for AD [7]. Our method with which Aβ is detected in urine could also be employed in estimating the efficacy of these novel therapies. Alterations in the pathological processes of the brain would result in an increase in Aβ degradation or its clearance from this organ. The change could be demonstrated by the second rise in the level of urinary Aβ after introduction of the disease-modifying therapy.

In a future study, we intend to improve our method for quantitative analysis of Aβ in the urine and to extend the range of sampling to clarify the age- and severity-dependent tendencies both in AD patients and healthy individuals.

Acknowledgements

We are grateful to all patients, their families and to the healthy volunteers who understood the purpose of this study and gave their informed consent for urine sampling. We also thank Dr. William Campbell and Catherine Campbell for English editing of the manuscript.

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Journal of Neuroscience Research 86:3427–3434 (2008)

Involvement of Independent Mechanism Upon Poly(ADP-ribose) Polymerase (PARP) Activation in Methylmercury Cytotoxicity in Rat Cerebellar Granule Cell Culture

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Poly(ADP-ribose) polymerase (PARP) activation plays a role in repairing injured DNA, while its overactivation is involved in various diseases, including neuronal degradation. In the present study, we investigated the use of a PARP inhibitor, 3,4-dihydro-5-[4-(1-piperidinyl)-butoxy]-1(2*H*)-isoquinolinone (DPQ), whether methylmercury-induced cell death in the primary culture of cerebellar granule cells involved PARP activation. DPQ decreased the methylmercury-induced cell death in a dose-dependent manner. Unexpectedly, this protective effect was DPQ specific; none of the other PARP inhibitors—1,5-dihydroxyisoquinoline, 3-aminobenzamide, or PJ34—affected neuronal cell death. Methylmercury-induced cell death involves the decrease of glutathione (GSH) and production of reactive oxygen species. Therefore, to understand the mechanism by which DPQ inhibits cytotoxicity, we first studied the effect of DPQ on buthionine sulfoximine– or diethyl maleate–induced death of primary cultured cells and human neuroblastoma IMR-32 cells, both of which are mediated by GSH depletion. DPQ inhibited the cell death of both cultured cells, but it did not restore the decrease of cellular GSH by buthionine sulfoximine to the control level. Second, we evaluated the antioxidant activity of PARP inhibitors by methods with ABTS (2-2′-azinobis(3-ethylbenzothiazoline 6-sulfonate) or DPPH (1,1-diphenyl-2-picrylhydrazyl) used as a radical because antioxidants also efficiently suppress methylmercury-induced cell death. The antioxidant activity of DPQ was the lowest among the tested PARP inhibitors. Taken together, our results indicate that DPQ effectively protects cells against methylmercury- and GSH depletion–induced death. Furthermore, they suggest that DPQ exerts its protective effect through a mechanism other than PARP inhibition and direct antioxidation, and that PARP activation is not involved in methylmercury-induced neuronal cell death.

Key words: methylmercury; cytotoxicity; cerebellar granule cells; PARP inhibitor; IMR-32

Methylmercury is well known as a potent neurotoxin. Methylmercury causes neuronal cell death by disrupting intracellular homeostasis, changing the intracellular Ca^{2+} concentration, inhibiting microtubule assembly, reducing intracellular glutathione (GSH) content, and increasing reactive oxygen species (ROS) production (Miura and Imura, 1987; Sarafian and Verity, 1991; Sarafian et al., 1994; Marty and Atchison, 1997; Sakaue et al., 2003). Cerebellar granule cells (CGCs) have provided a good model for the analysis of methylmercury-induced neuronal cell death because the cells are particularly vulnerable to methylmercury-induced cell death in vivo and in vitro (Nagashima et al., 1996; Sakaue et al., 2005). The results of previous studies have shown that

Contract grant sponsor: Grant-in-Aid for Young Scientists (B), The Ministry of Education, Cultures, Sports, Science and Technology, Japan; Contract grant number: 18790101 (to M.S.); Contract grant sponsor: Kitasato University Research Grant for Young Researchers (to M.S.).

