Effect of Chromium on Lipid Peroxidation in Isolated Rat Hepatocytes

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(Received 15 May 1987/Accepted 21 September 1987)

ABSTRACT. To study the effects of hexavalent and trivalent chromium on lipid peroxidation, isolated rat hepatocytes were incubated with different concentrations of chromium compounds at 37°C for 60 min. Lipid peroxidation was determined using thiobarbituric acid (TBA)-reacting materials. Cellular injury was observed as a leakage of lactate dehydrogenase (LDH) from hepatocytes into incubation medium. The contents of reduced glutathione (GSH) in hepatocytes were also assessed. Results obtained were as follows: (1) Lipid peroxidation of isolated rat hepatocytes which was expressed as TBA-reactant formation was inhibited by trivalent chromium at the range of concentrations tested in this experiment (125-1000μM). Hexavalent one inhibited lipid peroxidation at low concentration (125μM), but facilitated that at high concentration (1000μM). (2) LDH-leakage was facilitated by the addition of hexavalent chromium (K2Cr2O7, 125-1000μM), on the other hand trivalent one (Cr(NO3)3) inhibited it significantly at concentrations more than 250μM. (3) The hexavalent chromium-induced lipid peroxidation was inhibited by antioxidants such as N,N'-diphenylp-phenylenediamine (DPPD), α-tocopherol and diethyl dithiocarbamate (DDTC). However, LDH-leakage from hepatocytes was not inhibited by these antioxidants. (4) After the pretreatment with GSH depleting agent such as diethyl maleate (DEM) on isolated hepatocytes, the lipid peroxidation induced by hexavalent chromium was significantly enhanced and was dependent on the chromium concentrations in the incubation medium. Furthermore this enhancement of lipid peroxidation was canceled by the addition of GSH (10mM). (5) The lipid peroxidation induced by ascorbate was significantly inhibited by the addition of hexavalent chromium as well as trivalent one. This effect of hexavalent chromium might be essentially due to the reduction of hexavalent chromium to trivalent one by ascorbate. These results suggest that chromium in trivalent form inhibits lipid peroxidation in isolated hepatocytes, while hexavalent one facilitates it but is not necessarily correlated to cellular injury.—KEY WORDS: cell injury, chromium, glutathione, lipid peroxidation, rat hepatocyte.

It has been considered that lipid peroxidation may be a cause of liver injury induced by some chemicals [15,16]. It is known that liver injury caused by carbon tetrachloride occurs when lipid peroxidation is induced by a free radical formation [17]. Rana and Kumar reported that the enhancement of lipid peroxidation was observed in rat liver after heavy metal poisoning such as mercury, molybdenum, copper, chromium and manganese [14]. The formation of lipid peroxides may occur in association with heavy metal intoxications, since cadmium [13] and mercuric chloride [20] decrease the activity of GSH peroxidase, which catalyzes the destruction of organic hydroperoxides [10]. Furthermore, these formations of lipid peroxides occur as a result of the decrease in intracellular GSH contents[2,8].

Many investigations have been carried out on the relationship between lipid peroxidation and cellular injury. However, relatively few reports are available on the toxicity and lipid peroxidation by chromium. The present study was designed, therefore, to compare the effects of hexava-
lent chromium to those of trivalent one on lipid peroxidation and cellular injury in isolated rat hepatocytes. Furthermore, the effects of various antioxidants on the chromium-induced lipid peroxidation were studied, and the relationship between the chromium-induced lipid peroxidation and the intracellular GSH levels in isolated hepatocytes, including the influence of depletion or addition of GSH, was also investigated.

