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사람의 신경교세포에서 수은에 의한 세포 손상에 미치는 활성산소기 제거제 및 항산화제의 효과

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Effects of Radical Scavengers and Antioxidants on $HgCl_2$-Induced Cell Injury in Human Glioma Cells

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Abstract

Background Mercury exerts a variety of toxic effects on both neurons and glia and also may play a role in pathophysiological mechanisms of Alzheimer's disease in neuroblastoma cells. Studies in vivo and in vitro have shown that mercury generates reactive oxygen species (ROS) and increases lipid peroxidation in various tissues including brain, suggesting that oxidative stress may contribute to the development of neurodegenrative disorders caused by mercury intoxication. However, whether lipid peroxidation plays an important role in mercury cytotoxicity is not clear ***Methods*** The present study was undertaken to determine (1) the involvement of individual ROS in mediating mercury cytotoxicity and (2) whether mercury-induced cell death is resulted from lipid peroxidation in human glioma cells. $HgCl_2$ caused the loss of cell viability in a dose- and time-dependent manner ***Results*** The loss of viability was prevented by the hydroxyl radical scavenger dimethylthiourea, but the superoxide scavenger superoxide dismutase and the hydrogen peroxide scavengers catalase and pyruvate showed no beneficial effect. The potent antioxidant DPPD exerted partial protective effect, but BHA, Trlox, and melatonin were not effective $HgCl_2$-induced loss of viability was prevented by the ferrous iron chelator phenanthroline, but no the ferric iron chelator deferoxamine $HgCl_2$ cytotoxicity was effectively prevented by thiols (dithiothreitol and glutathione). $HgCl_2$ caused ATP depletion, which was prevented by the agents protecting HgCl2-induced cell death. ***Conclusion*** These results suggest that (1) $HgCl_2$-induced cell death is associated with generation of hydroxyl radicals resulting from an iron-dependent mechanism and (2) lipid peroxidation dose not play a critical role in $HgCl_2$ cytotoxicity in human glioma cells

Key Words: Inorganic mercury, Alzheimer disease

Introduction

Mercury exerts a variety of toxic effects on both neurons and glia[1,2] and also may play a role in pathophysiological mechanisms of Alzheimer's disease in neuroblastoma cells.[3] Mercury is capable of inhibiting sulfhydryl-containing enzyme systems, ion channels of cell membranes, and mitochondria presumably by binding to sulfhydryl groups, thereby contributing to the cascade of cell injury.

Studies in vivo and in vitro have shown that mercury generates reactive oxygen species (ROS) in various tissues including brain,[4-7] suggesting that oxidative stress may contribute to the development of neurodegenrative disorders caused by mercury intoxication. Biological membranes are particularly susceptible to peroxidative attacks by oxidants, resulting in lipid peroxidation.[8]

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Therefore, lipid peroxidation has been used as indirect marker of oxidant-induced cell injury.[9] Although mercury has been reported to increase lipid peroxidation in brain tissues,[10,11] whether lipid peroxidation is involved in the pathogenesis of mercury-induced cell injury remains controversial. Several investigators have reported that lipid peroxidation is not involved in the pathogenesis of mercury-induced cell injury in hepatocytes,[7,12] human epidermal keratinocytes,[13] and kidney.[14]

This study was thus performed to determine (1) which species of ROS plays a critical role in mediating cell death and (2) whether lipid peroxidation plays a critical role in mercury-induced cell death using established human glioma cell line A171. In brain, mercury is predominantly localized in astrocytes, which then lost their physiological functions, resulting in neuronal lesions.[15,16] To achieve inhibition of lipid peroxidation, an antioxidant DPPD was chosen on the basis of its predominant ability to prevent lipid peroxidation. DPPD has been extensively employed in in vivo and in vitro studies to specifically inhibit lipid peroxidation.[17,18]

Materials and Methods

Culture of A172 cells

A172 cells were obtained from the American Type Culture Collection (Rockville, MD) and maintained by serial passages in 75-cm^2 culture flasks (Costar, Cambridge, MA). The cells were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco,BRL) containing 10% heat inactivated fetal bovine serum (Gioco,BRL) at 37° C in humidified 95% air/5% CO_2 incubator. When the cultures reached confluence, subculture was prepared using a 0.02% EDTA-0.05% trypsin solution. The cells were grown on 24-well tissue culture plates and used 1-2 days after plating when a confluent monolayer culture was achieved.

