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Superoxide dismutase as a target of clioquinol-induced neurotoxicity

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ABSTRACT

Subacute myelo-optico-neuropathy (SMON) is a progressive neurological disorder affecting the spinal cord, peripheral nerves and optic nerves. Although it has been assumed that SMON was caused by intoxication of clioquinol, the mechanism underlying clioquinol-induced neurotoxicity is not fully understood. This study aimed to clarify the relevance of oxidative stress to clioquinol-induced neurotoxicity and the cause of the enhanced oxidative stress. Clioquinol induced cell death in human-derived neuroblastoma cell line, SH-SY5Y, in a dose-dependent manner. This process was accompanied by activation of caspase-3 and enhanced production of reactive oxygen species (ROS). We examined whether clioquinol inhibited the activity of superoxide dismutase-1 (SOD1), based on its metal chelating properties. Clioquinol inhibited activities of purified SOD1 in a dose-dependent manner. Cytosolic SOD activities were also inhibited in SH-SY5Y cells treated with clioquinol. Finally, addition of exogenous SOD1 to the culture significantly reduced enhanced ROS production and cell death induced by clioquinol in SH-SY5Y cells. These findings suggested that enhanced oxidative stress caused by inhibition of SOD1 undelay clioquinol-induced neurotoxicity and was relevant to the pathogenesis of SMON.

1. Introduction

Subacute myelo-optico-neuropathy (SMON) is a neurological disorder that develops with acute abdominal symptoms, such as abdominal pain and diarrhea, followed by subacute ascending dysesthesia, paresthesia, and muscle weakness of bilateral lower extremities [1–5]. Bilateral visual impairment may occur in severe cases. The syndrome caught attention of physicians particularly in Japan, where it was more prevalent since the middle of 1950's, compared with other areas of the world. According to the 1972 national survey, some 11,000 individuals had been affected by SMON in Japan [2]. The hypothesis that SMON might have been caused by clioquinol, which was popular as an oral drug for amoebic and bacterial dysentery at that time, rose from the discovery of the green hairy tongue in SMON patients. It was confirmed by a remarkable decrease of new SMON cases after the ban on its clinical use in 1970 in Japan [5]. The high prevalence of SMON observed in Japan was attributed to longer-term use of higher doses of clioquinol.

Clioquinol is a derivative of 8-hydroxyquinolone with high lipophilicity and an ability to chelate bivalent metals, particularly copper and zinc. SMON-like pathology was reproduced in experimental animals upon repeated oral administration of clioquinol, and induction of apoptosis in neuronal cells by clioquinol was demonstrated in in vitro cell cultures of neuronal cell lines as well as organotypic cultures of neural tissues [4,6–9]. However, most of studies on SMON are not satisfactory for the current demand of molecular pathobiology, since they were performed in the old times.

Recent efforts to develop a new therapy for Alzheimer disease (AD) have shed new light on a beneficial aspect of clioquinol in clinical use [10,11]. Clioquinol induced a rapid decrease in β-amyloid deposition in a mouse model of AD, and results of a phase II clinical trial of clioquinol in AD patients were promising. Now, clioquinol has been considered as having therapeutic potential on neurodegenerative disorders, not only AD but also Huntington disease and Parkinson's disease [12,13]. To elucidate the mechanism of clioquinol-induced neurotoxicity is becoming more essential beyond the scope of the SMON research.

In this study, we report the involvement of reactive oxygen species (ROS) in clioquinol-induced apoptosis in neuronal cells. We demonstrate that clioquinol inhibits superoxide dismutase-1 (SOD1) activity and enhances ROS production. Furthermore, exogenous SOD1 attenuates ROS production as well as cell death induced by clioquinol.

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2. Materials and methods

2.1. Materials

Clioquinol was purchased from Calbiochem (Billerica, MA). Clioquinol was dissolved in DMSO, to make 10 mmol/L stock solution. The stock solution was further diluted in DMSO before being added to culture medium, so that the concentration of DMSO in clioquinol-containing medium in this study was kept at 1%. Diethyldithiocarbamate (DDC) and SOD1 purified from bovine erythrocytes were from Wako (Osaka, Japan) and Sigma–Aldrich (St. Louis, MO), respectively.

