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# Involvement of Ceramide in the Mechanism of Cr(VI)-induced Apoptosis of CHO Cells

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Mitochondria reduce Cr(VI) to Cr(V) with concomitant generation of reactive oxygen species, thereby exhibiting cytotoxic effects leading to apoptosis in various types of cells. To clarify the mechanism by which Cr(VI) induces apoptosis, we examined the effect of Cr(VI) on Chinese hamster ovary (CHO) cells. Cr(VI) increased cellular levels of ceramide by activating acid sphingomyelinase (ASMase) and inhibiting the phosphorylation of pleckstrin homology domain-containing protein kinase B (Akt). Cr(VI) also induced cyclosporin A- and trifluoperazine-sensitive depolarization of mitochondria and activated caspase-3, 8 and 9, thereby causing fragmentation of cellular DNA. The presence of desipramine, an inhibitor of ASMase, and membrane permeable pCPT-cAMP suppressed the Cr(VI)-induced activation of caspases and DNA fragmentation. These results suggested that accumulation of ceramide play an important role in the Cr(VI)-induced apoptosis of CHO cells through activation of mitochondrial membrane permeability transition.

*Keywords*: Apoptosis; Ceramide; Chromium; Mitochondrial permeability transition; p-Akt

*Abbreviations*: Akt, pleckstrin homology domain-containing protein kinase B; Ac-DEVD-MCA, acetyl-Asp-Glu-Val-Asp-MCA; Ac-IETD-MCA, acetyl-Ile-Glu-Thr-Asp-MCA; Ac-LEHD-MCA, acetyl-Leu-Glu-His-Asp-MCA; CHO, Chinese hamster ovary; Cr, chromium; CsA, cyclosporin A; DCFH-DA, 2′, 7′-dichlorofluorescein diacetate; Hoechst 33342, 2′-(4-Ethoxyphenyl)-5-(4-methyl-1-piperazinyl)-2, 5′-bi-1H-benzimidazole; JC-1, 5, 5′, 6, 6′-tetrachloro-1, 1′, 3, 3′-tetraethylbenzimidazolyl carbocyanine iodide; MPT, membrane permeability transition; PI, propidium iodide; ROS, reactive oxygen species; TFZ, trifluoperazine dihydrochloride; z-VAD-fmk,z-Val-Ala-Asp (OMe) fluoromethylketone; pCPT-cAMP, 8-(4-chlorophenylthio) adenosine 3′:5′-cyclic monophosphate

## INTRODUCTION

Certain types of chromium, particularly Cr(VI), are toxic and/or carcinogenic.[1] Cr(VI) is transported into cells and reduced to Cr(V) by mitochondria with concomitant generation of genotoxic metabolites.[2–4] Isolated rat liver mitochondria also reduce Cr(VI) by an NADH-dependent reaction with concomitant production of reactive oxygen species (ROS).[5–8] Production of excess ROS often induces DNA fragmentation and apoptosis.[1,2,8,9] It was found that ROS generation by chromium compounds caused DNA-strand breakage and has an important role in chromium-induced carcinogenesis.[10,11] It has been reported that mitochondrial membrane permeability transition (MPT) play important roles in the induction of apoptosis.[12–14] In fact, cyclosporin A (CsA), an inhibitor of MPT, inhibited the mitochondrial depolarization and cytochrome c release in Cr(VI)-treated Chinese hamster ovary (CHO) cells.[8,9] However, the mechanism by which Cr(VI) induces MPT remains to be elucidated.

Ceramide induces differentiation and/or apoptosis of various types of cells.[15] In this context, ROS have been postulated to play a role in dihydroceramide-induced cytochrome c release from mitochondria[16] and ceramide-induced cell death.[17] Furthermore, ceramide has been known to inhibit phospho-Akt (p-Akt) that activates

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caspase-8.[18–22] Because apoptosis is the predominant response of cultured CHO cells to Cr(VI),[23–26] we examined its effect on ceramide generation and p-Akt activity of CHO cells. The results clearly showed that the accumulation of ceramide play critical roles in the mechanism of Cr(VI)-induced apoptosis of CHO cells.

## MATERIALS AND METHODS

### Chemicals

Desipramine, 8-(4-chlorophenylthio) adenosine 3′, 5′-cyclic monophosphate (pCPT-cAMP), CsA, 2′-(4-ethoxyphenyl)-5-(4-methyl-1-piperazinyl)-2,5′-bi-1H-benzimidazole (Hoechst 33342), trifluoperazine dihydrochloride (TFZ), propidium iodide (PI), ribonuclease A and proteinase K were obtained from Sigma Chemical Co. (USA). Antibody against p-Akt, 5,5′6,6′-tetrachloro-1,1′,3,3′-tetraethyl-benzimidazolylcarbocyanine iodide (JC-1) were purchased from Cell Signaling Technology (USA) and Molecular Probes (USA), respectively. Fluorogenic tetrapeptide substrates, such as acetyl-Asp-Glu-Val-Asp-MCA (Ac-DEVD-MCA for caspase-3), acetyl-Ile-Glu-Thr-Asp-MCA (Ac-IETD-MCA for caspase-8) and acetyl-Leu-Glu-His-Asp-MCA (Ac-LEHD-MCA for caspase-9), and z-VAD-fmk, an universal caspase inhibitor, were obtained from Peptide Institute (Japan). All other chemicals were of analytical grade and obtained from Nacalai Tesque (Japan).

### Cell Line

CHO cells were maintained in minimum essential Eagle medium (MEM) supplemented with 10% heat-inactivated fetal bovine serum (Sigma), 10 mM HEPES (pH 7.4), 1 mM sodium pyruvate, 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were grown in a humidified incubator at 37°C under 5% $CO_2$/95% air and used for assays during the exponential phase of their growth. Cells were counted and their viability was assayed by the trypan blue exclusion method.[27]

### Effect of Cr(VI) on Cell Growth and Nuclear Condensation

CHO cells were plated onto 35 mm dishes ($1.7 \times 10^5$ cells per dish) containing 1.5 ml of MEM and 10% fetal bovine serum and incubated for 16 h before treatment with various reagents. After incubation with Cr(VI) for varying times, the number of viable cells was determined. After incubation with Cr(VI) for 24 h, the cells were stained with 1 μM of Hoechst 33342 for 15 min. The fluorescence intensity of the dye was measured using an Olympus IX 70 fluorescence microscope with excitation and emission wavelengths at 340 and 510 nm, respectively.