Cell viability assay

Cell viability was evaluated using the MTT assay . Tetrtazolium salts such as MTT are metabolized by mitochondrial dehydrogenases to form a blue formazan dye and are therefore useful for the measurement of cell viability. The cells were gently washed with Hanks' balanced salt solution (HBSS, Sigma Co, USA), and exposed to $HgCl_2$. After washing the cells, culture medium containing 0.5 mg/ml of MTT was added to each well. The cells were incubated for 2 hr at 37° C, the supernatant was removed and the formed formazan crystals in viable cells were solubilized with 110 l of dimethyl sulfoxide. A 100 l aliquot of each sample was then translated to 96-well plates and the absorbance of each well was measured at 550 nm with ELISA Reader(Bio-Tek instrument,EL,311). Data were expressed as a percentage of control measured in the absence of $HgCl_2$. Unless stated otherwise, cells were treated with 0.02 mM $HgCl_2$ for 60 min. Test reagents were added to the medium 30 min before $HgCl_2$ exposure.

To determine the protective effect of catalase on H_2O_2 cytotoxicity, cells were exposed to exogenous H_2O_2 directly or glucose oxidase (2.5 U/ml)/ glucose (100 mg/dl) for 120 min in the presence or absence of catalase.

Measurement of ATP content

ATP levels were measured on OK cells with a luciferin-luciferase assay. After an exposure to oxidant stress, the cells were solubilized with 500 l of 0.5 % Triton X-100 and acidified with 100l of 0.6 M perchloric acid and placed on ice. Then cell suspension was diluted with 10 mM potassium phosphate buffer containing 4 mM $MgSO_4$ (pH 7.4), and 100 l of 20 mg/ml luciferin-luciferase was added to 10 l of diluted sample. Light emission was recorded at 20 sec with a luminometer (MicroLumat LB96P, Berthold, Germany). Protein content was determined on a portion of the cell sample.

Reagents

Catalase, superoxide dismutase (SOD), deferoxamine, 1,10-phenanthroline, dithiothreitol (DTT), glutathione

(GSH), butylated hydroxylanisole (BHA), hydrogen peroxide (H2O2), Trolox, melatonin, and (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma Chemical (St. Louis, MO). N,N'-diphenyl-p-phenylenediamine (DPPD) and dimethyithiourea (DMTU) were purchased from Aldrich Chemical (Milwaukee WI). All other chemicals were of the highest commercial grade available.

Statistical analysis

The data are expressed as mean SE and the difference between two groups was evaluated using Student's t-test. A probability level of 0.05 was used to establish significance.

Results

HgCl2-induced injury to A172 cells

The loss of cell viability was measured by the MTT assay. $HgCl_2$ caused the loss of cell viability in a dose-dependent manner after exposure of cells to various concentrations of $HgCl_2$ (0-50 M) for 60 min (Fig. 1A) To determine the time course of $HgCl_2$-induced cell death, the cells were exposed to 20 M $HgCl_2$, and the loss of cell viability was measured at various time points (0-90). A significant loss of cell viability was present 10 min after exposure of cells to $HgCl_2$ and increased up to 90 min at that the cell viability was 16.92 2.26% (Fig. 1B). In subsequent experiments, cells were treated with 0.02 mM $HgCl_2$ for 60 min. Similar data were obtained by a trypan blue exclusion method (data not shown).

Effects of radical scavengers and antioxidants

Although ROS have been reported to be involved in the pathogenesis of mercury cytotoxicity in brain tissues,[4,5] the exact chemical species responsible for the cytotoxicity is not known. Thus, we first examined the effects of agents scavenging each radical species. The

