Enhancement of Lipid Peroxidation by Chromium(IV) and Chromium(V) is Remarkable Compared to That by Chromium(VI) and is Effectively Suppressed by Scavengers of Reactive Oxygen Species

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It is assumed that oxidative stress due to reactive oxygen species (ROS) such as superoxide anion ($\cdot O_2^-$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($\cdot OH$) may result in oxidative damage to lipids and DNA. On the other hand, chromium(VI) compounds, potent carcinogens, are known to induce lipid peroxidation. However, it is not clear how Cr(VI) induces lipid peroxidation and DNA damage. In this article, in order to clarify the mechanism by which Cr(VI) enhances lipid peroxidation, the lipid-peroxidizing activity of Cr(IV) and Cr(V), Cr intermediates possibly produced in the reductive metabolism of Cr(VI), were compared with that of Cr(VI) and Cr(III), and the inhibitory effects of ROS scavengers on lipid peroxidation induced by Cr(IV) and Cr(V) were examined by incubating mouse liver homogenate with Cr and scavenger compounds. Cr(IV), Cr(V) and Cr(VI) compounds effectively enhanced lipid peroxidation of homogenate in this order. The lipid-peroxidizing effect of Cr(IV) was suppressed more remarkably by superoxide dismutase, catalase and formate, specific scavengers of $\cdot O_2^-$, H_2O_2 and $\cdot OH$, respectively, than that of Cr(V), which is indicative of the involvement of $\cdot O_2^-$, H_2O_2 and $\cdot OH$ in lipid peroxidation stimulated by these Cr species. These results suggest the possible participation of Cr(IV), Cr(V), $\cdot O_2^-$, H_2O_2 and $\cdot OH$ in Cr(VI)-enhanced lipid peroxidation.

Key words —— lipid peroxidation, chromium(IV), chromium(V), chromium(VI), reactive oxygen species, mouse liver homogenate

INTRODUCTION

Lipid peroxidation is a major contributor to membrane damage in cells and has been implicated as a cause and effect of an extraordinary range of pathological processes associated with oxygen and free radical toxicity.[1] It is assumed that oxidative stress due to reactive oxygen species (ROS) such as superoxide anion ($\cdot O_2^-$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($\cdot OH$) may result in oxidative damage to lipids and DNA.[2] In particular, $\cdot OH$ is one of the most powerful DNA-damaging radicals and one of the most potent oxidants known and is usually assumed to be the initiating oxidant in lipid peroxidation.[1] Lipid peroxidation may play an important role in the induction of tissue injuries by heavy metals.[3] Chromium(VI) compounds are seriously toxic and carcinogenic[4] and are known to induce lipid peroxidation.[5–11] In contrast, Cr(III) compounds are relatively nontoxic and noncarcinogenic. It has been proposed that reactive intermediates such as free radicals, ROS, Cr(IV) and Cr(V), generated during metabolic reduction of Cr(VI) to Cr(III) may be responsible for DNA damage and cancer induced by Cr(VI).[12] However, which oxidation state(s) of Cr and which free radicals and ROS formed in these reactions is (are) involved in Cr(VI)-induced DNA damage are not yet known. In particular, there are not many studies on the role of Cr(IV) and Cr(V).[13] In addition, the mechanisms by which Cr(VI) induces lipid peroxidation are not clear. In

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this article, we studied using mouse liver homogenate (i) the lipid-peroxidizing effects of Cr(IV) and Cr(V), Cr intermediates produced possibly in the reductive metabolism of Cr(VI) and (ii) the inhibitory effects of ROS scavengers on lipid peroxidation induced by Cr(IV) and Cr(V). We used superoxide dismutase (SOD), catalase and formate as specific scavengers of $\cdot O_2^-$, H_2O_2 and $\cdot OH$, respectively.