2.2. Cell culture and cell viability assay

SH-SY5Y human neuroblastoma cells were purchased from the American Type Culture Collection (Manassas, VA). Cells were grown in Dublbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS (GIBCO, Carlsbad, CA) and maintained at 37 °C in an environment containing 5% of CO_2.

Cell viability was analyzed using CellQuanti-Blue™ Cell Viability Assay Kit (BioAssay Systems, Hayward, CA). 10,000 SH-SY5Y cells were plated in each well of a 96-well culture plate with 100 μl of culture medium containing clioquinol or DDC and cultured for 24 h. 10 μl of CellQuanti-Blue™ Reagent was added for the last four hours. Fluorescent intensity was measured for each well at 535/595 nm (excitation/emission), using an Infinite® F200 microplate reader (Tecan, Männedorf, Switzerland). Data are presented as a percentage of the values obtained from cells cultured under the same conditions in the absence of clioquinol.

2.3. Caspase-3 activity assay

Caspase-3 activities were measured using EnzChek® Caspase-3 assay kit #2 (Molecular Probes, Eugene, OR). Cell lysates were prepared from 1×10^6 SH-SY5Y cells pretreated in the presence or absence of 50 μmol/L of clioquinol for 8 h. Caspase-3 activities of the lysates were determined by fluorimetric detection at 485/535 nm (excitation/emission) of conversion of the rhodamine 110 bis-(*N*-CBZ-L-aspartyl-L-glutamyl-L-valyl-L-aspartic acid amide) (Z-DEVD-R110) to fluorescent R110 caused by enzymatic cleavage of DEVD sequence of the substrate. In order to confirm that the observed fluorescence signal was due to caspase-3 activity, the reversible aldehyde inhibitor Ac-DEVD-CHO was used.

2.4. ROS production assay

ROS production by SH-SY5Y cells treated with clioquinol was assessed in two ways: CellMeter™ Fluorimetric Intracellular Total ROS Activity Assay (AAT Bioquest®, Sunnyvale, CA) and 2′,7′-dichlorfluorescein-diacetate (DCFH-DA) assay (Molecular Probes, Eugene, OR). In the former assay, 20,000 SH-SY5Y cells plated in each well of a 96-well culture plate with 100 μl of medium were cultured with indicated concentrations of clioquinol or 100 μmol/L H_2O_2 for 8 h. 20 μl of Amplite™ ROS Green was added for the last one hour. Fluorescent intensity was measured for each well at 535/595 nm (excitation/emission). In the latter, SH-SY5Y cells pretreated with 50 μmol/L of clioquinol for 16 h in chamber slides were stained with MitoTracker® Red CMXRos (Molecular Probes, Eugene, OR), and Hoechst 33342 (Invitrogen, Carlsbad, CA). Immediately after loaded with 20 μmol/L of DCFH-DA, fluorescence was analyzed, using a confocal microscope, C2si (Nikon, Tokyo, Japan) and the image analysis system, NIS Elements (Nikon). Relative ROS signals were calculated by dividing DCF (green) signal intensity by CMXRos (Red) intensity in individual cells. Total 200 cells randomly selected were quantitatively analyzed.