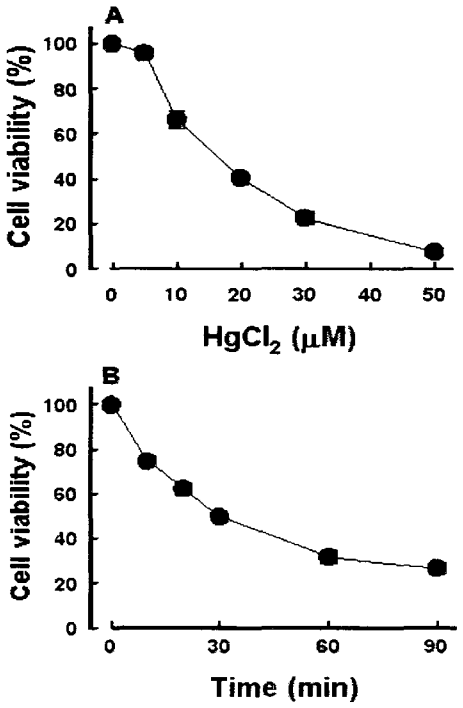

Fig. 1. A. Concentration-dependent effect of $HgCl_2$ on cytotoxicity in human glioma cells Cells were treated with various concentrations of $HgCl_2$ for 60 min. Data are mean SE of four determinations. B, Time-dependent effect of $HgCl_2$ on cytotoxicity human glioma cells Cells were incubated with 0.02 mM $HgCl_2$ for various times. The cytotoxicity was estimated by MTT assay. Data are mean SE of four determinations.

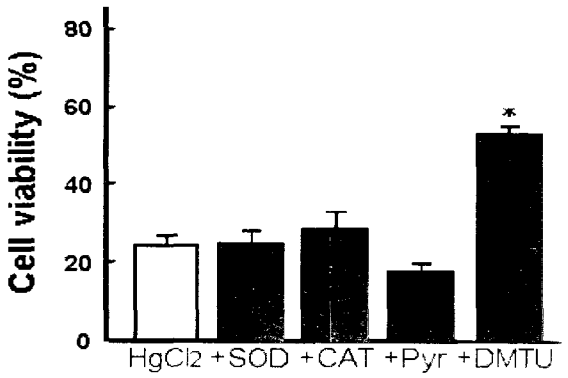

Fig. 2. Effects of radical scavengers on $HgCl_2$-induced cytotoxicity in human glioma cells Cells were treated with 0.02 mM $HgCl_2$ for 60 min in the presence or absence of superoxide dismutase (SOD, 500 Units/ml), catalase (CAT, 800 Units/ml), pyruvate (Pyr, 10 mM), and dimethylthiourea (DMTU, 30 mM) The cytotoxicity was estimated by MTT assay. Data are mean SE of six determinations. *$p<0.05$ compared with $HgCl_2$ alone

results are summarized in Fig. 2. The hydroxyl radical scavenger DMTU exert protective effect, whereas the superoxide scavenger SOD and the H_2O_2 scavengers catalase and pyruvate did not affect $HgCl_2$-mediated loss of cell viability. The failure of H_2O_2 scavengers to prevent $HgCl_2$ toxicity could be due to the inability of these agents to scavenge H_2O_2 in A172 cells. To test this possibility, we examined the effect of scavengers on H_2O_2-induced cell death. Cells were exposed to reagent H_2O_2 directly or H_2O_2 generated by glucose oxidase and glucose. Exposure of cells to glucose oxidase/glucose induced a significant loss of cell viability, which was significantly prevented by catalase and pyruvate (Fig. 3). When cells were exposed to 0.5 mM H_2O_2 in the presence of catalase and pyruvate, the cell death was completely prevented (data not shown).

In order to determine whether lipid peroxidation plays a critical role in the pathogenesis of $HgCl_2$ cytotoxicity, we examined the efficacy of antioxidants on $HgCl_2$-mediated loss of cell viability. The potent antioxidant DPPD at 0.01 mM produced only a slight protection and BHA at 0.1 mM was not significantly effective (Fig. 4). Even when DPPD concentration was increased to 0.05 mM, the potency of protective effect was not different from that by 0.01 mM (data not shown). Trolox (0.1 mM), a water-soluble vitamine E compound, also was not effective in preventing $HgCl_2$-induced cell death (46.10 2.46 vs. 53.45 3.05, $p>0.10$, n=6). Melatonin also did not provide protective effect (42.67 2.13 vs. 45.31 2.29, $p>0.20$, n=4). The concentration of antioxidants used in the present study was similar to or higher than the concentrations that have effectively prevented oxidant-induced cell injury and lipid peroxidation in brain tissues.[19,20,21]