### Analysis of Apoptotic Cells by Flow Cytometry

The number of cells in each phase of cell cycle and that showing apoptosis were determined by using a FACScan flow cytometer after staining DNA with PI as described previously.[28] Briefly, CHO cells ($\sim 2 \times 10^6$) were washed twice with PBS and resuspended in PBS (0.2% Triton X-100). Following treatment with 0.1 mg/ml RNase A, the cells were stained with 50 μg/ml PI. Flow cytometric analysis was carried out with argon laser excitation at 488 nm (Becton Dickinson). Fluorescence (FL2;DNA) analysis was carried out using a 564–606 nm band pass filter. Twenty thousand cells were analyzed in each sample.

### Analysis of DNA Fragmentation

The extent of DNA fragmentation was determined spectrophotometrically by the diphenylamine method.[29] After incubation with Cr(VI), cells were lysed in 200 μl of a lysis buffer [10 mM Tris–HCl buffer (pH 7.4), 10 mM EDTA and 0.5% Triton X-100] at 4°C for 30 min. The lysate was centrifuged at $13,000 \times g$ and 4°C for 20 min to separate intact and fragmented chromatins. Both the pellet and the supernatant were treated with 6% perchloric acid at 4°C for 30 min followed by centrifugation at $13,000 \times g$ and 4°C for 20 min. The DNA specimens were incubated at 70°C for 20 min in 50 μl of 6% perchloric acid, and then mixed with 100 μl of diphenylamine solution [1.5% (w/v) diphenylamine, 1.5% sulfuric acid and 0.01% acetaldehyde in glacial acetic acid]. After incubation for overnight at 30°C in the dark, optical density was measured at 600 nm, and the percentage of DNA fragmentation was calculated as the ratio of DNA in the supernatant to the total DNA.

DNA fragmentation was analyzed by agarose gel electrophoresis.[27] The lysate was treated with 400 μg/ml RNase A at 37°C for 1 h and 400 μg/ml proteinase K at 37°C for 1 h. The DNA was precipitated with an equal volume of isopropanol, and then electrophoresed at 50 V on a 2% agarose gel in TBE buffer [90 mM Tris-borate buffer (pH 8.3) containing 2 mM EDTA]. DNA bands were visualized under ultraviolet illumination and photographed using a Polaroid 667 film.

### Western Blot Analysis

Cells ($\sim 10^7$) were dissolved in SDS-sample buffer [125 mM Tris–HCl (pH 6.8), 4% SDS, 10% mercaptoethanol, 20% glycerol and 0.002% bromophenol blue]

and subjected to SDS-PAGE as described elsewhere.[28,30] Proteins in the gel were transferred onto an Immobilon filter (Millipore Co.) and the filter was incubated with the primary antibody (1:1000 dilution) and subsequently with horseradish peroxidase-linked secondary antibody (1:2000 dilution) and analyzed by using an ECL plus kit (Amersham). Protein concentration was determined by the method of Bradford [31] using bovine serum albumin as a standard.

### Assay for Caspase Activity

Activity of caspase was determined in 20 mM HEPES buffer (pH 7.5) containing 0.1 M NaCl and 5 mM DTT at 37°C using Ac-DEVD-MCA, Ac-IETD-MCA or Ac-LEHD-MCA (all 10 μM), for caspase-3, 8, and 9, respectively.[30] One unit of the activity was defined as the amount of enzyme required for the liberation of 1 nmol of 7-amino-4-methylcoumarin (AMC) in 1 h.

### Assay for Mitochondrial Membrane Potential in Cells

After incubation with 50 μM Cr(VI) for 24 h, cells were washed twice with PBS and stained with 5 μg/ml of JC-1 at 37°C for 30 min. Then, the cells were analyzed using a FACS Calibur flow cytometer (Becton Dickinson) to determine the mitochondrial membrane potential.[32]

### Assay for Sphingomyelinase Activity

Catalytic activities of neutral (NSMase) and acid sphingomyelinase (ASMase) were determined by the method of Wiegmann.[33] To measure NSMase, cells ($2 \times 10^6$) were washed with PBS and the pellet was resuspended in 200 μl of 20 mM HEPES buffer (pH 7.4) containing 10 mM $MgCl_2$, 2 mM EDTA, 5 mM DTT, 0.1 mM $Na_3VO_4$, 10 mM β-glycerophosphate, 750 μM ATP, 1 mM PMSF, 10 μM leupeptin, 10 μM pepstatin, and 0.2% Triton X-100. After incubation at 4°C for 5 min, cells were sonicated and the protein concentration was measured. Proteins (50–100 μg) were incubated at 37°C for 1 h in 100 μl of 20 mM HEPES buffer (pH 7.4) containing 1 mM $MgCl_2$ and 2.3 μl of [N-methyl-$^{14}$C]SM (0.2 μCi/ml; specific activity of 56.6 mCi mmol). To measure ASMase, the washed cells were resuspended in 200 μl of 0.2% Triton X-100, incubated at 4°C for 15 min, and sonicated prior to the determination of protein. Proteins (50–100 μg) were incubated at 37°C for 1 h in 100 μl of 250 μM sodium acetate buffer (pH 5.0) containing 1 mM EDTA and 2.25 μl of [N-methyl-$^{14}$C]SM. Radioactive phosphorylcholine produced from [$^{14}$C] SM was extracted with 1.5 ml of chloroform/methanol (2:1). The [$^{14}$C] phosphorylcholine produced in the aqueous phase was measured by a scintillation counter.

### Assay for Ceramide

Ceramide was determined using the diacylglycerol kinase assay method.[34] Briefly, $2 \times 10^6$ cells were lysed in chloroform/methanol (2:1), and their lipids were extracted by the method of Bligh and Dyer.[35] Aliquots were dried under nitrogen and used for the analysis of ceramide and phosphate. Lipids were solubilized in β-octylglucopyranoside/dioleoyl-phosphatidylglycerol mixed micelles, and incubated at 25°C for 30 min in the presence of 2 mM DTT, 1 μg of diacylglycerol kinase and 2 mM ATP (mixed with 2 μCi of [gamma-$^{32}$P]ATP) in a final volume of 100 μl. The reaction was quenched in chloroform/methanol (2:1) and the lipids were extracted by the method of Bligh and Dyer.[35] After evaporation of the chloroform phase under nitrogen, phosphorylated lipids were suspended in chloroform and spotted on silica gel 60 TLC plates. The plates were developed in a solvent system consisting of chloroform/acetone/methanol/acetic acid/water (10:4:3:2:1). After autoradiography, the location of ceramide 1-phosphate was determined based on an Rf value of 0.48 for the phosphorylated standard sample of ceramide.