2.5. SOD activity analysis

SOD activity was assayed using the xanthine oxidase/xanthine/cytochrome C method as previously described [14]. Briefly, the control reaction was started by adding 0.005 unit of xanthine oxidase to a reaction mixture containing 10.4 μmol/L cytochrome C, 50.4 μmol/L xanthine, and 99.9 μmol/L EDTA (pH 7.8) at 25 °C in a 3.5 ml cuvette. The absorbance of the cuvette was read every 30 s for up to 5 min at 550 nm, and ΔOD_{550}/min was calculated as rate of the control reaction. ΔOD_{550}/min was obtained for each reaction containing one unit of SOD1 purified from bovine erythrocyte either alone or with indicated concentrations of clioquinol, and percent inhibition of the control reaction rate, which correlated with SOD activity, was determined. Substitution of 100 μg of cell lysates prepared from SH-SY5Y cells for the purified SOD1 allowed the analysis of inhibition of SOD activity in cell lysates by clioquinol. Finally, SOD activity in 100 μg of cell lysates from SH-SY5Y cells cultivated for 20 h in the presence and absence of 50 μmol/L of clioquinol or 100 μmol/L of DDC was analyzed in the same way. Cytosolic protein concentrations were determined with Coomassie Plus (Bradford) assay (Thermo Scientific, Waltham, MA).

2.6. Statistics

Differences among groups of data were assessed using Wilcoxon signed ranks test or one-way ANOVA followed by Bonferroni/Dunn test with $P < 0.05$ as the level of statistical significance.

3. Results

3.1. Clioquinol enhances production of ROS and induces apoptosis in SH-SY5Y cells

Incubation of human neuroblastoma-derived cell line, SH-SY5Y cells, with clioquinol for 24 h reduced their cell viability (Fig. 1A, $P < 0.01$). Reduction of the cell viability was apparent at 10 μmol/L of clioquinol and increased up to 55% at 50 μmol/L in a dose-dependent manner. To determine whether the cell death was accompanied by apoptosis, caspase-3 activities in the cell lysates from SH-SY5Y cells treated with 50 μmol/L of clioquinol for 8 h were measured (Fig. 1B). Caspase-3 activity in SH-SY5Y cells was significantly up-regulated by clioquinol ($P < 0.01$), as shown by generation of higher fluorescent signals from enzymatic cleavage of DEVD sequence in the Z-DEVD-R110 substrate in clioquinol-treated cells and complete abrogation of the signals by addition of the Ac-DEVD-CHO inhibitor ($P < 0.01$).

Next, we decided to examine the relationship between apoptotic cell death and ROS production. After 8-h incubation with 50 μmol/L of clioquinol, intracellular ROS production was significantly enhanced, compared with that of DMSO-treated, control cells (Fig. 1C, $P < 0.01$). Although dose-dependency was not clear, the enhancement of ROS production was apprent at 10 μmol/L of clioquinol, which was the lowest cytotoxic concentration of clioquinol to SH-SY5Y cells. We examined the intracellular localization of ROS using a ROS probe, DCFH-DA. DCF signals were greatly enhanced throughout the cell including the nucleus, mitochondria, and cytosol, when cells were treated with clioquinol (50 μM, 16 h) (Fig. 1D, upper panels). Relative DCF signal to mitochondrial signal was significantly increased in cells with clioquinol treatment (Fig. 1D, lower panel, $P < 0.01$).