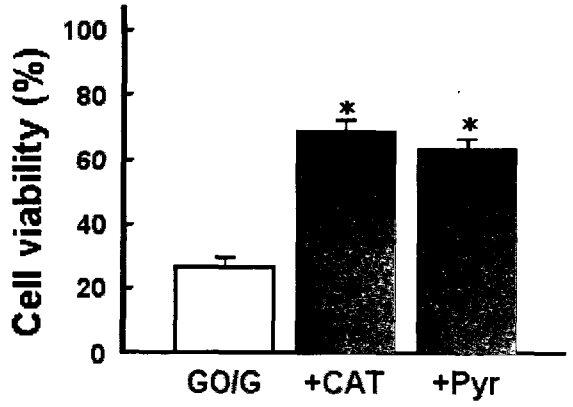

Fig. 3. Effects of H_2O_2 radical scavengers on cytotoxicity human glioma cells exposed to H2O2 generated by glucose oxidase/glucose (GO/G). Cells were treated with glucose oxidase (2.5 Units/ml) and glucose (100 mg/dl) for 120 min in the presence or absence of catase (CAT, 800 Units/ml) and pyruvate (Pyr, 10 mM). The cytotoxicity was estimated by MTT assay. Data are mean SE of four determinations. *$p<0.05$ compared with GO/G alone.

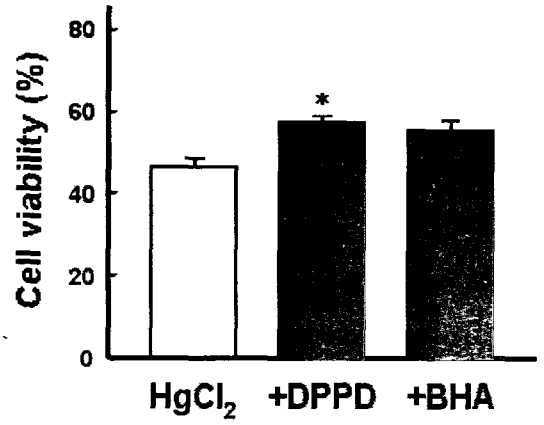

Fig. 4. Effects of antioxidants on $HgCl_2$-induced cytotoxicity in human glioma cells. Cells were treated with 0.02 mM $HgCl_2$ for 60 min in the presence or absence of N,N'-diphenyl-p-phenylenediamine (DPPD, 0.02 mM) and butylated hydroxylanisole (BHA, 0.1 mM). The cytotoxicity was estimated by MTT assay. Data are mean SE of seven determinations. *$p<0.05$ compared with $HgCl_2$ alone.

The role of iron in $HgCl_2$-mediated cell death was examined in cells pretreated with iron chelators deferoxamine and phenanthroline. $HgCl_2$-induced cell death was not affected by 2 mM deferoxamine, a membrane impermeable iron chelator,[22] but it was effectively prevented by 0.2 mM phenanthroline, a membrane permeable iron chelator (Fig. 5A). These results indicate that $HgCl_2$ causes the loss of cell viability via an iron-dependent mechanism.

To determine if thiols exert protective effect against $HgCl_2$-mediated loss of cell viability, DTT and GSH were added to the incubation medium 30 min before exposure of cells to $HgCl_2$. The loss of cell viability induced by