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Received 2 March 2008; Revised 11 April 2008; Accepted 17 April 2008

Published online 14 July 2008 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/jnr.21780

methylmercury-induced death of rat CGCs in vitro occurs even at a low concentration of methylmercury via a caspase-independent pathway (Castoldi et al., 2000; Dare et al., 2000, 2001), and antioxidants, including vitamin E, catalase, L-cysteine, and GSH, prevent methylmercury from leading cultured neurons to cell death (Park et al., 1996; Gassó et al., 2001). GSH is the major intracellular antioxidant. Depletion of GSH, a cellular model of oxidative stress, contributes to a time-dependent accumulation of endogenous ROS and subsequent oxidative stress in neurons (Murphy et al., 1989; Kane et al., 1993). Distinct GSH depletion by inhibition of GSH synthesis with buthionine sulfoximine (BSO) or by conjugation of GSH with diethyl maleate (DEM) also causes an increase in ROS and oxidative cell death in neural cell cultures (Kane et al., 1993; Sarafian et al., 1994; Li et al., 2003).

Poly(ADP-ribose) polymerase (PARP) plays a physiological role in maintaining genomic integrity, repairing DNA strand breaks (Satoh and Lindahl, 1992) and regulating gene transcription activity (Ju et al., 2006). However, the overactivation of PARP, which has nicotinamide adenine dinucleotide (NAD^+) as a substrate, causes a rapid depletion of intracellular energy sources such as NAD^+ and adenosine triphosphate, leading to cell death. Previous reports show that neuronal degeneration in some in vivo and in vitro models is attenuated by PARP inhibitors (Takahashi et al., 1999; Aito et al., 2004; Zhang and Rosenberg, 2004; Besson et al., 2005; Tanaka et al., 2005; Haddad et al., 2006; Fossati et al., 2007). The compound 3,4-dihydro-5-[4-(1-piperidinyl)butoxy]-1(2*H*)-isoquinolinone (DPQ) belongs to the dihydroisoquinolinones that was synthesized and selected as PARP inhibitors (Suto et al., 1991, 1993), which is one of the second-generation PARP inhibitors, because DPQ has a lower 50% inhibitory concentration value than 3-aminobenzamide (3-AB), a widely used PARP inhibitor (Banasik et al., 1992). Not only DPQ and 3-AB, but also N-(6-oxo-5,6-dihydrophenanthridin-2-yl)-N,N-dimethylacetamide,hydrochloride (PJ34), the third-generation PARP inhibitor, and 1,5-dihydroxyisoquinoline are used as PARP inhibitors in some experiments. Overactivation of PARP can be induced by generation of or exposure to ROS or oxidative stress, leading to cell death. Methylmercury-induced cell death of T lymphocytes is prevented by inhibition of PARP (Guo et al., 1998). It remains unclear whether methylmercury-induced death of neurons involves PARP activation, although neurons are the main target of methylmercury.

The purpose of the present study was to elucidate whether PARP activation is involved in methylmercury-induced neuronal death. However, because we found that only DPQ among the PARP inhibitors had a protective effect against cell death, we investigated further, and we found protective effects of DPQ on other cell death models via GSH reducers like methylmercury-, BSO-, or DEM-induced cell death. Our current investigation indicates that the mechanism of methylmercury-induced cell death in neurons can exclude PARP activation, and that DPQ has a protective effect against methylmercury neurotoxicity by means of some activity other than PARP inhibition.