MATERIALS AND METHODS

Chemicals —— 2-Thiobarbituric acid was obtained from E. Merck (Darmstadt, Germany). Chromic nitrate ($Cr(NO_3)_3$) and potassium chromate (K_2CrO_4) purchased from Nacalai Tesque (Kyoto, Japan) were used as Cr(III) and Cr(VI) compounds, respectively. Triamminechromium tetroxide ($(NH_3)_3CrO_4$) was synthesized as a Cr(IV) compound according to the method of Riesenfeld.[14] Sodium bis(2-hydroxy-2-methylbutyrato)oxochromate (CrHMBA) and sodium bis(2-ethyl-2-hydroxybutyrato)oxochromate (CrEHBA) were synthesized as Cr(V) compounds according to the method of Krumpolc and Roček.[15] Potassium tetraperoxochromate (K_3CrO_8) was also synthesized as another Cr(V) compound according to the method of Riesenfeld *et al.*[16]

The elemental composition (%) and magnetic moment μ(B.M.) of the four compounds synthesized were determined as follows: $(NH_3)_3CrO_4$, *Anal.* Calcd for $H_9N_3O_4Cr$: H, 5.43; N, 25.15; Cr, 31.12; μ, 2.83. Found: H, 5.16; N, 24.89; Cr, 30.6; μ, 2.73. CrHMBA, *Anal.* Calcd for $C_{10}H_{16}NaO_7Cr$: C, 37.16; H, 4.99; Cr, 16.09; μ, 1.73. Found: C, 37.65; H, 5.48; Cr, 15.5; μ, 1.72. CrEHBA, *Anal.* Calcd for $C_{12}H_{22}NaO_8Cr$: C, 39.03; H, 6.00; Cr, 14.08; μ, 1.73. Found: C, 39.31; H, 6.08; Cr, 13.6; μ, 1.80. K_3CrO_8, *Anal.* Calcd for K_3O_8Cr: Cr, 17.49; μ, 1.73. Found: Cr, 18.1; μ, 1.58. These data confirmed that Cr is tetravalent in $(NH_3)_3CrO_4$ and is pentavalent in CrHMBA, CrEHBA and K_3CrO_8. Magnetic moments were measured according to the NMR method of Adams.[17]

Preparation of Mouse Liver Homogenate —— Male ddY mice weighing 29—31 g were given laboratory chow (Funakoshi F-2, Funabashi Farms, Chiba, Japan). Mice were sacrificed by scission of the throat vessels. Liver was then removed, perfused, and homogenized in 10 vol of 0.10 M acetate buffer solution (pH 7.4) with a Potter-Elvehjem homogenizer and motor-driven Teflon pestle under ice cooling.

Incubation of Mouse Liver Homogenate with Cr Compounds —— The incubation mixture contained 1.5 ml of the liver homogenate and 0.5 ml of the acetate buffer solution of Cr compound. In a control experiment, the Cr compound was absent from the incubation mixture. Incubations were carried out in a shaking water bath at 37℃ for 10 min.

In ROS scavenger experiment, 1.5 ml of the liver homogenate were incubated with 0.25 ml of the buffer solution of superoxide dismutase (incubation level: 300 U per ml, Sigma, U.S.A.), catalase (incubation level: 3000 U per ml, Funakoshi, Japan) or sodium formate (incubation level: 800 mM, Nacalai Tesque, Kyoto, Japan) at 37℃ for 10 min, and thereafter were incubated with 0.25 ml of the buffer solution of Cr compound at 37℃ for 10 min.

Measurement of Thiobarbituric Acid Reactive Substances (TBARS) —— Malondialdehyde (MDA), an end product of lipid peroxidation, was measured as TBARS to assess lipid peroxidation. The contents of TBARS were measured according to the method of Masugi and Nakamura.[18] 0.25 ml of the homogenate mixture after incubation was mixed with 0.25 ml of the buffer solution, and then treated with 0.2 ml of 7% sodium dodecyl sulphate (SDS) solution, 2.0 ml of 0.1 M hydrochloric acid solution, 0.3 ml of 10% phosphotungstic acid solution, and 1 ml of 0.5% thiobarbituric acid solution. The whole mixture was heated for 45 min in boiling water bath. The mixture was then cooled, and the colored pigment was extracted with 5 ml of *n*-butanol. The butanol phase was centrifuged for 10 min at 3000 rpm, and the optical density (OD) of the organic phase was determined at 532 nm. Tetraethoxypropane was used as a standard of MDA. TBARS value was expressed as nmol MDA per g liver. In ROS scavenger experiment, the rate of inhibition by ROS scavengers is expressed as follows:

$$\frac{(\text{buf}+\text{Cr})-(\text{scavenger}+\text{Cr})}{(\text{buf}+\text{Cr})-(\text{buf}+\text{buf})}\times 100\ (\%)$$

where the terms (buf+Cr), (scavenger+Cr) and (buf+buf) denote MDA levels after homogenate solution was incubated with buffer, Cr and buffer solutions for 10 min and thereafter with Cr, Cr and buffer solutions for 10 min, respectively.