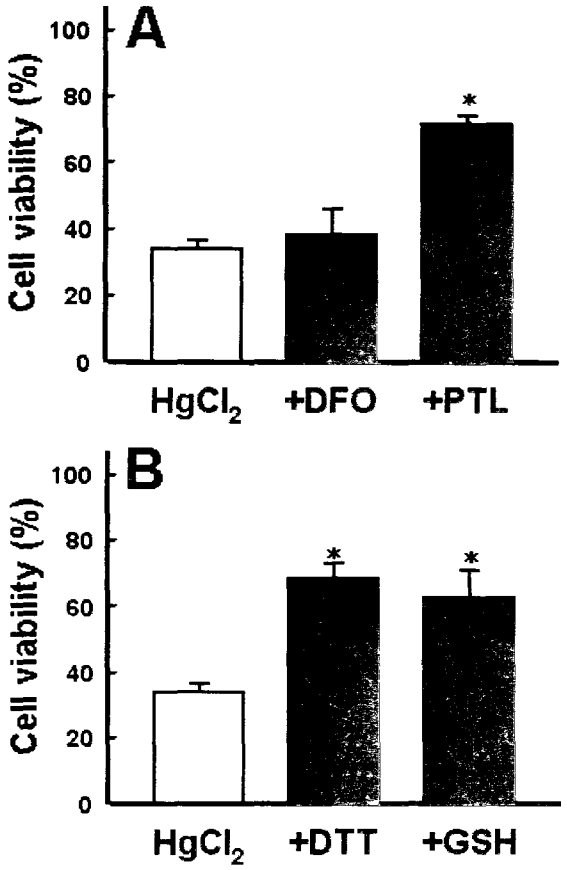

Fig. 5 A. Effects of iron chelators on $HgCl_2$-induced cytotoxicity in human glioma cells. Cells were treated with 0.02 mM $HgCl_2$ for 60 min in the presence or absence of deferoxamine (DFO, 3 mM) and phenanthroline (PTL, 0.2 mM). Data are mean SE of four determinations. *p<0.05 compared with $HgCl_2$ alone. B. Effects of thiols on $HgCl_2$-induced cytotoxicity in human glioma cells. Cells were treated with 0.02 mM $HgCl_2$ for 60 min in the presence or absence of dithiothreitol (DTT, 3 mM) and glutathione (GSH, 3 mM). The cytotoxicity was estimated by MTT assay. Data are mean SE of four determinations. *p<0.05 compared with $HgCl_2$ alone.

$HgCl_2$ was significantly prevented by these thiols (Fig. 5B), suggesting that thiol groups are involved in $HgCl_2$ cytotoxicity.

Effects of various agents on $HgCl_2$-induced ATP depletion

It has been reported that $HgCl_2$ causes an early and rapid depletion of ATP which leads to cell death in

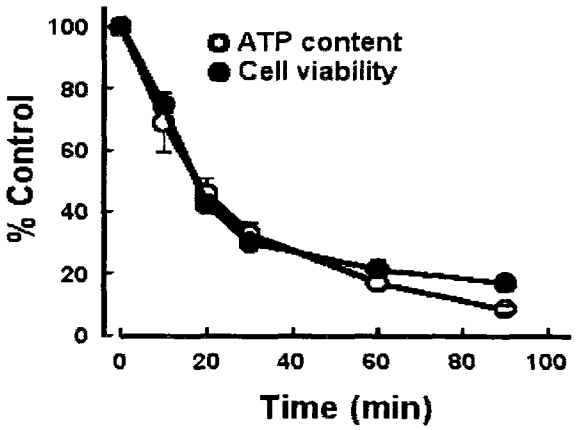

Fig. 6. Time course of $HgCl_2$-induced ATP depletion in human glioma cells. Cells were treated with 0.02 mM $HgCl_2$ for various times. Data are mean SE of four determinations. For comparison, changes in cell viability (data of Fig. 1) were plotted.

Table 1. Effects of various agents on $HgCl_2$-induced ATP depletion in A172 cells. Cells were pretreated with various agents for 30 min and exposed to $HgCl_2$ for 60 min at 37 °C. Data are mean SE of four experiments. *p<0.05 compared with control; #p<0.05 compared with $HgCl_2$ alone.

Treatment	ATP content (nmol/mg protein)
Control	4.65 ± 0.70
$HgCl_2$(0.02mM)	0.81 ± 0.17*
+ †DTT(2mM)	4.84 ± 0.41#
+ ‡GSH(2mM)	3.85 ± 0.59#
+ §SOD(500U/ml)	1.00 ± 0.32*
+ Catalase(800U/ml)	
+ ‖DMTU(30mM)	3.85 ± 0.57
+ Phenanthroline(0.2mM)	2.80 ± 0.67*#
+ ¶DPPD(0.01mM)	

†DTT dithiothreitol
‡GSH glutathione
§SOD superoxide dismutase
‖DMTU dimethylthiourea
¶DPPD N,N-diphenyl-p-phenylenediamine

hepatocytes.[23] We therefore examined the tine course of ATP depletion and compared with that of the loss of cell viability in exposed to $HgCl_2$. $HgCl_2$-induced ATP depletion occurred at the same rate and extent as the loss of cell viability (Fig. 6). Effects of various agents on ATP depletion were examined and summarized in Table 1. The ATP depletion was prevented by thiols, DMTU, and phenanthroline, agents that prevent HgCl2-induced

loss of cell viability. But SOD, catalase and DPPD were not effective in preventing ATP depletion.