MATERIALS AND METHODS

Cell Cultures and Treatment

Primary cultures of CGCs were prepared from Wistar rats (Jcl:Wistar; Clea Co., Tokyo, Japan) within 24 hr after birth. Cerebella were removed from the pups and incubated in trypsin solution containing DNase for 13 min at room temperature and then were minced by mild trituration with a Pasteur pipette in DNase solution after three washes with Hanks' balanced salt solution (Gibco BRL, Grand Island, NY). CGCs were seeded in Eagle's minimal essential medium (Gibco BRL) containing 1 mg/mL bovine serum albumin, 10 μg/mL bovine insulin, 0.1 nM thyroxin, 0.1 mg/mg human transferrin, 1 μg/mL aprotinin, 30 nM Na_2SeO_3, 0.25% glucose, 100 U/mL penicillin, and 135 μg/mL streptomycin on poly-L-lysine–coated dishes and cultured for 2 days at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. All cell culture supplements were purchased from Sigma Chemical Company (St. Louis, MO). Then the cells were treated with methylmercuric chloride (Tokyo Kasei Kogyo Co. Ltd., Tokyo, Japan), BSO, or DEM for 12 to 72 hr with or without DPQ (Sigma), 1,5-dihydroxyisoquinoline (DHIQ, Sigma), Trolox (Sigma), PJ34 (Calbiochem, San Diego, CA), or 3-AB (Sigma).

The neuroblastoma cell line, IMR-32, used in this study was from the American Type Culture Collection (Rockville, MD). IMR-32 cells were cultured in Dulbecco modified Eagle medium (Nacalai Tesque Inc., Kyoto, Japan) with 10% fetal bovine serum (v/v, HyClone, Logan, UT), penicillin (100 U/mL), and streptomycin (100 μg/mL) (Gibco BRL). Cultures were maintained in a humidified incubator at 37°C in 5% CO_2 and 95% air.

The number of viable CGCs and IMR-32 cells was estimated by crystal violet staining. Cells were fixed in 1% glutaraldehyde and stained in 0.2 M 2-(N-morpholino)ethanesulfonic acid (pH 6.8, Nacalai) containing 0.1% crystal violet (Wako Pure Chemical Industry, Osaka, Japan) for 10 min. The cells were washed and dried at 60°C after being washed well with distilled water, then incubated in 10% acetic acid to extract the dye that had stained the cells. Optical absorption of the extract was assayed at 595 nm with a Bio-Rad microplate reader model 550 (Bio-Rad Laboratories, Hercules, CA). Dead cells mostly detach from the culture plate bottom, so attached cells were regarded as living cells. Because the crystal violet stains cells that remain attached to the bottom of the culture dish, the concentration of the crystal violet contained in the extract indicates the number of attached cells; thus, we can estimate the ratio of treatment group cells to controls on the basis of the optical density (at 595 nm) of the extract. All experiments were performed in accordance with the Kitasato University Guidelines for Animal Care and Experimentation.

Fluorescence Microscopy and Counting Nucleus Number

To detect morphological alteration of the nucleus as an index of cytotoxicity, we fixed cells with 4% formalin

(Nacalai) for 10 min at room temperature and then stained them with 0.2 mM Hoechst 33258 (Sigma). The morphology of nuclei was visualized in micrographs made from images taken with a confocal microscope (Zeiss, Oberkochen, Germany). At least 200 nuclei containing condensed, fragmented, and normal nuclei were counted on the three micrographs randomly chosen per culture coverslip, which was independently repeated three times. The data expressed the ratio of morphologically abnormal nuclei against the total number of nuclei.

Antioxidant Assay

We used two methods to determine the direct antioxidant activity of compounds. Test compounds in these assays were Torolox, DPQ, DHIQ, PJ34, 3-AB, vitamin E (Wako), (−)-epigallocatechin-3-O-gallate (EGCG, Wako), N-acetyl-L-cysteine (NAC, Sigma), vitamin K_1 (VK_1, Sigma) and menaquinone-4 (MK4, Sigma). Torolox, vitamin E, EGCG, and NAC were used as a positive control, and VK1 and MK4 were used as a negative control that does not have direct antioxidant activity in these assays. In the first method, we eliminated the activity of the test compound as a free radical scavenger according to the method reported by Miller et al. (1993) using radical cation formation of 2-2′-azinobis(3-ethylbenzothiazoline 6-sulfonate) ($ABTS^{+\bullet}$). The inhibition of the absorbance of the radical cation was assayed at 665 nm after incubation at 30°C for 4 min in phosphate-buffered saline (pH 7.4) containing 150 μM ABTS (Sigma), metmyoglobin, 75 μM H_2O_2, and the test compounds at the indicated final concentrations. In the second method, we detected the disappearance of the optical absorbance of stable free radical 1,1-diphenyl-2-picrylhydrazyl (DPPH, Sigma) after reaction with test compounds at 520 nm (Blois, 1958). Test compounds were incubated in reaction ethanol solution containing 30 μM DPPH as a final concentration at room temperature for 20 min. Then, for evaluation of radical scavenging activity of the test compounds, the optical absorbance of the solution was measured at 520 nm with the model 550 microplate reader.