MATERIALS AND METHODS

**Chemicals:** The chemicals used were; K$_2$Cr$_3$O$_7$, Na$_2$Cr$_2$O$_7$, Na$_2$CrO$_4$, Cr(NO$_3$)$_3$, Cr$_2$(C$_2$O$_4$)$_3$, CrCl$_3$, K$_2$Cr$_2$(SO$_4$)$_3$ (Kanto Chemical Co., Inc.), K$_2$CrO$_4$, Cr(CH$_3$COO)$_3$, 5, 5'-dithio-bis-2-nitrobenzoic acid (Wako Pure Chemical Industries, Ltd.), DPPD (Tokyo Kasei Industry Co. Ltd.), DDTC (Eastman Kodak Co.), L-ascorbic acid, DL-a-tocopherol, DEM (Kanto Chemical Co., Inc.), GSH (Sigma) and thiobarbituric acid (E. Merck. Co.). All chemicals and reagents employed were of commercial reagent-grade quality.

**Preparation of isolated rat hepatocytes:** Male rats of the Wistar strain, kept on a standard laboratory diet (rat chow MF; Oriental Yeast Co., Ltd.), were used as liver donors. Rats (250–300g B. W.) were lightly anesthetized with ether and surgical procedures were performed at about 9:00 a.m. for each experiment. Hepatocytes were isolated essentially by the method of Stacey and Klaassen [18] with some modifications. The preperfusion of the liver for 10 min with 50 ml of Ca$^{2+}$ free buffer (137 mM NaCl, 5.4 mM KCl, 0.5 mM Na$_2$HPO$_4$, 0.44 mM KH$_2$PO$_4$, 5.55 mM Glucose, 4.2 mM NaHCO$_3$, 0.5 mM EGTA and 10 mM HEPES; equilibrated with 95% O$_2$ and 5% CO$_2$; pH7.3) via the portal vein was performed, with the effluent running to waste. After the preperfusion, enzyme perfusion was carried out with 50 ml of perfusion medium (0.05% W/V collagenase type IV Sigma, 1.26 mM CaCl$_2$ and 0.41 mM MgSO$_4$ were added to the above preperfusion medium). The enzyme perfusion continued for 10–15 min under the pressure of 25–30 cm water height. Temperature of perfusion medium was maintained close to 37°C throughout the procedure. After the perfusion, the liver was dispersed into 50 ml preperfusion medium. The resulting suspension was filtered through nylon mesh. Hepatocytes were separated by centrifugation (400 rpm for 5 min at 4°C) from cellular debris and washed twice with fresh preperfusion medium. A third washing was performed with incubation medium (131 mM NaCl, 5.2 mM KCl, 0.9 mM MgSO$_4$, 1 mM CaCl$_2$, 3 mM Na$_2$HPO$_4$, and 10 mM Tris; pH 7.4) before final resuspension to approximately 1.0×10$^6$ cells per ml of suspension.

**Lipid peroxidation:** A 5 ml of hepatocytes suspension was pipetted into 50 ml Erlenmeyer flasks. Chemicals to be added in the suspension of hepatocytes were previously dissolved in distilled water and added to the flasks in a volume of 0.05 ml. The incubation mixture was incubated at 37°C for 60 min in a water bath shaking at 100 oscillations per minute (Yamato, water bath incubator, Model BT-31).

**Determination of Lipid peroxidation:** Lipid peroxidation was measured by the thiobarbituric acid (TBA) reaction [5]. Aliquots of 1 ml of incubated suspension were pipetted and added to a mixture of 2 ml of TBA reagent (15g CCl$_3$COOH, 0.375g TBA, 25 ml 1N HCl in 100 ml of distilled water). The mixture was placed in a boiling water bath for 15 min, and then cooled in ice cold water, and centrifuged (3000 rpm for 10 min at 20°C). The optical density of supernatant fluid was read at 535 nm, and the concentration of TBA-reactant was shown at nmol malondialdehyde (MDA) per mg.
protein.

Determination of LDH-leakage: Leakage of the cytoplasmic enzyme (LDH) from isolated hepatocytes into incubation medium, which was recognized as an indicator of cellular injury [3], was measured by UV-method. The enzymatic activities in the supernatant fluid of centrifuged samples (1000 rpm for 5 min at 4°C) were assessed by use of LDH-UV test Wako (Wako Pure Chemical Industries, Ltd.).