Discussion

Although there is an increasing recognition of the importance of mercury intoxication in neurodegenerative disorders, the exact mechanisms by which mercury induces cell injury is not clearly defined. Mercury has been reported to increase the generation of ROS, which may be a mechanism underlying the mercury toxicity in brain tissues in vivo and in vitro.[4,5] Since the membrane lipids are most susceptible to oxidative stress, oxidative damage to membrane lipids, i.e., lipid peroxidation in biological systems, has been recognized as an evidence of oxidative cell injury.[24,25] Therefore, mercury may induce cell injury via lipid peroxidation in the nervous systems. In fact, mercury has been demonstrated to increase lipid peroxidation in brain tissues in vivo and in vitro.[10,11] However, whether lipid peroxidation plays an important role in mercury-induced cell injury is not clear.

Although mercury increases the generation of ROS, which may contribute to mercury cytotoxicity in brain tissues, the exact chemical species responsible for mercury-induced cell injury is not clear. In the kidney, $HgCl_2$ increases $HgCl_2$ generation via depolarization of the mitochondrial inner membrane.[6,26] However, the involvement of and precise role for individual ROS in mediating the loss of cell viability are not well understood. Thus, in the present study, the role of each species was examined using radical scavengers. While the hydroxyl radical scavenger DMTU exert a significant protective effect, the superoxide scavenger SOD and the H_2O_2 scavengers catalase and pyruvate were not effective. These results suggest that hydrogen peroxide is not involved in $HgCl_2$-induced loss of cell viability in human glioma cells. Catalase, because of its large molecular size, may not have access to the interior of the cell. However, pyruvate, another H_2O_2 scavenger that is permeable to cell membrane,[27] also did not alter $HgCl_2$-induced loss of cell viability (Fig. 2). Catalase prevented cell death induced by glucose oxidase/glucose, a H_2O_2 generating system (Fig. 3). The data of the present study with hydrogen peroxide scavengers are different those reported in kidney cells in which $HgCl_2$-induced cell injury is significantly prevented by catalase (880U/ml) and pyruvate (4 mM).[28]

Mercury has been known to produce reactive oxygen species (ROS)[4,5] and increases lipid peroxidation in brain tissues.[10,11] Indeed, in the present study, the hydroxyl radical scavenger DMTU provided beneficial effect (Fig. 2), suggesting involvement of hydroxyl radicals in $HgCl_2$-induced cell death, and lipid peroxidation increased after $HgCl_2$ exposure (Fig. 6). Since hydroxyl radicals are potent initiators of lipid peroxidation,[25] these data could support notion that lipid peroxidation resulting from oxidative stress plays a critical role in cell death caused by $HgCl_2$. Recently, however, we observed in renal epithelial cells that H_2O_2-induced injury was not associated with lipid peroxidation.[29] Similar dissociation of cell injury and lipid peroxidation was reported in cultured endothelial cells[30,31] and murine fibroblasts.[32] Thus, although $HgCl_2$ induces ROS generation in human glioma cells, lipid peroxidation may not be involved in $HgCl_2$-induced loss of cell viability. Lipid peroxidation can be a result of cell injury or an epiphenomenon of cell death rather than as a cause of cell injury.[19] Thus, we determined whether $HgCl_2$ causes cell death through a lipid peroxidation-dependent mechanism. We examined the effects of antioxidants on $HgCl_2$-induced cell death. If $HgCl_2$-induced cell death were resulted from lipid peroxidation, the cell death would be prevented by the potent antioxidants. In the present study, although the antioxidants DPPD, Trolox, and melatonin were ineffective in preventing cell death following exposure to $HgCl_2$ These data suggest that lipid peroxidation does not play a critical role in $HgCl_2$-induced loss of viability and thus may be a result of cell death rather than pathogenic in cells exposed to $HgCl_2$. Similar results have been reported in hepatocytes[7] and human epidermal keratinocytes.[13]

In the present study, the ferric iron chelator deferoxamine was not effective in preventing H_2O_2-induced loss of viability, but the ferrous iron chelator phena-

nthroline was effective (Fig. 5A). Similar results have been shown in the cytotoxicity induced by tBHP in cultured mammalian cells[33] and lectin-dependent cytotoxicity in human lymphocytes.[34] The inability of deferoxamine to protect H_2O_2-induced loss of viability may be because deferoxamine does not penetrate cells rapidly.[22] The protective effect of phenanthroline demonstrates the importance of iron catalyzed formation of hydroxyl or iron containing radicals in cellular injury.