GSH Assay

Primary cultured cells from rat cerebella and IMR-32 cells were collected and washed twice with phosphate-buffered saline. After freezing and thawing the cells twice to burst the cells in 10 mM HCl, the cell suspension was centrifuged at 15,000 rpm for 10 min. The protein concentration in each sample was determined with a Bio-Rad Protein Assay Kit. For protein precipitation, 5-sulfosalicylic acid (final concentration, 5%) was added to the supernatant, and the sample was centrifuged again. Total levels of GSH in each sample were measured by the 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB)-GSH reductase recycling method (Kondo, 1994) as follows: a reaction solution was made of the supernatant and reaction mixture, which contained 6.25 mM phosphate buffer (pH 7.5), 0.31 mM EDTA, 0.25 mM NADPH, 0.625 mM DTNB, and 0.75 U/mL GSH reductase at final concentrations, and then the optical absorbance after incubation for 20 min at room temperature was determined at 412 nm with the microplate reader. The content of GSH in the samples was quantified by comparing absorption with a standard curve constructed with known amounts of GSH and standardized by protein concentration. Data are expressed as percentages of the GSH amount compared with the control group 12 hr after treatment.

Statistical Analysis

Statistical differences in the means between the treatment groups were assessed by one-way analysis of variance followed by the post hoc Bonferroni/Dunn test. The differences were considered to be statistically significant at the $P < 0.05$ level.

RESULTS

Protective Effect of DPQ Against Methylmercury-induced Cell Death

The cell viability was investigated in rat CGC culture after treatments to evaluate the effect of DPQ on methylmercury-induced cell death. The methylmercury treatment decreased the cell viability of rat CGCs in a concentration-dependent manner. The cell viabilities at 30, 100, and 300 nM of methylmercury were 71.6% ± 9.9%, 51.4% ± 6.7%, and 36.7% ± 3.3%, respectively. The decrement of cell viability via methylmercury was significantly attenuated to 100% ± 1.0%, 88.8% ± 4.0%, and 58.0% ± 4.6% with 30 μM DPQ cotreatment (Fig. 1A). Further, the protective effect of DPQ was shown to be dose dependent (Fig. 1B). Against the cytotoxicity at 30 and 100 nM of methylmercury, DPQ cotreatment showed almost complete protection. Because DPQ is a PARP inhibitor, we further determined whether other PARP inhibitors, DHIQ, PJ34, and 3-AB, protect cultured cells from the cytotoxicity of methylmercury. Unexpectedly, these compounds did not restore the cell viability decreased through methylmercury treatment (Fig. 1C,D,E). On the other hand, Torolox, a widely used antioxidant and a water-soluble derivative of vitamin E, reversed methylmercury-induced cell death at 50 μM (Fig. 1F).

The effect of DPQ on methylmercury-induced morphological alterations of nuclei was also investigated by means of fluoromicroscopy to reevaluate the result of the cell viability assay because fragmentation and condensation of nuclei were observed in degraded cells. The fluoromicroscopy in the primary cultures of CGCs showed that the morphologically altered nuclei were significantly multiplied in methylmercury-treated cells compared with control cells, while these methylmercury-induced alterations of nuclei were diminished by cotreatment with DPQ, but not by DHIQ, another PARP inhibitor, at 48 hr after treatment (Fig. 2A). As shown in Figure 2B,C, further, there was a significant time-dependent increase in the number of altered nuclei by methylmercury incubation for 48 to 72 hr. DPQ cotreatment significantly reduced the numbers of altered nuclei to the control level in each incubation time, but DHIQ treatment tended to promote rather than inhibit the methylmercury-induced increment in numbers of