## RESULTS

### Effect of Cr(VI) on Cell Growth, DNA Synthesis and Nuclear Condensation

Figure 1 shows that Cr(VI) inhibited the growth of CHO cells in a concentration-dependent manner. A complete suppression was observed at Cr(VI) concentration of 25 μM. Nuclear condensation was observed in stained cells with Hoechst 33342 at 24 h after treatment with 50 μM Cr(VI) (Fig. 1B). The DNA content of cells was quantitated by flow cytometry of PI-stained cells that allowed simultaneous evaluation of their cell cycle and the number of apoptotic cells. The PI-stained cells increased during the incubation with Cr(VI) at concentrations higher than 75 μM (data not shown). The number of apoptotic cells also increased during the incubation with 50 μM Cr(VI) (Fig. 1C).

### Effect of Cr(VI) on Nuclear DNA

Cr(VI) induced DNA fragmentation of CHO cells in a time- and concentration-dependent manner (Fig. 2). Kinetic analysis revealed that DNA fragmentation became apparent at 20–30 h after incubation with

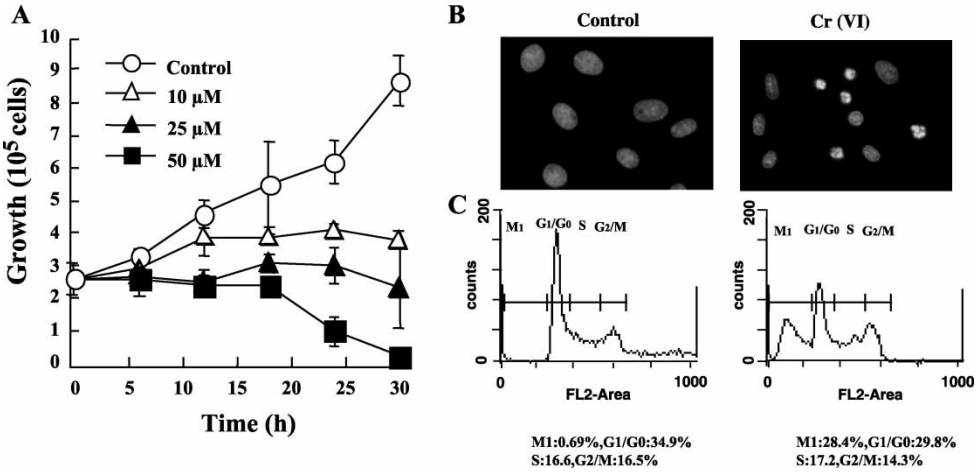


FIGURE 1 Effect of Cr(VI) on the growth and nuclear DNA status of CHO cells. (A) CHO cells were exposed to various concentrations of Cr(VI) and their growth assessed. Data are Mean ± SD derived from triplicate experiments. Nuclear condensation of cells stained with Hoechst 33342 was apparent after incubation with 50 μM Cr(VI) for 24 h (B). Flow cytometric analysis of the distribution of DNA content of PI-stained cells is shown in (C). Similar results were obtained in three separate experiments.



50 μM Cr(VI). These results indicated that Cr(VI) induced apoptotic cell death. To obtain further insight into the mechanism of DNA-fragmentation, effect of z-VAD-fmk on the Cr(VI)-induced DNA fragmentation was examined. As shown in Fig. 3, fragmentation of cellular DNA was suppressed by the presence of z-VAD-fmk (Fig. 3). Since MPT plays a crucial role in the process of apoptosis, effects of CsA and TFZ on Cr(VI)-induced DNA fragmentation were investigated. Both CsA and TFZ suppressed the fragmentation of DNA. These results indicate that Cr(VI)-induced apoptosis occurred through opening of MPT pore followed by activation of caspases.

### Effect of Cr(VI) on the Caspase Cascade

Since Cr(VI)-induced apoptosis was suppressed by z-VAD-fmk, we examined the activity of various caspase-like proteases, such as caspase-3, 8, and 9, in CHO cells. Incubation of cells with Cr(VI) for 24 h significantly increased the caspase-3 activity in the cells (Fig. 4). Cr(VI) also increased caspase-8 and 9 activity.

### Effect of CsA and TFZ on Mitochondrial Depolarization

MPT plays a critical role in the mechanism of apoptosis.[36] To gain further insight into the mechanism of Cr(VI)-induced changes in mitochondrial functions, its effect on mitochondrial membrane potential was analyzed with CHO cells. FACScan analysis revealed that, during the incubation with Cr(VI), mitochondria-associated red fluorescence in JC-1-stained cells decreased with concomitant increase in cytosolic green fluorescence,

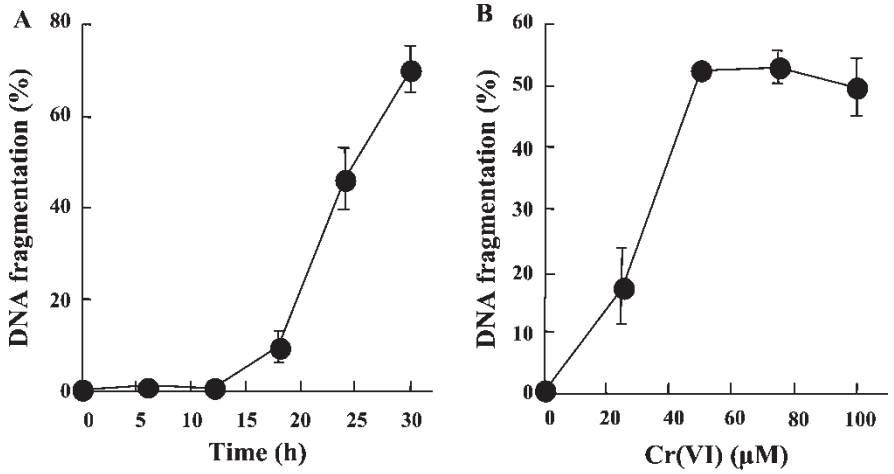


FIGURE 2 Effect of Cr(VI) on nuclear DNA of CHO cells. The cells were treated with 50 μM Cr(VI) and the percentage of fragmented DNA was determined by the diphenylamine method. Time course (A) of DNA-fragmentation and its concentration dependency after 24 h (B) were determined. Data show Means ± SD from triplicate experiments.