Statistical Analysis —— Results are expressed as means±standard deviation (S.D.) of four experiments. Significance between means was assessed by one-way analysis of variance (ANOVA) followed by Fisher's protected least significance difference test for multiple comparison. Comparison between two groups was made using Student's *t*-test. The level of statistical significance was $p<0.05$.

RESULTS AND DISCUSSION

Lipid peroxidation levels in mouse liver homogenate reached maximum at 5 to 10 min and fell significantly at 30 min after incubation with K_2CrO_4, Cr(VI) compound (Fig. 1). Therefore, lipid peroxidation was assayed at 10 min after incubation of homogenate with Cr compounds throughout the present experiments.

We next examined lipid peroxidation levels in mouse liver homogenate at 10 min after incubation with different doses of Cr(VI) (Fig. 2). The levels increased significantly at 0.50 mM of Cr(VI) and reached maximum at 5.00 mM. Thus, lipid peroxidation was examined at 10 min after incubation of homogenate with 5.00 mM of Cr compounds throughout the present experiments.

The lipid-peroxidizing effects of Cr(IV) and Cr(V) compounds on mouse liver homogenate were compared with those of Cr(VI) and Cr(III) ($Cr(NO_3)_3$) compounds (Fig. 3). Cr(IV), Cr(V) and Cr(VI) compounds significantly enhanced lipid peroxidation compared to control. The effect of Cr(IV) compound was the most potent. The effect of all Cr(V) compounds was stronger than that of Cr(VI) compound. Cr(III) compound failed to enhance lipid peroxidation.

Inhibitory effects of preincubation of mouse liver homogenate for 10 min with ROS scavengers on lipid peroxidation levels in the homogenate incubated for 10 min with 5 mM of Cr(IV) or Cr(V) (CrEHBA) compound, which were more effective in enhancing lipid peroxidation than Cr(VI) compound (Fig. 3), were examined (Fig. 4). All ROS scavengers employed, SOD, catalase and formate, significantly prevented lipid peroxidation enhancement by Cr(IV) and Cr(V). This strongly indicates that $\cdot O_2^-$, H_2O_2 and $\cdot OH$ are responsible for lipid peroxidation stimulated by Cr(IV) and Cr(V). The inhibitory effects of ROS scavengers on lipid peroxidation promotion by Cr(IV) were about twice those by Cr(V) whatever scavenger is used. This reflects that the amounts of ROS produced in homogenate by Cr(IV) are twice those by Cr(V).

Our results are suggestive of the possible participation of Cr(IV) and Cr(V) in Cr(VI)-enhanced lipid peroxidation because Cr(IV) and Cr(V) may be formed partly and temporarily in the process of reduction of Cr(VI) by cellular reductants, leading to damage cells.[12]

The direct oxidizing power of Cr species decreases in the order of Cr(VI), Cr(V) and Cr(IV) because in the presence of reductants they can undergo 3, 2 and 1-electron reductions, respectively, to give the same end product Cr(III).[19] In contrast to the oxidizing powers of Cr species, their activities to induce lipid peroxidation de-

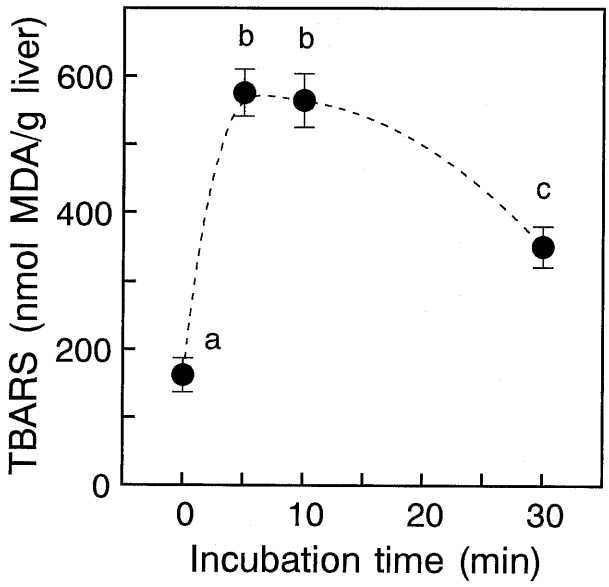

Fig. 1. Lipid Peroxidation Levels in Mouse Liver Homogenate at Different Times after Incubation with 5 mM of Cr(VI) Compound

Means not sharing a common superscript are significantly different ($p<0.05$).