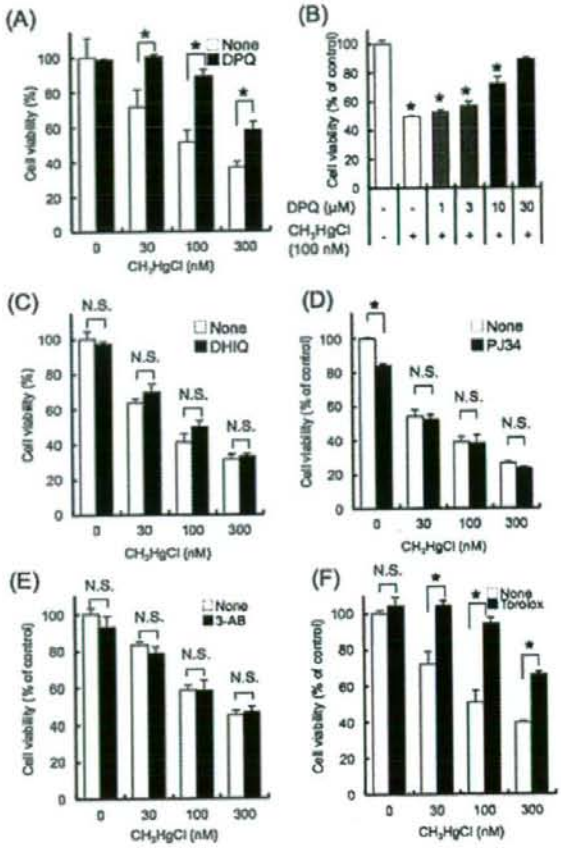

Fig. 1. Effects of PARP inhibitors on methylmercury-induced cytotoxicity in primary culture of rat CGCs. CGC viability was assayed 48 hr after treatment of methylmercury. Reagents, DPQ at 30 μM (**A**,**B**), DHIQ at 30 μM (**C**), PJ34 at 10 μM (**D**), 3-AB at 1 mM (**E**), and Torolox at 50 μM (**F**), were treated 30 min before methylmercury treatment and then were coincubated with methylmercury for 48 hr. Data are expressed as the percentage of cell viability compared with the control group, mean ± SD ($n = 3$). Asterisks indicate data that are significantly different ($P < 0.05$). n.s. in figures indicates that there is no statistically significant difference between the data.