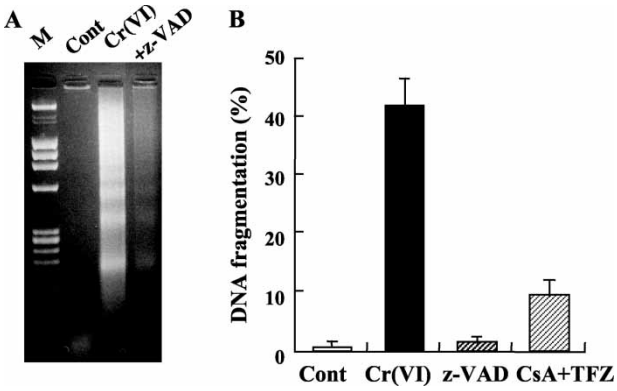


FIGURE 3 Cr(VI)-induced DNA fragmentation and its sensitivity to agents. (A) DNA fragmentation of CHO cells was analyzed by agarose gel electrophoresis. Cells were incubated in the presence or absence of 50 μM z-VAD-fmk for 30 min and subsequently with 50 μM Cr(VI) for 24 h. (B) DNA fragmentation of CHO cells was analyzed by difenylamine method. Cells were incubated in the presence of either 50 μM z-VAD-fmk or 5 μM CsA + 10 μM TFZ for 30 min and subsequently with 50 μM Cr(VI) for 24 h. Similar results were obtained in triplicate experiments in (A) and data in (B) show the Mean ± SD derived from triplicate experiments.

indicating the depolarization of mitochondria (Fig. 5A). Cr(VI) depolarization by a mechanism that was suppressed by the presence of CsA and TFZ (Fig. 5B). These results support the hypothesis that the activation of caspases and MPT underlie the mechanism for Cr(VI)-induced apoptosis of CHO cells.

### Effect of Desipramine on Cr(VI)-induced Caspase-3 Activation and DNA Fragmentation

It has been known that ganglioside (GD3) synthesized in Golgi apparatus from ceramide interacts with mitochondrial membranes and induces cytochrome c release through MPT pores.[16,37] Since ceramide is generated from sphingomyelin by ASMase, [37,38] effects of desipramine, an inhibitor of the enzyme, on the Cr(VI)-induced caspase activation and DNA fragmentation were examined. Both DNA fragmentation and caspase-3 activation were suppressed by desipramine in a concentration-dependent manner (Fig. 6). These results indicated that Cr(VI)-induced generation of ceramide play an important role in the signaling pathway leading to apoptosis.

### Effect of Cr(VI) on ASMase and Ceramide Generation

Since desipramine suppressed the Cr(VI)-induced apoptosis of CHO cells, we assessed the cellular levels of ASMase and ceramide during the incubation with Cr(VI). The activity of ASMase, but not NSMase, was increased by treating cells with Cr(VI) in a time-dependent manner (Fig. 7). Cellular levels of ceramide also increased during the incubation with Cr(VI).

### Effect of Cr(VI) on p-Akt in CHO Cells

Recent studies showed that ceramide inhibited cell proliferation by inactivating Akt and induced apoptosis of various cells.[19,21,22] Furthermore, p-Akt plays an important role in the activation of caspase-8.[18,20] Thus, effect of Cr(VI) on cellular levels of Akt and p-Akt was examined by Western blotting using anti-Akt and anti-p-Akt antibodies. Cellular level of Akt remained unchanged during the incubation with Cr(VI) while that of p-Akt transiently increased after 6 h and then decreased thereafter (Fig. 8).

### Effect of pCPT-cAMP on Cr(VI)-induced Depolarization, DNA Fragmentation and Caspase Activation

It has been shown that cellular levels of p-Akt are regulated by cAMP.[39,40] To gain further insight

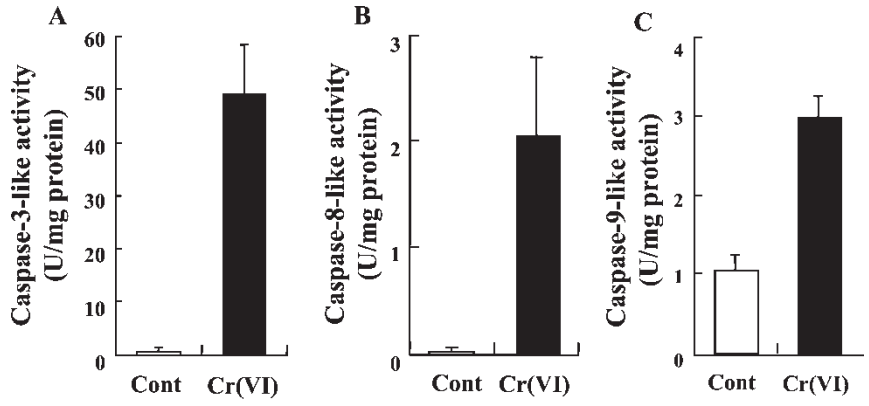


FIGURE 4 Effect of Cr(VI) on caspases in CHO cells. After incubation of CHO cells with 50 μM Cr(VI) for 24 h, the cytosol (50 μl) was incubated with 10 μM Ac-DEVD-MCA, Ac-IETD-MCA and Ac-LEHD-MCA, fluorogenic substrates for caspase-3 (A), 8 (B) and 9 (C), respectively. Data show the Mean ± SD derived from triplicate experiments.