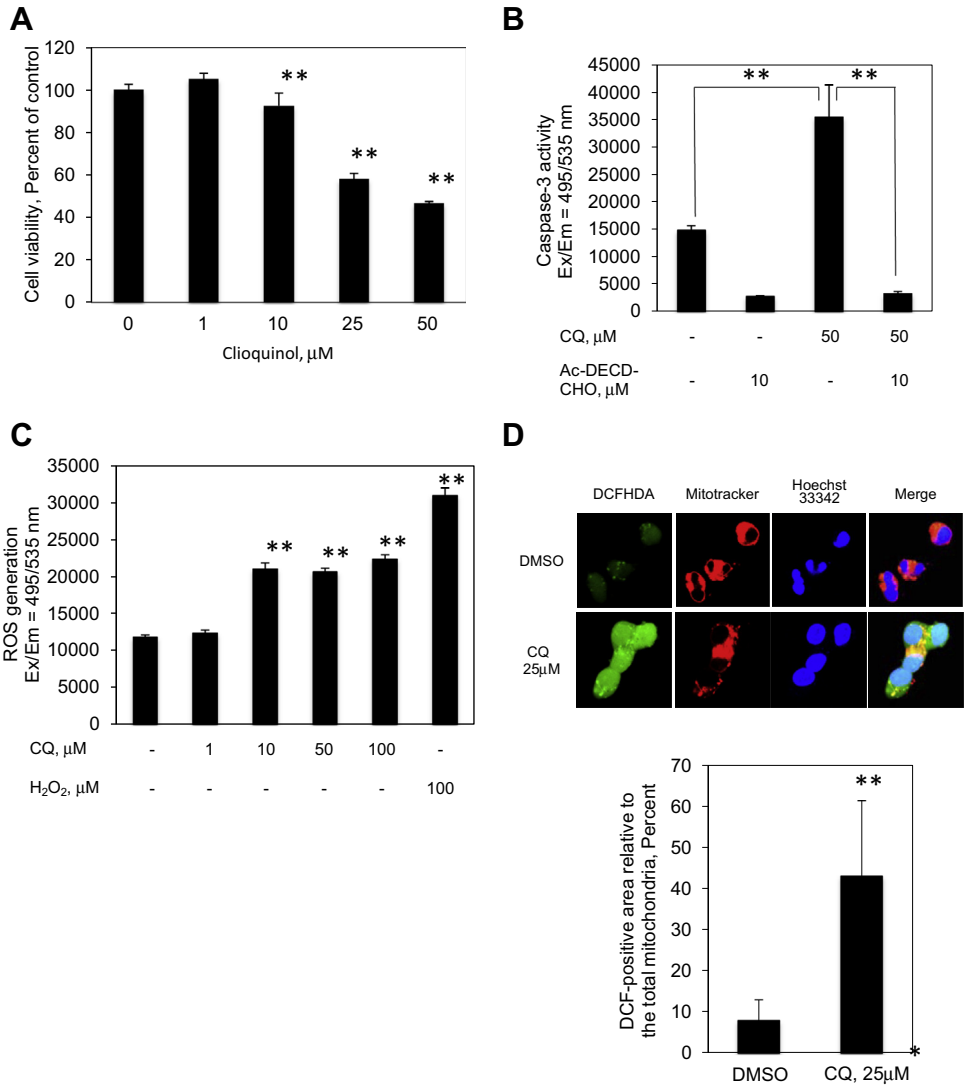

Fig. 1. Clioquinol enhances production of ROS and induces apoptosis in SH-SY5Y cells. (A) cytotoxicity of clioquinol in SH-SY5Y cells. SH-SY5Y cells were plated in a 96-well culture plate at 1×10^4 cells per well and incubated with indicated concentrations of clioquinol for 24 h. Cells were loaded with 10 μl of CellQuanti-Blue™ reagent for the last four hours, and cell viability was analyzed fluorometrically using a microplate reader (**$P < 0.01$, compared with control; one-way ANOVA followed by Bonferroni/Dunn test). (B) Activation of caspase-3 in SH-SY5Y cells treated with clioquinol. Cell lysates were prepared from 1×10^6 SH-SY5Y cells treated with 50 μmol/L of clioquinol for 8 h. Caspase-3 activity in the lysates was determined in a fluorometric assay dependent on DEVD-specific cleavage of the Z-DEVD-R110 substrate. The Ac-DECD-CHO inhibitor was used to confirm that the generated fluorescence signal was due to the activity of caspase-3 (**$P < 0.01$, compared with control; one-way ANOVA followed by Bonferroni/Dunn test). (C) And (D) enhancement of ROS production by clioquinol in SH-SY5Y cells. 2×10^4 SH-SY5Y cells were incubated with indicated concentrations of clioquinol or 100 μM H_2O_2 for 8 h. Intracellular ROS production was determined in a fluorometric analysis, using CellMeter™ Fluorimetric Intracellular Total ROS Activity Assay Kit (C) (**$P < 0.01$, compared with control; one-way ANOVA followed by Bonferroni/Dunn test). SH-SY5Y cells pretreated with 50 μmol/L of clioquinol for 16 h in chamber slides were stained with MitoTracker® Red CMXRos, Hoechst 33342 and 4′,6-diamidino-2-phenylindole (DAPI). Fluorescence microscope images were taken, immediately after the cells were loaded with 20 μmol/L of DCFH-DA (D). Relative ROS signals were calculated by dividing DCF (green) signal intensity by CMXRos (Red) intensity in individual cells for randomly selected two hundred cells. Representative data (mean ± SD) in three to six independent experiments are shown (**$P < 0.01$, compared with control; Wilcoxon signed ranks test). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.2. Clioquinol inhibits SOD1 activity