Assay for GSH: GSH was determined by the method of Beutler et al. [4]. One ml of each supernatant fraction which was deproteinized by addition of metaphosphoric acid was transferred to another tube containing 4 ml of 0.3 M sodium phosphate buffer (pH 8.0), 0.5 ml of 10 mM 5,5'-dithio-bis-2-nitrobenzoic acid was added. The absorbances were read at 412 nm.

Assay for protein: Protein was measured by the method of Lowry et al. [11].

In this paper, the values were expressed as mean±standard error of results from three separate experiments. Comparisons of the means of control and treated suspensions were made by Student’s t-test with p<0.05 as a limit of significance.

RESULTS

The concentration of TBA-reactant in isolated rat hepatocytes before incubation was 0.167±0.007 nmol MDA per mg protein. After incubation at 37°C for 60 min, this value was increased to 0.631±0.118 nmol MDA per mg protein without the addition of chromium compounds. Effects of the nine kinds of chromium compounds on lipid peroxidation were compared (Fig. 1). At low concentration (125 μM), hexavalent chromium compounds depressed lipid peroxidation in isolated rat hepatocytes, but produced the increase of TBA-reactant formation and LDH-leakage in suspensions of isolated rat hepatocytes after the incubation at 37°C for 60 min.

Table 1. Effects of chromium on TBA-reactant formation and LDH-leakage in suspensions of isolated rat hepatocytes after the incubation at 37°C for 60 min.

<table>
<thead>
<tr>
<th>Concentration of chromium</th>
<th>TBA-reactant formation (%) of control</th>
<th>LDH-leakage (%) of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>CrCl₃</td>
<td>125μM</td>
<td>64.0±3.7(a)</td>
</tr>
<tr>
<td></td>
<td>250</td>
<td>77.3±3.2(d)</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>97.8±5.0</td>
</tr>
<tr>
<td></td>
<td>1000</td>
<td>140.2±7.2(e)</td>
</tr>
<tr>
<td>Cr(NO₃)₃</td>
<td>125</td>
<td>53.9±3.8(d)</td>
</tr>
<tr>
<td></td>
<td>250</td>
<td>51.8±3.6(d)</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>56.8±4.0(d)</td>
</tr>
<tr>
<td></td>
<td>1000</td>
<td>58.1±5.0(b)</td>
</tr>
</tbody>
</table>

a) The average value of TBA-reactant formation in control hepatocytes was 0.631 nmol MDA/mg protein.
b) The average control LDH-leakage value obtained was 0.808 LDH units/ml incubation medium.
c) Mean±S.E.
d) Significant decrease in TBA-reactant formation and LDH-leakage compared to control (p<0.05).
e) Significant increase in TBA-reactant formation and LDH-leakage compared to control (p<0.05).
formation at high concentration (1000 μM). On the other hand, trivalent one did not facilitate lipid peroxidation at the range of concentrations tested in this experiment (125~1000 μM). Na₂Cr₂O₇ manifested the strongest activity to induce lipid peroxidation among hexavalent chromium compounds tested. However, no difference in the effects of trivalent chromium compounds could be detected.

The following experiments hereinafter was carried out by use of K₂Cr₂O₇ as a hexavalent chromium compound and Cr(NO₃)₃ as a trivalent one.

As shown in Table 1, hexavalent chromium (K₂Cr₂O₇) facilitated LDH-leakage from isolated hepatocytes into incubation medium even at low concentration (125 μM) which inhibited TBA-reactant formation, while trivalent one (Cr(NO₃)₃) inhibited it significantly at the concentrations more than 250 μM.

The effects of DPPD, α-tocopherol and DDTC on lipid peroxidation induced by hexavalent chromium (K₂Cr₂O₇, 1000 μM) were studied. As shown in Fig. 2, all of antioxidants used in this experiment depressed TBA-reactant formation induced by high concentration of hexavalent chromium. These values of TBA-reactant were less than those of control which was incubated without chromium and antioxidants. On the other hand, these antioxidants could not inhibit LDH-leakage from isolated hepatocytes into incubation medium, which was induced by hexavalent chromium (data not shown).