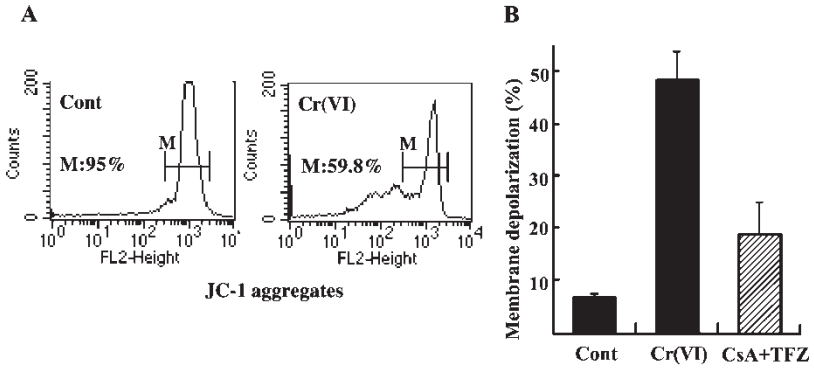


FIGURE 5 Cr(VI)-induced depolarization of mitochondria in CHO cells. After incubation of CHO cells with 50 μM Cr(VI) for 24 h, cells were stained with JC-1. (A) Flow cytometric analysis of JC-1-stained cells was carried out 24 h after incubation with Cr(VI). Similar results were obtained in triplicate experiments. (B) In the presence of 5 μM CsA + 10 μM TFZ, the percent of depolarized cells was calculated. Data show the Mean ± SD derived from triplicate experiments. M, polarized cells.



into the mechanism of Cr(VI)-induced activation of caspase-8, effect of membrane permeable pCPT-cAMP on the Cr(VI)-induced apoptosis was examined. As shown in Fig. 9, pCPT-cAMP suppressed the Cr(VI)-induced membrane depolarization, DNA fragmentation and caspase activation in CHO cells. Cr(VI)-induced activation of caspase-8 was inhibited completely by the presence of pCPT-cAMP. These results support the hypothesis that Cr(VI) suppressed cellular p-Akt to activate caspase-8.

## DISCUSSION

The present work shows that incubation of CHO cells with Cr(VI) activated ASMase and increased ceramide content with concomitant suppression of p-Akt expression in the cells, thereby triggering mitochondrial depolarization to activate the caspase cascade and DNA fragmentation. We also showed that Cr(VI) induced DNA fragmentation of CHO cells via the mitochondria-dependent apoptotic pathway described by Pritchard.[9] Because CsA and TFZ suppressed the Cr(VI)-induced DNA fragmentation while membrane permeable cAMP analog markedly suppressed the activation of caspase-8, Cr(VI) might induce apoptosis of CHO cells through MPT-dependent pathway by suppressing PI3kinase.

Ceramide is a bioactive sphingolipid metabolized to ganglioside (GD3) which modulates a variety of cell functions including mitochondrial MPT and apoptosis.[41] Cellular levels of ceramide are determined either by *de novo* synthesis or generation by ASMase via the desipramine-inhibitable mechanism. Both pathways have been shown to participate in the mechanism of apoptosis.[38] Preliminary experiments in this laboratory revealed that a low concentration of vero-toxin (Shiga-toxin) stimulated

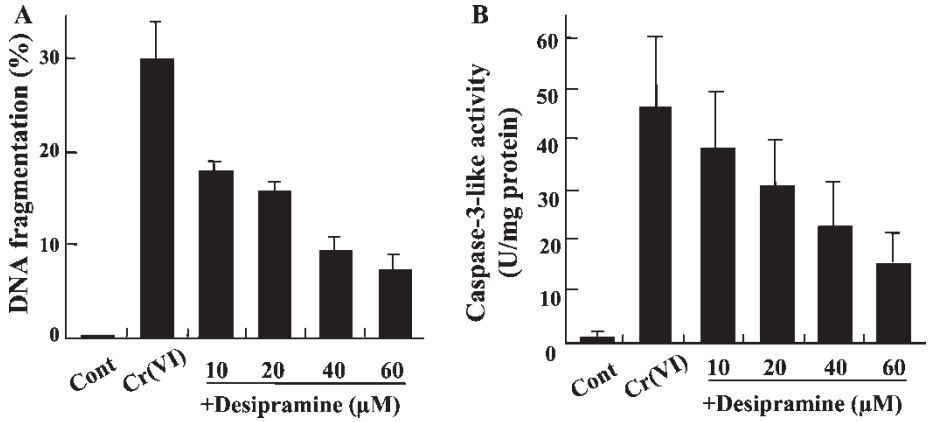


FIGURE 6 Effect of Cr(VI) and desipramine on cellular DNA and caspase-3. Cells were incubated with various concentrations of desipramine for 1 h and subsequently with 50 μM Cr(VI) for 24 h. Then, DNA-fragmentation and caspase-3 activity were determined. Data show the Means ± SD derived from triplicate experiments.

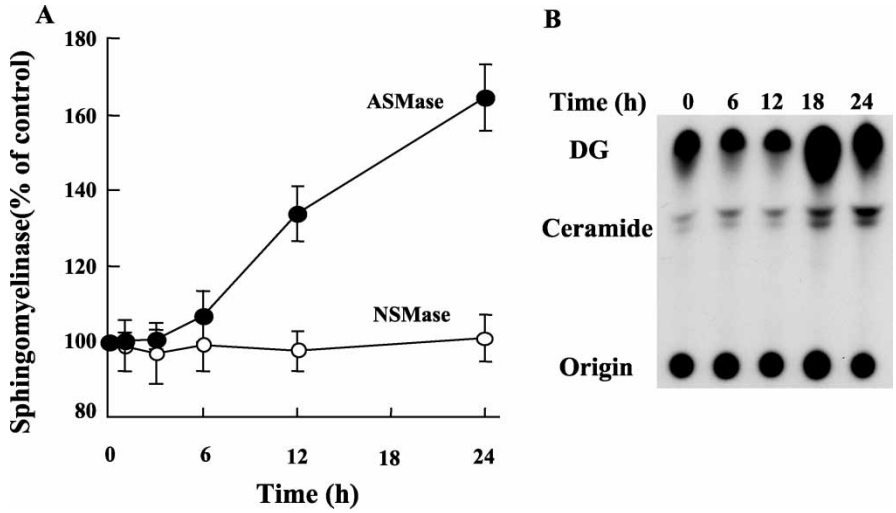


FIGURE 7 Effect of Cr(VI) on sphingomyelinase and ceramide in CHO cells. CHO ($2 \times 10^6$) cells were incubated in the presence or absence of 50 μM Cr(VI) for 24 h. (A) The acid and neutral sphingomyelinases in the cytosolic fraction were measured using [$^{14}$C] sphingomyelin. Data show the Means ± SD derived from triplicate experiments. (B) Ceramide was assayed by the diacylglycerol kinase method using thin layer chromatography as described in the text. DG, 1,2-diacylglycerol. Similar results were obtained in triplicate experiments.



the activity of ASMase in Vero cells within 30 min with concomitant accumulation of ceramide. The present work also shows that Cr(VI) stimutated the activity of ASMase in CHO cells with concomitant increase in ceramide by a desipramine-sensitive mechanism. These results suggested that Cr(VI) stimulated ASMase to increase ceramide, which was converted to GD3 that induced mitochondrial MPT.