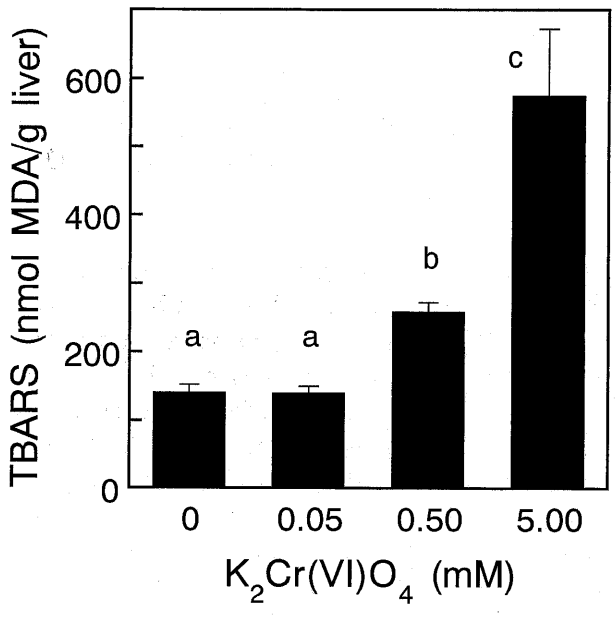

Fig. 2. Lipid Peroxidation Levels in Mouse Liver Homogenate at 10 min after Incubation with Different Doses of Cr(VI) Compound

Means not sharing a common superscript are significantly different ($p<0.05$).

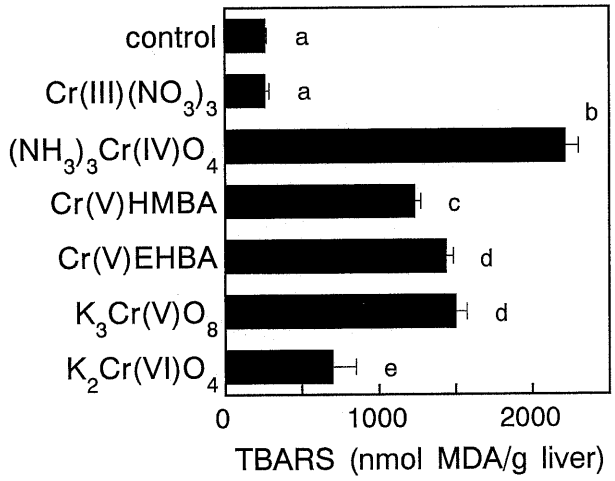

Fig. 3. Lipid Peroxidation Levels in Mouse Liver Homogenate at 10 min after Incubation with 5 mM of Cr(III), Cr(IV), Cr(V) or Cr(VI) Compounds

Means not sharing a common superscript are significantly different ($p<0.05$).

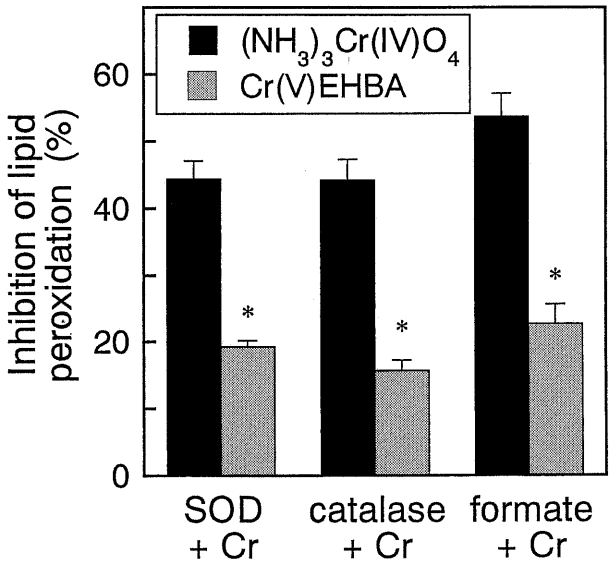

Fig. 4. Inhibitory Effects of Preincubation of Mouse Liver Homogenate for 10 min with ROS Scavengers on Lipid Peroxidation Levels in the Homogenate Incubated for 10 min with 5 mM of Cr(IV) or Cr(V) Compounds