We analyzed the effect of clioquinol on SOD activity, using the xanthine oxidase/xanthine/cytochrome C method [14]. As shown in Fig. 2A, one unit of SOD1 purified from bovine erythrocytes inhibited by 40.8% the rate of reduction of oxidized cytochrome c by superoxide radicals. Clioquinol suppressed this inhibition to 28.2% at 10 μmol/L ($P < 0.01$), and the suppression by clioquinol was augmented in a dose-dependent manner. Similarly, dose-dependent suppression by clioquinol of SOD activity in cell lysates prepared from SH-SY5Y cells was apparent at 50 μmol/L (Fig. 2B, $P < 0.05$). In order to make sure that inhibition of SOD takes place in cells under treatment with clioquinol, we compared SOD activity in cell lysates from cells cultivated for 20 h in the presence and absence of 50 μmol/L of clioquinol (Fig. 2C). SOD activity in cell lysate from clioquinol-treated cells was significantly suppressed, compared with that of control, DMSO-treated cells (% inhibition of DMSO-treated and clioquinol-treated cells; 46.8% and 30.6%, $P < 0.01$), and the suppression was half as much of that of DDC, a potent copper chelator commonly used as an SOD1 inhibitor.

3.3. Exogenous SOD1 attenuates clioquinol-induced apoptosis

We then determined whether the reduced SOD activity is causal or consequent phenomenon in clioquinol-induced neurotoxicity. When exogenous SOD1 was added into culture medium, ROS