Ceramide has been shown to inhibit cellular p-Akt and activate caspase-8 to induce apoptosis of cells.[19,21] The present work also shows that Cr(VI) suppressed the cellular p-Akt and activated caspases-8 by a pCPT-cAMP-inhibitable mechanism. Caspase-8 has been shown to convert Bid to its truncated form (tBid) which releases cytochrome c by a CsA-insensitive mechanism.[42–44] These results indicated that caspase-8 was activated as a result of p-Akt suppression occurred during the early stages of Cr(VI)-treatment. Because pCPT-cAMP partially suppressed DNA fragmentation and activation of caspase-3, Cr(VI) seems to activate caspases thereby inducing DNA fragmentation both through CsA-sensitive and insensitive MPT pores.

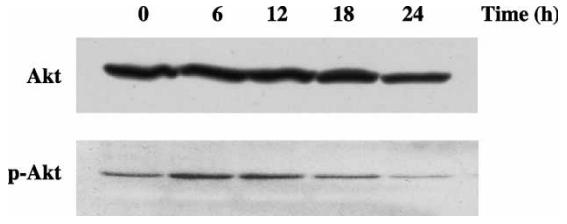


FIGURE 8 Changes in p-Akt content of CHO cells induced by Cr(VI). At various times after treatment with 50 μM Cr(VI), p-Akt in CHO cells were detected by Western blot analysis with anti-p-Akt antibody. Similar results were obtained in three separate experiments.

It has been well known that mitochondria reduce Cr(VI) to Cr(V) by an NADH-dependent mechanism with concomitant generation of ROS.[5–8] In this context, Balamurugan suggested that tyrosine kinase in the Src-family underlies the mechanism of lymphocyte apoptosis elicited by various stimuli that generate ROS.[45] It has been reported that ultraviolet C- and $H_2O_2$-induced apoptosis involves the activation of ASMase [46] and that ceramide and ROS enhance the degradation of Akt/protein kinase B.[47] On the contrary, the ability of ASMase to induce oxidative stress has been postulated to be a consequence of apoptosis[48] and that TNF-α rapidly enhances the ceramide-dependent generation of ROS by mitochondria.[49] However, preliminary experiments in this laboratory revealed that ROS generation increased during the early stage of Cr(VI)-induced apoptosis when ASMase activation and ceramide accumulation were not apparent. Therefore, relationship between Cr(VI)-induced ROS generation and ceramide synthesis remained in obscure. Thus, Cr(VI) stimulate the generation of ceramide by activated ASMase to decrease p-Akt and activate caspase-8, thereby increasing mitochondrial membrane permeability leading to apoptosis of Cr(VI)-treated cells via the desipramine-sensitive mechanism.

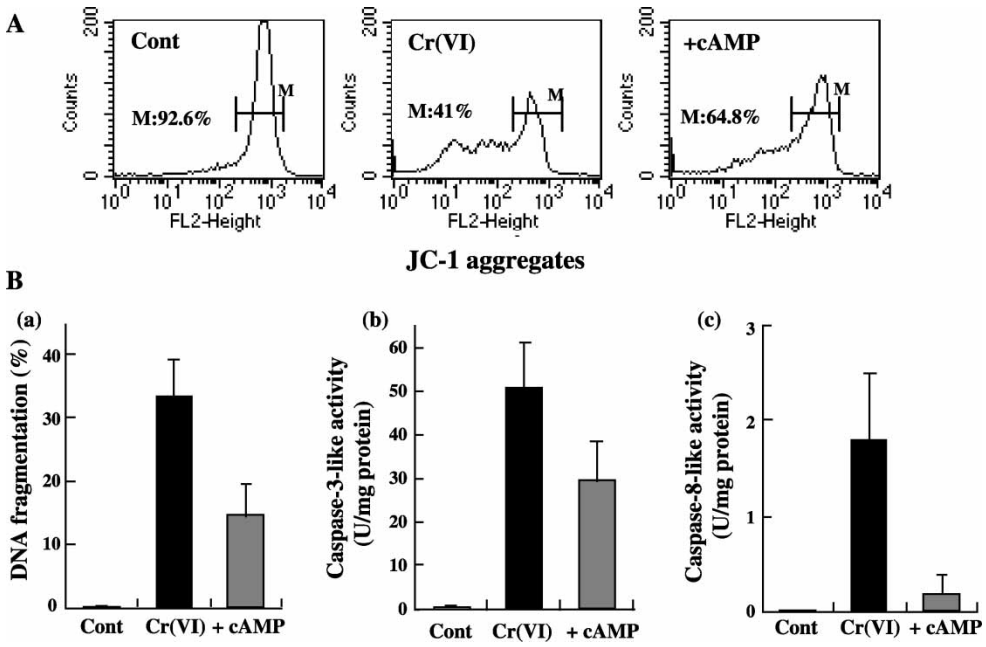


FIGURE 9 Effect of Cr(VI) and pCPT-cAMP on CHO cells. Cells were incubated in the presence or absence of 100 μM pCPT-cAMP for 1 h and subsequently with 50 μM Cr(VI) for 24 h. (A) show membrane depolarization assay as described in Fig. 5. Similar results were obtained in three separate experiments. (B) show the DNA-fragmentation (a), caspase-3 like activity (b) and caspase-8-lile activity (c). Data show the Mean ± SD derived from triplicate experiments.