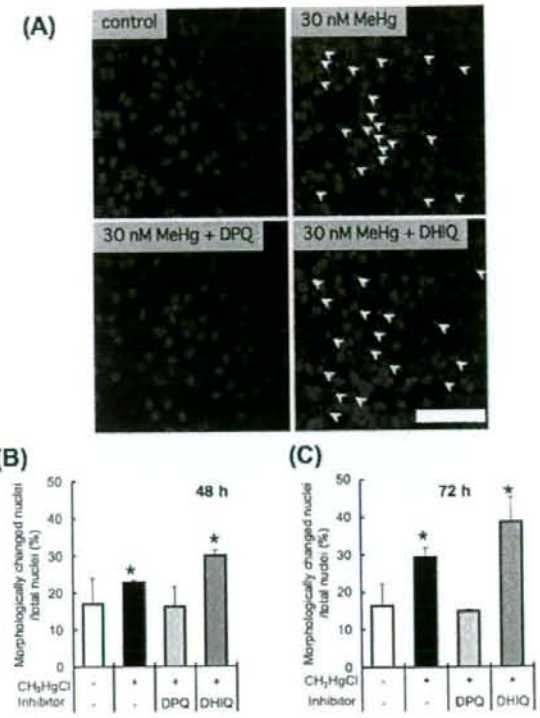

Fig. 2. Protective effects of DPQ on the methylmercury-induced morphological changes of nuclei in CGCs. Primary cultures of rat CGC were exposed at 30 nM methylmercury 30 min after PARP inhibitor treatments with DPQ and DHIQ. (**A**) Microphotographs of the cells stained by Hoechst 33258 48 hr after methylmercury treatment. Arrowheads indicate the condensed and fragmented nuclei. Scale bar = 50 μm. (B,C) Ratio of morphologically changed nuclear number to total nuclear number. These conditions of treatment were determined in preliminary experiments. Results are shown (**B**) 48 hr and (**C**) 72 hr after treatment with methylmercury. Data are expressed as percentage of abnormal nuclei compared with the total number of nuclei, mean ± SD ($n = 3$). Asterisks indicate data that are significantly different compared with control ($P < 0.01$).

morphologically abnormal nuclei. These results indicate that the protective effect of DPQ against methylmercury-induced cell death involves mechanisms other than PARP inhibition.

Effect of DPQ on GSH Depletion-induced Cell Death and Intracellular Total GSH Level

A decrease of GSH is observed in methylmercury-treated cells and accelerates the methylmercury-induced cell death of CGCs (Kaur et al., 2006). GSH, the major intracellular antioxidant, reduces ROS and methylmercury cytotoxicity in vitro. Then we investigated the effect of DPQ on a cell death model of CGCs and IMR-32 cells via treatment of GSH reducers, BSO or DEM. BSO at 300 μM for 72 hr or DEM at 200 μM for 24 hr treatment caused a decrease of cell viability in the primary culture of CGCs to 54.0% and 54.7%, respectively (Fig. 3A,B). The viabilities of IMR-32 cells treated with BSO at 300 μM for 48 hr or DEM at 50 μM for 24 hr were also decreased compared with that of control cells to 11.8% or 43.7% (Fig. 3C,D), respectively. DPQ cotreatment significantly affected the decrease of cell viability levels caused by BSO/DEM treatment, which were restored to approximately 80% and 90% in CGCs and IMR-32 cells (Fig. 3A–D), respectively. On the other hand, neither DHIQ nor 3-AB affected BSO- or DEM-induced cytotoxicity in CGCs and IMR-32 cells. Because DPQ had a protective effect against GSH reducers, we hypothesized that DPQ

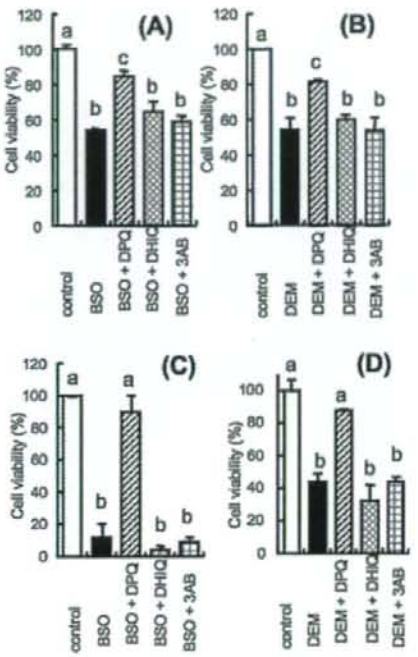

Fig. 3. Effects of PARP inhibitors on GSH depletion-induced cytotoxicity in vitro. Primary cultures of rat CGCs (**A,B**) and human neuroblastoma IMR-32 cells (**C,D**) were incubated in medium containing PARP inhibitors and GSH reducers buthionine-sulfoximine (BSO) (A,C), DEM (B,D). The treatment of BSO or DEM is (A) at 300 μM for 72 hr, (B) at 200 μM for 24 hr, (C) at 300 μM for 48 hr and (D) at 50 μM for 24 hr. These conditions of treatment were determined in preliminary experiments. The cells were pretreated with PARP inhibitors, DPQ (30 μM), DHIQ (30 μM) and 3-AB (1 mM), 1 hr before BSO or DEM treatment. The concentrations of PARP inhibitors were the highest concentration at which the PARP inhibitors had no affect effect on cell viability. Data are expressed as the percentage of cell viability compared with the control group, mean ± SD ($n = 3$). Bars with different lowercase letters are significantly different ($P < 0.05$) from one another.