As shown in Fig. 3, the contents of intracellular GSH in isolated hepatocytes were clearly diminished by the addition of hexavalent chromium (K₂Cr₂O₇), and were reduced to 50% of control by its addition of 1000 μM. On the other hand, no effect was observed in those by trivalent one (Cr(NO₃)₃) at the range of concentrations tested (125~1000 μM).

It was observed that lipid peroxidation induced by hexavalent chromium (K₂Cr₂O₇) was enhanced remarkably in isolated hepatocytes pretreated with GSH depleting agent, such as DEM for 5 min, compared with control without this pretreatment.
Fig. 4. The effects of GSH depleting agent, DEM (61 mM), on lipid peroxidation induced by hexavalent chromium (K₂Cr₂O₇) and the inhibitory effects of GSH on those induced by the cooperation of DEM and hexavalent chromium in suspension of isolated rat hepatocytes after the incubation at 37°C for 60 min. Data represent the mean±S.E. mean of results from three separate experiments. ○, Cr⁶⁺; ●, Cr⁶⁺ with DEM (61 mM); □, Cr⁶⁺ with DEM (61 mM) and GSH (10 mM).

Under this condition the value of TBA-reactant was increased with dependence on the concentration of hexavalent chromium (125~1000 µM, Fig. 4).

On the other hand, this enhanced lipid peroxidation in isolated hepatocytes pretreated with DEM was depressed less than 10% by the addition of GSH (10 mM) at the range of hexavalent chromium concentrations (Fig. 4).

The lipid peroxidation induced by ascorbate in isolated hepatocytes was depressed remarkably by the addition of hexavalent (K₂Cr₂O₇) or trivalent chromium (Cr(NO₃)₃) at the concentration above 125 µM respectively. There was the tendency that this effect of trivalent chromium was stronger than that of hexavalent one (Fig. 5).

**DISCUSSION**

Effects of hexavalent and trivalent chromium on lipid peroxidation were studied.

Hexavalent chromium of 1000 µM had an augmentative effect on TBA-reactant formation in isolated rat hepatocytes. In contrast, the lower concentration (125 µM) of these materials showed an inhibitory effect on lipid peroxidation as well as trivalent one over the range of 125~1000 µM. On the other hand, cellular injury which was estimated as LDH-leakage from isolated hepatocytes into incubation medium, was induced by the addition of hexavalent chromium (K₂Cr₂O₇) at the concentrations more than 125 µM, whereas TBA-reactant formation was inhibited by the presence of the 125 µM hexavalent chromium in the isolated hepatocytes suspension. Furthermore, antioxidants could inhibit chromium-induced lipid peroxidation, but they could not prevent chromium-induced cellular injury.

According to the report by Stacey and Klaassen [18], mercury and copper among heavy metals were the most effective for producing TBA-reactant. They also reported that lipid peroxidation associated with mercury was not necessarily responsi-
ble for the loss of cell viability induced by this metal. Also in our experiment, lipid peroxidation by hexavalent chromium (K₂Cr₂O₇) did not directly relate to cellular injury, but trivalent one (Cr(NO₃)₃) which could not induce cellular injury, could prevent lipid peroxidation in isolated hepatocytes. The effects of hexavalent and trivalent chromium on LDH-leakage were consistent with the data that hexavalent chromium was more toxic than trivalent one [12].

Yonaha et al. [21] have reported that both hexavalent and trivalent chromium, at lower concentrations in the range of 1~100 µM, inhibited lipid peroxidation induced by ascorbate and NADPH in rat liver microsomes. They have observed that hexavalent chromium above 1 mM caused lipid peroxide formation in the same preparations. Lipid peroxidation in isolated hepatocytes was inhibited by trivalent chromium at the range of concentrations tested and hexavalent one at lower concentration. It was considered that the latter effect partly resulted from the decreasing of hexavalent chromium, because hexavalent chromium was reduced to trivalent one by intracellular reducing agents such as GSH [1]. Further, it has been also demonstrated that ascorbate induces lipid peroxidation in isolated rat hepatocytes [9]. This effect of ascorbate was inhibited by the addition of hexavalent chromium or trivalent one. Since ascorbate reduces hexavalent chromium to trivalent one, the inhibitory effect of hexavalent chromium on lipid peroxidation might essentially be due to the reduction of hexavalent chromium to trivalent one by ascorbate. These results suggest the possibility of trivalent chromium as a radical scavenger, since the decrease of TBA-reactant formation was considered a depression against the lipid peroxidation occurred by the incubation at 37°C for 60 min.