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## *References*

[1] Stohs, S.J., Bagchi, D., Hassoun, E. and Bagchi, M. (2000) "Oxidative mechanisms in the toxicity of chromium and cadmium ions", *J. Environ. Pathol. Toxicol. Oncol.* **19**, 201–213.
[2] Rossi, S.C., Gorman, N. and Wetterhahn, K.E. (1988) "Mitochondrial reduction of the carcinogen chromate: formation of chromium (V)", *Chem. Res. Toxicol.* **1**, 101–107.
[3] Wetterhahn, K.E., Hamilton, J.W., Aiyar, J., Borges, K.M. and Floyd, R. (1989) "Mechanism of chromium (VI) carcinogenesis. Reactive intermediates and effect on gene expression", *Biol. Trace Elem. Res.* **21**, 405–411.
[4] Klein, C.B., Frenkel, K. and Costa, M. (1991) "The role of oxidative processes in metal carcinogenesis", *Chem. Res. Toxicol.* **4**, 592–604.
[5] Rossi, S.C. and Wetterhahn, K.E. (1989) "Chromium(V) is produced upon reduction of chromate by mitochondrial electron transport chain complexes", *Carcinogenesis* **10**, 913–920.
[6] Ryberg, D. and Alexander, J. (1990) "Mechanisms of chromium toxicity in mitochondria", *Chem. Biol. Interact.* **75**, 141–151.
[7] Shi, X., Chiu, A., Chen, C.T., Halliwell, B., Castranova, V. and Vallyathan, V. (1999) "Reduction of chromium (VI) and its relationship to carcinogenesis", *J. Toxicol. Environ. Health Part B Crit. Rev.* **2**, 87–104.
[8] Ye, J., Wang, S., Leonard, S.S., Sun, Y., Butterworth, L., Antonini, J., Ding, M., Rojanasakul, Y., Vallyathan, V., Castranova, V. and Shi, X. (1999) "Role of reactive oxygen species and p53 in chromium(VI)-induced apoptosis", *J. Biol. Chem.* **274**, 34974–34980.
[9] Pritchard, D.E., Singh, J., Carlisle, D.L. and Patierno, S.R. (2000) "Cyclosporin A inhibits chromium (VI)-induced apoptosis and mitochondrial cytochrome c release and restores clonogenic survival in CHO cells", *Carcinogenesis* **21**, 2027–2033.
[10] Leonard, S.S., Vallyathan, V., Castranova, V. and Shi, X. (2002) "Generation of reactive oxygen species in the enzymatic reduction of PbCrO4 and related DNA damage", *Mol. Cell. Biochem.* **234,235**, 309–315.
[11] Kawanishi, S., Inoue, S. and Sano, S. (1986) "Mechanism of DNA cleavage induced by sodium chromate(VI) in the presence of hydrogen peroxide", *J. Biol. Chem.* **261**, 5952–5958.
[12] Green, D.R. and Reed, J.C. (1998) "Mitochondria and apoptosis", *Science* **281**, 1309–1312.
[13] Nishikimi, A., Kira, Y., Kasahara, E., Sato, E.F., Kanno, T., Utsumi, K. and Inoue, M. (2001) "Tributyltin interacts with mitochondria and induces cytochrome c release", *Biochem.J.* **356**, 621–626.
[14] Petronilli, V., Penzo, D., Scorrano, L., Bernardi, P. and Di Lisa, F. (2001) "The mitochondrial permeability transition, release of cytochrome c and cell death. Correlation with the duration of pore openings *in situ*", *J. Biol. Chem.* **276**, 12030–12034.
[15] Perry, D.K. (1999) "Ceramide and apoptosis", *Biochem. Soc. Symp.* **27**, 399–404.
[16] Richter, C. and Ghafourifar, P. (1999) "Ceramide induces cytochrome c release from isolated mitochondria", *Biochem. Soc. Symp.* **66**, 27–31.
[17] Andrieu-Abadie, N., Gouaze, V., Salvayre, R. and Levade, T. (2001) "Ceramide in apoptosis signaling: relationship with oxidative stress", *Free. Radic. Biol. Med.* **31**, 717–728.
[18] Rytomaa, M., Lehmann, K. and Downward, J. (2000) "Matrix detachment induces caspase-dependent cytochrome c release from mitochondria: inhibition by PKB/Akt but not Raf signalling", *Oncogene* **19**, 4461–4468.
[19] Kim, D.S., Kim, S.Y., Moon, S.J., Chung, J.H., Kim, K.H., Cho, K.H. and Park, K.C. (2001) "Ceramide inhibits cell proliferation through Akt/PKB inactivation and decreases melanin synthesis in Mel-Ab cells", *Pigm. Cell Res.* **14**, 110–115.
[20] Zhang, L., Himi, T., Morita, I. and Murota, S. (2001) "Inhibition of phosphatidylinositol-3 kinase/Akt