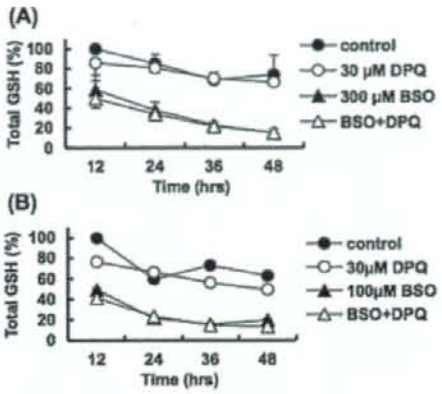

Fig. 4. Cellular total GSH levels in the BSO- and DPQ-treated cells, (**A**) rat CGCs, and (**B**) human neuroblastoma IMR-32 cells. The cells were treated with DPQ or BSO for the times indicated. Data are indicated as percentage compared with control group at 12 hr, mean ± SD ($n = 3$).

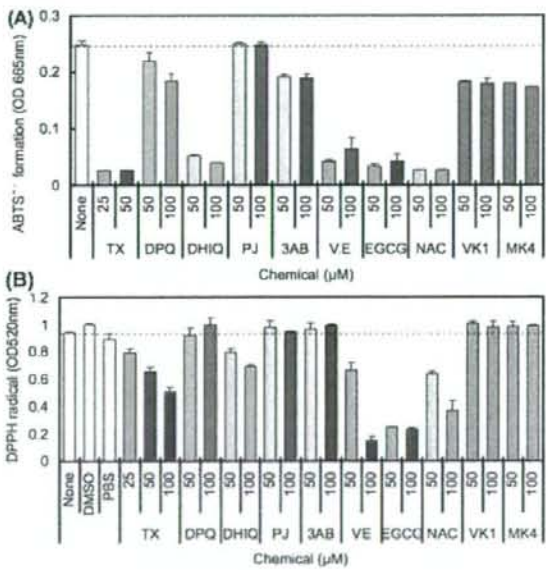

Fig. 5. Radical scavenging activities of PARP inhibitors. (**A**) Antioxidant activity assay by using ABTS cation ($ABTS^{+\bullet}$) formation with H_2O_2 as the oxidant trigger. $ABTS^{+\bullet}$ was measured by optical absorbance at 665 nm. (**B**) Free radicals, which have absorption at 520 nm, are generated by DPPH in solution. The scavenging of the radicals was assayed spectrophotometrically after the addition of DPQ, other PARP inhibitors or antioxidants at 520 nm. Data are expressed as mean ± SD ($n = 3$).

has the ability to increase cellular GSH level, then tested the hypothesis by measuring total GSH in BSO-treated cells and controls, and those cotreated with DPQ. BSO treatment significantly decreased the GSH level compared with the control level at each incubation time in CGCs and IMR-32 cells (Fig. 4). Unexpectedly, DPQ did not significantly bring the BSO-induced decrement of GSH level back to the control level in CGCs and IMR-32, although DPQ did show a protective effect against cell death caused by GSH depletion, as shown in Figure 3A,C.

Direct Antioxidant Activity of DPQ Against Radicals

Involvement of ROS in the toxicity of methylmercury has been established (Ganther, 1978; Sarafian and Verity, 1991). Moreover, antioxidants inhibit the cytotoxicity of methylmercury in primary neuronal cultures (Park et al., 1996; Gassó et al., 2001). If DPQ directly scavenges ROS, that would account for the protective

effect of DPQ on the methylmercury cytotoxicity in the primary culture.