Mercury is known to be extremely reactive toward sulfhydryl groups,[35] which could cause consequent glutathione depletion and oxidative stress.[36] Therefore, thiols are thought to play a pivotal role in protecting cells against mercury cytotoxicity. Indeed, the present study showed that thiols DTT and GSH effectively prevented $HgCl_2$-induced loss of cell viability (Fig. 5B). A number of studies have demonstrated that mitochondria are a principal target of $HgCl_2$, as evidenced by mitochondrial swelling[37,38] and impairment of oxidative phosphorylation.[39,40] Thus, ATP depletion in the present study may appear to be the consequence of loss of oxidative phosphorylation.[23] reported in hepatocytes that ATP depletion after $HgCl_2$ follows mitochondrial depolarization but precedes cell death. In the present study, ATP depletion followed closely the loss of cell viability (Fig. 7). Moreover, ATP depletion was prevented by agents protecting $HgCl_2$-induced cell death (Table 1). However, it is unclear whether ATP depletion is a cause of cell death in human glioma cells. Several studies have shown that the cell injury caused by oxidants and anoxia in renal cells and hepatocytes is dissociated with ATP depletion.[41,42,43]

Conclusion

$HgCl_2$ induced hydroxyl radical generation, lipid peroxidation, and ATP depletion in human glioma cells. The cell death caused by $HgCl_2$ was not affected or partially prevented by potent antioxidants. The cell death and ATP depletion by $HgCl_2$ were prevented by iron chelator (phenanthroline) and thiols (DTT and GSH), but not by antioxidants. These results suggest that $HgCl_2$ induces cell death through a lipid peroxidation- independent and iron-dependent mechanism.

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국문초록

배경 수은은 신경조직에서 여러 가지 독성을 나타내며 알츠하이머병의 병태생리학적 기전에 관련되어 있는 것으로 알려져 있다. 생체 및 시험관내 실험을 통하여 신경을 포함한 여러 조직세포에서 수은이 반응성 산소기를 발생시키고 지질의 과산화를 유발하는 것으로 밝혀짐으로서 산화성 스트레스가 수은에 의해 유발되는 여러 가지 신경성 질환에 관여할 것으로 예상된다. 그러나 지질의 과산화가 수은에 의한 세포독성에 중요한 역할을 하는 지에 대해서는 분명하지 않다.

방법 본 연구에서는 수은에 의한 세포손상에 어떤 반응성 산소기가 관여하며, 지지의 관산화가 수은에 의한 세포독성에 기여하고 있는 지를 인간 신경교종세포인 A172세포를 이용하여 조사하고자 하였다.

결과 수은은 농도와 처리시간에 비례하여 세포사망을 일으다. 이러한 변화는 Hydroxyl radical scavenger인 dimethylthiourea에 의해 방지 되었으나, superoxide scavenger인 superoxide dismutase와 hydrogen peroxide scavenger인 catalase와 pyruvate에 의해서는 영향을 받지 않았다. 황산화제인 DPPD, Trolox 및 melatonin은 방지효과를 나타내지 못했다. 수은에 의한 세포손상은 iron chelator인 phenanthroline과 sulfhydryl group 방지제인 dithiothreitol과 glutathione에 의해 방지되었다. 수은은 세포내 ATP농도를 감소시켰으며, 이러한 효과는 수은에 의한 세포손상을 방지하는 약물들에 의해 억제되었다.

결론 철이온에 의존적인 기전을 통해 발생된 hydroxyl radical이 수은에 의한 세포손상에 관련되어 있으며, 지질의 관산화는 수은에 의한 세포독성에 중요한 역할을 하지 못하는 것으로 생각된다.