or mitogen-activated protein kinase signaling sensitizes endothelial cells to TNF-alpha cytotoxicity", *Cell Death Differ.* **8**, 528–536.
[21] Zinda, M.J., Vlahos, C.J. and Lai, M.T. (2001) "Ceramide induces the dephosphorylation and inhibition of constitutively activated Akt in PTEN negative U87 mg cells", *Biochem. Biophys. Res. Commun.* **280**, 1107–1115.
[22] Bourbon, N.A., Sandirasegarane, L. and Kester, M. (2002) "Ceramide-induced inhibition of Akt is mediated through protein kinase Czeta: implications for growth arrest", *J. Biol. Chem.* **277**, 3286–3292.
[23] Blankenship, L.J., Manning, F.C., Orenstein, J.M. and Patierno, S.R. (1994) "Apoptosis is the mode of cell death caused by carcinogenic chromium", *Toxicol. Appl. Pharmacol.* **126**, 75–83.
[24] Manning, F.C., Blankenship, L.J., Wise, J.P., Xu, J., Bridgewater, L.C. and Patierno, S.R. (1994) "Induction of internucleosomal DNA fragmentation by carcinogenic chromate: relationship to DNA damage, genotoxicity, and inhibition of macromolecular synthesis", *Environ. Health. Perspect* **102**(Suppl. 3), 159–167.
[25] Shimada, H., Shiao, Y.H., Shibata, M. and Waalkes, M.P. (1998) "Cadmium suppresses apoptosis induced by chromium", *J. Toxicol. Environ. Health* **54**, 159–168.
[26] Yuan, C., Kadiiska, M., Achanzar, W.E., Mason, R.P. and Waalkes, M.P. (2000) "Possible role of caspase-3 inhibition in cadmium-induced blockage of apoptosis", *Toxicol. Appl. Pharmacol.* **164**, 321–329.
[27] Yabuki, M., Inai, Y., Yoshioka, T., Hamazaki, K., Yasuda, T., Inoue, M. and Utsumi, K. (1997) "Oxygen-dependent fragmentation of cellular DNA by nitric oxide", *Free Rad. Res.* **26**, 245–255.
[28] Arita, K., Utsumi, T., Kato, A., Kanno, T., Kobuchi, H., Inoue, M., Akiyama, J. and Utsumi, K. (2000) "Mechanism of Dibucaine-Induced Apoptosis in Promyelocytic Leukemia Cells (HL-60)", *Biochem. Pharmacol.* **60**, 905–915.
[29] Yabuki, M., Kariya, S., Inai, Y., Hamazaki, K., Yoshioka, T., Yasuda, T., Horton, A.A. and Utsumi, K. (1997) "Molecular mechanisms of apoptosis in HL-60 cells induced by a nitric oxide-releasing compound", *Free Rad. Res.* **27**, 325–335.
[30] Kariya, S., Ogawa, Y., Yoshida, S., Yabuki, M., Imajo, Y. and Utsumi, K. (1999) "X-irradiation enhances the expression of Bcl-2 in HL-60 cells: The resulting effects on apoptosis and radiosensitivity", *Int. J. Mol. Med.* **3**, 145–152.
[31] Bradford, M.M. (1976) "A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding", *Anal. Biochem.* **72**, 248–254.
[32] Salvioli, S., Ardizzoni, A., Franceschi, C. and Cossarizza, A. (1997) "JC-1, but not DiOC6(3) or rhodamine 123, is a reliable fluorescent probe to assess delta psi changes in intact cells: implications for studies on mitochondrial functionality during apoptosis", *FEBS Lett.* **411**, 77–82.
[33] Wiegmann, K., Schutze, S., Machleidt, T., Witte, D. and Kronke, M. (1994) "Functional dichotomy of neutral and acidic sphingomyelinases in tumor necrosis factor signaling", *Cell* **78**, 1005–1015.
[34] Okazaki, T., Bielawska, A., Bell, R.M. and Hannun, Y.A. (1990) "Role of ceramide as a lipid mediator of 1 alpha,25-dihydroxyvitamin D3-induced HL-60 cell differentiation", *J. Biol. Chem.* **265**, 15823–15831.
[35] Bligh, E.G. and Dyer, W.J. (1959) "A rapid method of total lipid extraction and purification", *Can. J. Biochem. Physiol.* **37**, 911–917.
[36] Zoratti, M. and Szabo, I. (1995) "The mitochondrial permeability transition", *Biochim. Biophys. Acta* **1241**, 139–176.
[37] Malisan, F. and Testi, R. (1999) "Lipid signaling in CD95-mediated apoptosis", *FEBS Lett.* **452**, 100–103.
[38] Colell, A., Morales, A., Fernandez-Checa, J.C. and Garcia-Ruiz, C. (2002) "Ceramide generated by acidic sphingomyelinase contributes to tumor necrosis factor-alpha-mediated apoptosis in human colon HT-29 cells through glyco-sphingolipids formation.Possible role of ganglioside GD3", *FEBS Lett.* **526**, 135–141.
[39] Sable, C.L., Filippa, N., Hemmings, B. and Obberghen, E.V. (1997) "cAMP stimulate protein kinase B in a Wortmannin-insensitive manner", *FEBS Lett.* **409**, 253–257.
[40] Li, J., Yang, S. and Billiar, T.R. (2000) "Cyclic nucleotides suppress tumor necrosis factor-αmediated apoptosis by inhibiting caspase activation and cytochrome c release in primary hepatocytes via a mechanism independent of Akt activation", *J. Biol. Chem.* **275**, 13026–13034.
[41] Inoki, Y., Miura, T., Kajimoto, T., Kawase, M., Kawase, Y., Yoshida, Y., Tsuji, S., Kinouchi, T., Endo, H., Kagawa, Y. and Hamamoto, T. (2000) "Ganglioside GD3 and its mimetics induce cytochrome c release from mitochondria", *Biochem. Biophys. Res. Commun.* **276**, 1210–1216.
[42] Schubert, K.M., Scheid, M.P. and Duronio, V. (2000) "Ceramide inhibits protein kinase B/Akt by promoting dephosphorylation of serine 473", *J. Biol. Chem.* **275**, 13330–13335.
[43] Rokhlin, O.W., Guseva, N.V., Tagiyev, A.F., Glover, R.A. and Cohen, M.B. (2002) "Caspase-8 activation is necessary but not sufficient for tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-mediated apoptosis in the prostatic carcinoma cell line LNCaP", *Prostate* **52**, 1–11.
[44] Singh, R., Pervin, S. and Chaudhuri, G. (2002) "Caspase-8-mediated Bid cleavage and release of mitochondrial cytochrome c during Nomega-hydroxy-L-arginine-induced apoptosis in MDA-MB-468 cells.Antagonistic effects of L-ornithine", *J. Biol. Chem.* **277**, 37630–37636.
[45] Balamurugan, K., Rajaram, R., Ramasami, T. and Narayanan, S. (2002) "Chromium (III)-induced apoptosis of lymphocytes: death decision by ROS and Src-family tyrosine kinases", *Free Radic. Biol. Med.* **33**, 1622–1640.
[46] Komatsu, M., Takahashi, T., Abe, T., Takahashi, I., Ida, H. and Takada, G. (2001) "Evidence for the association of ultraviolet-C and H(2)O(2)-induced apoptosis with acid sphingomyelinase activation", *Biochim. Biophys. Acta.* **1533**, 47–54.
[47] Martin, D., Salinas, M., Fujita, N., Tsuruo, T. and Cuadrado, A. (2002) "Ceramide and reactive oxygen species generated by H2O2 induce caspase-3-independent degradation of Akt/protein kinase B", *J. Biol. Chem.* **277**, 42943–42952.
[48] Garcia-Ruiz, C., Mari, M., Morales, A., Colell, A., Ardite, E. and Fernandez-Checa, J.C. (2000) "Human placenta sphingomyelinase, an exogenous acidic pH-optimum sphingomyelinase, induces oxidative stress, glutathione depletion, and apoptosis in rat hepatocytes", *Hepatology* **32**, 56–65.
[49] Corda, S., Laplace, C., Vicaut, E. and Duranteau, J. (2001) "Rapid reactive oxygen species production by mitochondria in endothelial cells exposed to tumor necrosis factor-alpha is mediated by ceramide", *Am. J. Respir. Cell Mol. Biol.* **24**, 762–768.