*Significantly different from Cr(IV) ($p<0.05$).

creased in an order of Cr(IV), Cr(V) and Cr(VI) (Fig. 3). Accordingly, we can explain this result not by the oxidizing powers of Cr species but by the amount of ROS generated from Cr species if ROS are responsible for lipid peroxidation enhancement by Cr species. Luo *et al.*[13,20] demonstrated that the reaction of Cr(IV) with H_2O_2 is able to generate ·OH with a concomitant formation of Cr(V) (reaction 1) and that the Cr(V) generated also reacts with H_2O_2 to produce an additional ·OH (reaction 2). The net result of reactions 1 and 2 is expressed as reaction 3:

$$Cr(IV)+H_2O_2 \rightarrow Cr(V)+\cdot OH+OH^- \quad (1)$$
$$Cr(V)+H_2O_2 \rightarrow Cr(VI)+\cdot OH+OH^- \quad (2)$$
$$Net\ Cr(IV)+2\ H_2O_2 \rightarrow Cr(VI)+2\ \cdot OH+2\ OH^- \quad (3)$$

Reactions 2 and 3 indicate that the total amount of ·OH generated from Cr(IV) must be twice that from Cr(V) and that Cr(VI) must fail to produce ·OH from H_2O_2. This agrees closely with our results that the inhibitory effect of formate on lipid peroxidation enhancement by Cr(IV) was twice that by Cr(V) (Fig. 4) and that the order of lipid-peroxidizing effect was Cr(IV)>Cr(V)>Cr(VI) (Fig. 3). The inhibitory effect of catalase on lipid peroxidation enhancement by Cr(IV) was twice that by Cr(V) (Fig. 4). This can be explained by the amount of H_2O_2 reacting with Cr(IV) must be twice that reacting with Cr(V) according to reactions 2 and 3.

Hydrogen peroxide (and $\cdot O_2^-$), the substrate(s) essential for the Fenton-like reactions 1 and 2, may originate from autoxidation of Cr(IV) and Cr(V). It is considered more likely that ROS arise *via* direct reduction of molecular oxygen by redox-active metal ions in low valence states as shown by the following reactions[21-23]:

$$Fe(II)+O_2 \rightarrow Fe(III)+\cdot O_2^- \quad (4)$$
$$Fe(II)+\cdot O_2^-+2\ H^+ \rightarrow Fe(III)+H_2O_2 \quad (5)$$

Thus, we may show the oxidation of Cr(IV) and Cr(V) by molecular oxygen to produce $\cdot O_2^-$ and H_2O_2 as follows:

$$Cr(IV)+O_2 \rightarrow Cr(V)+\cdot O_2^- \quad (6)$$
$$Cr(V)+O_2 \rightarrow Cr(VI)+\cdot O_2^- \quad (7)$$
$$Net\ Cr(IV)+\ 2O_2 \rightarrow Cr(VI)+2\cdot O_2^- \quad (8)$$

$$Cr(IV)+\cdot O_2^-+2H^+ \rightarrow Cr(V)+H_2O_2 \quad (9)$$
$$Cr(V)+\cdot O_2^-+2H^+ \rightarrow Cr(VI)+H_2O_2 \quad (10)$$
$$Net\ Cr(IV)+2\cdot O_2^-+4H^+ \rightarrow Cr(VI)+2H_2O_2 \quad (11)$$

Reactions 6 and 7 were also proposed as a Cr-dependent route to $\cdot O_2^-$ by O'Brien *et al.*[24] According to reactions 10 and 11, the amount of $\cdot O_2^-$ reacting with Cr(IV) must be twice that reacting with Cr(V), and Cr(VI) must fail to react with $\cdot O_2^-$ to produce H_2O_2. This can reasonably explain our results that the inhibitory effect of

SOD on lipid peroxidation promotion by Cr(IV) was twice that by Cr(V) (Fig. 4) and that the order of lipid-peroxidizing effect was Cr(IV)> Cr(V)>Cr(VI) (Fig. 3). Autoxidation reactions 7 and 8 are also compatible with the data of Fig. 3. Thus our results can be clearly accounted for by the reactions of the Cr species with H_2O_2, molecular oxygen and $\cdot O_2^-$ (reactions 1—3 and 6—11) if ROS are responsible for lipid peroxidation promotion by Cr. We propose that the lipid peroxidation of mouse liver homogenate may be enhanced by ROS generated from reactions of Cr species with molecular oxygen.