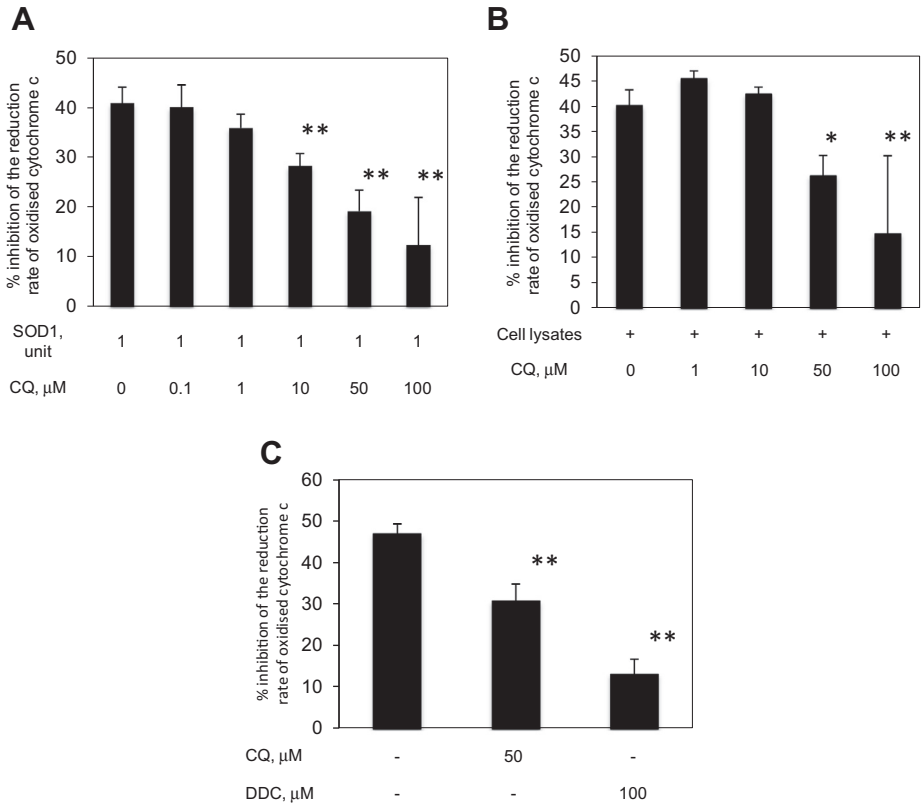

Fig. 2. Effects of clioquinol on SOD1 activity. SOD activity was measured with the xanthine oxidase/xanthine/cytochrome C method and shown as percent inhibition of the control reduction rate of oxidized cytochrome c by the superoxide radicals, as described in Section 2. Clioquinol inhibited enzymatic activity of SOD1 purified from bovine erythrocytes (A) and SOD activity in cell lysates from SH-SY5Y cell (B) in a dose-dependent manner. SOD activity was also inhibited in the cytosol of SH-SY5Y cells cultivated for twenty hours with clioquinol (50 μmol/L) or DDC (100 μmol/L) (C). Representative data (mean ± SD) in three to five independent experiments are shown ($^{*}P < 0.05$, $^{**}P < 0.01$, compared with control SOD activity, using one-way ANOVA followed by Bonferroni/Dunn test).

production induced by clioquinol was significantly reduced (Fig. 3A, $P < 0.01$). Furthermore, the SOD1-treatment significantly improved cell viability (Fig. 3B, $P < 0.01$).

4. Discussion

This study aimed to elucidate the molecular mechanism of clioquinol-induced neurotoxicity, using a human neuronal cell line. Major findings we obtained were as follows: (1) Clioquinol induced apoptotic cell death and augmentation of intracellular ROS production. (2) Clioquinol inhibited enzymatic activity of SOD1. (3) Exogenous SOD1 reduced ROS production and improved cell viability in cells treated with clioquinol. In the present study, clioquinol showed cytotoxicity against the cell at concentrations between 10 and 50 μmol/L. Although plasma concentrations of clioquinol in patients with SMON were not known, it had been

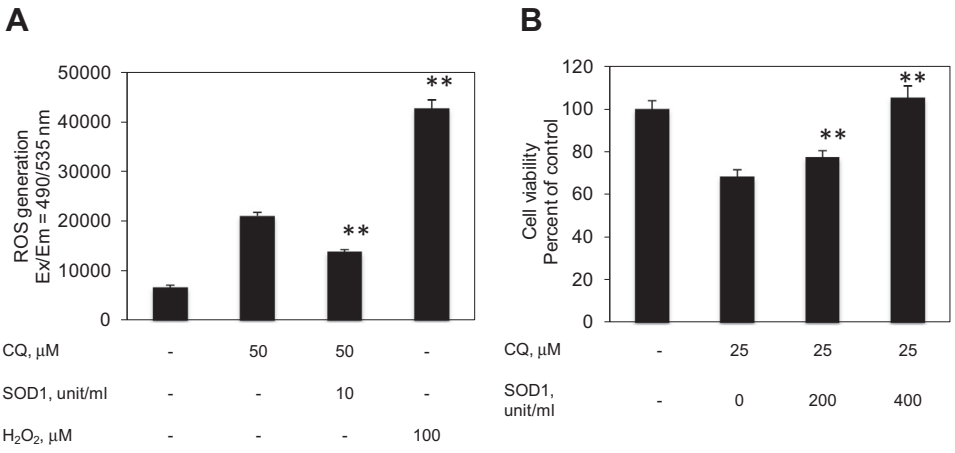

Fig. 3. Exogenous SOD1 inhibits ROS production and cell death induced by clioquinol in SH-SY5Y cells. (A) Simultaneous addition of 10 unit of bovine erythrocyte SOD1 suppressed ROS production in SH-SY5Y cells induced by 50 μmol/L of clioquinol during 8 h of incubation. (B) Exogenously added bovine erythrocyte SOD1 rescued SH-SY5Y cells from clioquinol-induced cell death in a dose-dependent manner ($^{**}P < 0.01$, compared with control treated with clioquinol alone, using one-way ANOVA followed by Bonferroni/Dunn test).