It has been known that lipid peroxidation is also associated with the contents of intracellular GSH. Anundi et al. reported that GSH deficiency per se could lead to lipid peroxidation and that this reaction caused the hepatocellular lysis [2]. GSH inhibits efficiently lipid peroxidation of unsaturated fatty acids by the stimulation of ascorbate in fresh microsomal fraction and mitochondria of rat liver [7]. GSH also diminished the chromium-induced cytotoxicity in HeLa cells [19]. Furthermore, it has been reported that GSH reduces hexavalent chromium to trivalent one [6]. In the present experiment, lipid peroxidation induced by hexavalent chromium was enhanced by the depletion of intracellular GSH levels in isolated hepatocytes pretreated with DEM. Under the same condition, this enhancement was canceled by the addition of GSH. It was also confirmed that intracellular GSH in isolated hepatocytes was consumed by the presence of hexavalent chromium, but trivalent one did not affect the levels of intracellular GSH. These observations also suggest that the TBA-reactant formation increased by hexavalent chromium is partly associated with the decrease of intracellular GSH levels.

However, since lipid peroxidation has the characteristics of a chain reaction, it may be impossible to make completely clear the mechanism of chromium-induced lipid peroxidation by means of a search for the effect of chromium on intracellular GSH levels alone. Consequently, it would be necessary to have a detailed investigation of the effects of chromium on other systems related to lipid peroxidation such as superoxide dismutase, glutathione peroxidase, glutathione S-transferase and so on. Furthermore, since chromium compounds have the different effects on lipid peroxidation and cellular injury according to their valences, the clarification of the difference in their mechanism of the influence on isolated hepatocytes may be also necessary.
REFERENCES


要約

遊離ラット肝細胞の脂質過酸化に及ぼすクロムの影響：上野俊治・澤佐信行・古川義宣・相川勝弘・板垣直織・小宮山敬・高島由佳（北里大学薬学部薬科）——遊離ラット肝細胞の脂質過酸化に対する6価（Cr VI）及び3価クロム（Cr III）の影響を調べたため、遊離肝細胞を種々濃度の各種クロム化合物とともに37℃、60分間培養し、脂質過酸化をチオバロールペール酸法で測定、細胞障害を培養液中のLDH遊出により検討した結果、次の成績を得た。1）遊離肝細胞の脂質過酸化は、125～1000 μM濃度のCr III添加により抑制された。Cr VIは低濃度（125 μM）で脂質過酸化を抑制したが、高濃度（1000 μM）では促進した。2）肝細胞からのLDH遊出は、Cr VI（K₂Cr₂O₇、125～1000 μM）により抑制されたが、Cr III（Cr（NO₃）₃）では250 μM以上の添加で有意に抑制された。3）Cr VI添加で誘起された脂質過酸化は、抗酸化剤N,N'-diphenyl-p-phenylenediamine α-tocopherol, diethyl dithiocarbamateで抑制されたが、LDH遊出は抑制されなかった。4）グルタチオン枯渇剤（diethyl maleate）で前処理した肝細胞においては、Cr VIによる脂質過酸化がCr VI濃度に依存して増強され、さらにこの増強作用は、GSHの添加により消失した。5）アスコルビン酸による脂質過酸化は、Cr VI及びCr IIIの添加で著しく抑制された。以上の成績から、遊離肝細胞における脂質過酸化はCr VIにより誘起され、Cr IIIにより抑制されることが明らかになり、さらに、Cr VIによる脂質過酸化は細胞内のGSHと一部関連するが、細胞障害とは直接関連していないことが示唆された。