Yonaha *et al.*[5] reported that the stimulatory effect of Cr(VI) on lipid peroxidation of isolated rat liver microsomes under air is not found substantially under nitrogen atmosphere. This strongly supports our proposal that ROS generated from the reactions of Cr compounds with molecular oxygen may peroxidize lipids in liver homogenate (reactions 1—3 and 6—11). Our results and the report of Yonaha *et al.*[5] strongly suggest that Cr(VI) may enhance lipid peroxidation in liver homogenate not by its potent direct oxidizing power but by ROS generated from the autoxidation and Fenton-like reactions (reactions 1—3 and 6—11) of Cr(IV) and Cr(V) formed partly and temporarily on biological reduction of Cr(VI); Cr(IV) may play more critical role than Cr(V) as Cr intermediates because inhibitory effects of ROS scavengers on lipid peroxidation stimulation by Cr(IV) were twice those by Cr(V) whatever scavenger is used (Fig. 4). Cr(III) exhibited no stimulatory effect on lipid peroxidation (Fig. 3). This may be due to the well-known chemical inertness of Cr(III)[19] although it, like Cr(IV) and Cr(V), can be subject to the autoxidation and Fenton-like reactions in principle; much longer incubation time may give lipid peroxidation-enhancing activity to Cr(III).

Inhibition of Cr(IV) and Cr(V)-stimulated lipid peroxidation by formate, a ·OH scavenger, (Fig. 4) is indicative of the involvement of ·OH in the lipid peroxidation. Hydroxyl radical is one of the most powerful DNA-damaging radicals and one of the most potent oxidants known and is usually assumed to be the initiating oxidant in lipid peroxidation.[1] In addition, it is the end product of the autoxidation and Fenton-like reactions of Cr species (reactions 1—3 and 6—11). After SOD decomposes 1.0 mol of $\cdot O_2^-$, the formation of 0.5 mol H_2O_2 from the enzymatic reaction of SOD and the suppression of generation of 1.0 mol H_2O_2 from 1.0 mol $\cdot O_2^-$ in reaction 9 or 10 will result, which leads to a decrease of 0.5 mol H_2O_2 on balance. This results in suppression of generation of 0.5 mol ·OH from 0.5 mol H_2O_2 by reactions 1 and 2. Accordingly, the inhibitory effect of SOD on the lipid peroxidation may be due to not only decomposing $\cdot O_2^-$ but also inhibiting ·OH generation from $\cdot O_2^-$. The inhibitory effect of catalase on the lipid peroxidation may be due to not only decomposing H_2O_2 but also inhibiting ·OH generation from H_2O_2 because Cr(V) or Cr(IV) may induce ·OH generation from H_2O_2 (reaction 2 or 3). Superoxide anion is a better reductant rather than oxidant and is relatively unreactive towards most biological molecules including lipids, DNA and proteins.[25] The direct reactivity of H_2O_2 is also relatively modest.[25] Much of the damage done by H_2O_2 and $\cdot O_2^-$ *in vivo* is thought to be due to their conversion into highly reactive species; it seems to be firmly established that one of these species is ·OH.[26] Thus, ·OH may be the ultimate toxicant for lipid peroxidation enhanced by Cr species.

In conclusion, (i) Cr(IV), Cr(V) and Cr(VI) compounds effectively enhanced lipid peroxidation of mouse liver homogenate in this order; (ii) the lipid-peroxidizing effect of Cr(IV) was suppressed more remarkably by specific scavengers of $\cdot O_2^-$, H_2O_2 and ·OH than that of Cr(V). These results indicate the participation of $\cdot O_2^-$, H_2O_2 and ·OH in lipid peroxidation promoted by Cr(IV) and Cr(V), and additionally suggest the possible involvement of Cr(IV), Cr(V), $\cdot O_2^-$, H_2O_2 and ·OH in Cr(VI)-enhanced lipid peroxidation.

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