shown in a pharmacokinetic study in healthy subjects that plasma concentrations of clioquinol reached around 30 μg/ml (98 μmol/L) on oral administration of clioquinol 3 × 0.5 g/day for three days [15]. Based on this finding, the cytotoxic concentrations of clioquinol identified and used in this study were considered physiologically relevant.

A previous study, which demonstrated production of malondialdehyde and cell death in neurons in clioquinol-treated murine cortical cultures, suggested a pro-oxidant effect of clioquinol and its association with neurotoxicity [9]. Since then, several fragments of evidence for clioquinol-induced neurotoxicity have been reported, including induction of DNA double-strand breaks (DSBs) followed by activation of p53 signaling, inhibition of Trk autophosphorylation and RNA synthesis induced by nerve growth factor, formation of clioquinol zinc chelates acting as a mitochondrial toxin, and disturbance of vitamin B12 homeostasis in the brain [7,8,16–18]. Some of these mechanisms are closely associated with oxidative stress. However, there are few reports showing the direct relationship with ROS. Our study is the first to show that a pro-oxidant effect of clioquinol is mediated by enhanced ROS production via SOD1 inhibition. The finding is supported by a report demonstrating induction of neuronal apoptosis by chronic inhibition of SOD with either antisense oligodeoxynucleotides or DDC in murine spinal cord organotypic cultures [19].

It is widely believed that ROS are involved in induction of apoptosis as key players under physiologic and pathologic conditions. Excess intracellular ROS are sufficient to trigger apoptosis by initiating the redox-sensitive ASK1/JNK pathway or opening mitochondrial permeability transition pore (PTP) through oxidative modification of PTP component proteins [20,21]. Some studies have reported the existence of ROS-independent apoptosis, which was induced by chemical agents, such as gossypol, myricetin, and 7,8-dihydroxy-4-methylcoumarin [22–24]. In apoptotic cells, ROS released from mitochondria, following the original stimulus, in turn, facilitate the apoptotic process [25,26]. Therefore, ROS may cause apoptosis and result from apoptosis. There was a possibility that enhanced ROS production we found in clioquinol-treated cells was not a cause of apoptosis, just an epiphenomenon of induction of apoptosis. We ruled out this possibility by showing that both cell death and enhancement of ROS production in clioquinol-treated cells were prevented by exogenous purified SOD1.

SOD, that requires metal cofactor for their enzymatic activities, catalyzes the dismutation of superoxide into oxygen and hydrogen peroxide [27]. In human, it is classified into three classes of SOD (SOD1–3), depending on the cofactor. SOD1 and SOD3, which are located in the cytoplasm and the extracellular matrix respectively, require copper and zinc, whereas SOD2 in the mitochondria requires manganese. It plays a crucial role for cellular antioxidant defense, together with catalase and glutathione peroxidase (GPX) [28]. Oxidative stress is generated from an imbalance between ROS production and scavenging caused by impairment of the antioxidant system and is involved in almost all disease, degenerative, vascular, and inflammatory. Recent studies demonstrated that impairment of SOD activities are related to neurological disorders [29,30]. Loss of function of DJ-1 decreases SOD1 expression, making dopaminergic cell more susceptible to ROS-induced cell death, in Parkinson's disease [29]. Furthermore, excess superoxide generated from SOD1 deficiency causes spontaneous intracranial hemorrhage through activation of matrix metaloproteinase-9 [30]. These findings as well as the present results suggest that oxidative stress mediated by SOD1 inhibition underlies the pathogenesis of clioquinol-induced neuronal damage, to which attention should be paid, when clioquinol or its derivatives are used for treatment of neurodegenerative disorders, such as AD.

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