *Food Chemistry and Toxicology*, **30**, 483–489.
[14] A.J. Nappi and E. Vass (1997) Comparative studies of enhanced iron-mediated production of hydroxyl radical by glutathione, cysteine, ascorbic acid, and selected catechols. *Biochimica et Biophysica Acta*, **1336**, 295–301.
[15] M.L. Hu, Y.K. Chen and Y.F. Lin (1995) The antioxidant and prooxidant activity of some B vitamins and vitamin-like compounds. *Chemico-Biological Interactions*, **97**, 63–73.
[16] M.A. Ali, K. Oykawa, M. Ohwada and T. Konishi (1997) Identification of Chlorella T-1 ingredient which enhances DMPO-OH adduct formation in Fenton reaction. *Biochemistry and Molecular Biology International*, **43**, 787–797.
[17] M.A. Ali and T. Konishi (1998) Enhancement of hydroxy radical generation in the Fenton reaction by alpha-hydroxy acid. *Biochemistry and Molecular Biology International*, **46**, 137–145.
[18] Y. Manevich, K.D. Held and J.E. Biaglow (1997) Coumarin-3-carboxylic acid as a detector for hydroxyl radicals generated chemically and by gamma radiation. *Radiation Research*, **148**, 580–591.
[19] S. Matsui, M. Nonaka, T. Nakai and T. Inouye (1997) Fast imaging of the second moment using magic echo trains. *Solid State Nuclear Magnetic Resonance*, **10**, 39–44.
[20] Y. Mizuta, T. Masumiza, M. Kohno, A. Mori and L. Packer (1998) Kinetic analysis of the Fenton reaction by ESR-spin trapping. *Biochemistry and Molecular Biology International*, **43**, 1107–1120.
[21] J.M.C. Gutteridge, L. Maidt and L. Poyer (1990) Superoxide dismutase and Fenton chemistry: Reaction of ferric-EDTA complex and ferric-bipyridyl complex with hydrogen peroxide without the apparent formation of iron(II). *Biochemical Journal*, **269**, 169–174.
[22] J.M.C. Gutteridge (1990) Superoxide dependent formation of hydroxyl radical from ferric complexes and hydrogen peroxide: An evaluation of fourteen iron chelates. *Free Radical Research Communications*, **9**, 119–125.
[23] H. Iwahashi, H. Morishita, T. Ishii, R. Sugata and R. Kido (1989) Enhancement by catechols of hydroxyl-radical formation in the presence of ferric ions and hydrogen peroxide. *Journal of Biochemistry*, **105**, 429–434.
[24] D.N.R. Rao and A.I. Cederbaum (1997) A comparative study of the redox-cycling of a quinone (rifamycin s)

and a uinonimine (rifabutin) antibiotic by rat liver microsomes. *Free Radical Biology and Medicine*, **22**, 439–446.

[25] B.E. Sandstrom, P. Svoboda, M. Granstrom, M.H. Ringdahl and L.P. Candeias (1993) H_2O_2-driven reduction of the Fe^{3+}-quin2 chelate and the subsequent formation of oxidizing species. *Free Radical Biology and Medicine*, **23**, 744–753.

[26] A.E. Harvey Jr., J.A. Smart and E.S. Amis (1955) Determination of Fe(II) and total Fe with 1,10-phenanthroline. *Analytical Chemistry*, **27**, 26–29.

[27] K. Reszka, J.W. Lown and C.F. Chignell (1992) Photosensitization by anticancer agents – 10. ortho-Semiquinone and superoxide radicals produced during anthrapyrazole-sensitized oxidation of catechols. *Photochemistry Photobiology*, **55**, 359–366.

[28] H. Hohl, L. Gille and A.V. Kozlov (1998) Antioxidant-derived prooxidant formation from ubiquinol. *Free Radical Biology and Medicine*, **25**, 666–675.

[29] M.S. Paller and B.E. Hedlund (1994) Extra cellular iron chelators protect kidney cells from hypoxia/reoxygenation. *Free Radical Biology and Medicine*, **17**, 597–603.