To determine the potential of DPQ as a direct scavenger against radicals, we used two antioxidant methods, the $ABTS^{+\bullet}$ formation method and the DPPH radical method. These methods use $ABTS^{+\bullet}$ or DPPH as radicals, respectively. Widely used antioxidants, e.g., vitamin E, NAC, EGCG, and Trolox, significantly diminished the radicals in both of these methods. Among PARP inhibitors such as DPQ, DHIQ, PJ34, and 3-AB, DHIQ clearly had the strongest scavenging potential against the radicals. Its potential was approximately five times (Fig. 5, top) and 1.3 times (Fig. 5, bottom) greater than that of DPQ in the $ABTS^{+\bullet}$ formation method and the DPPH radical method, respectively. The other compounds, PJ34, 3-AB, VK_1, and MK4, had almost the same radical scavenging activity as DPQ in both methods. In those assay, vitamin K was used as a negative control that has no ability to scavenge these radicals (Li et al., 2003).

DISCUSSION

Methylmercury at lower concentrations, ≤ 1 μM, induces cell death (apoptosis) in rat CGC culture, independent of the caspase cascade (Castoldi et al., 2000; Dare et al., 2000, 2001). PARP is activated via DNA damage and catalyzes the covalent attachment of ADP-ribose units from NAD^+ to chromatin-interacting proteins, such as histones, p53, DNA topoisomerase, and PARP-1 itself, which regulates nuclear homeostasis and assists in DNA repair (D'Amours et al., 1999). Additional stronger genotoxic stress than that in physiological state, however, causes the overactivation of PARP, resulting in hyper poly(ADP-ribosyl)ation- and PARP-dependent cell death. Thus, we hypothesized that the methylmercury-induced cell death of rat primary cultured CGCs at low concentrations is caused by PARP activation because a previous study showed that PARP is activated in when human lymphocytes die as a result of methylmercury treatment (Guo et al., 1998). In the present study, the involvement of PARP activation in cell death induced by methylmercury treatment was investigated by means of PARP inhibitors in primary cultured CGCs. If the cell death mechanism involved PARP activation, not only would DPQ treatment prevent cell death, but treatments by other PARP inhibitors (DHIQ, PJ34, and 3-AB) would as well. We found that among the PARP inhibitors, only DPQ inhibited cell death (Figs. 1 and 2). Additionally, Western blot test detected no poly(ADP-ribose) modification of PARP itself or of any other proteins in extracts of methylmercury-treated cells in the primary culture (data not shown). These results indicate that the methylmercury-induced cell death of CGCs does not involve PARP activation.

GSH is the most predominant intracellular thiol and acts as a major cellular antioxidant (Meister and Andersson, 1983). GSH modulators affect the methylmercury-treated cell viability in primary cultured neurons; NAC, a GSH precursor, increases the viability, and DEM, a reducer of GSH level, decreases it. In other words, the total amount of GSH is decreased in methylmercury-treated neural cells, primary cultured neurons, PC12 cells and GT1-7 cells (Sarafian et al., 1994; Kaur et al., 2006), and NAC treatment supplies the decrement of ROS to rescue primary cultured neurons from methylmercury-induced cell death (Park et al., 1996; Kaur et al., 2006). Therefore, in this study, we investigated the effect of DPQ on cell death of CGCs and IMR-32 cells through treatments of BSO and DEM. DPQ showed a protective effect against the cytotoxicity via treatment of GSH reducers in CGCs and IMR-32 cells. Furthermore, in DPQ's protection of CGCs, the BSO-induced reduction of cellular GSH was not restored (Figs. 3 and 4). These results exclude the influence of intracellular GSH levels from the protective effect of DPQ on GSH-depletion-induced cell death.

The involvement of oxidative stress in the process of methylmercury cytotoxicity has been hypothesized (Ganther, 1978; Sarafian and Verity, 1991). We found that Trolox, a widely used antioxidant, prevented methylmercury-induced cell death in CGC culture in this study (Fig. 1F), and results of previous reports showed that other antioxidants inhibited cell death in methylmercury-treated neuron cultures (Park et al., 1996; Gassó et al., 2001). To elucidate whether DPQ itself has the ability to scavenge radicals, we detected the antioxidant activity of DPQ by using $ABTS^{+\bullet}$ formation and DPPH radical methods. The potential of DPQ as a direct radical scavenger was considerably less than that of the other PARP inhibitors, while DHIQ had the strongest potential to scavenge radicals among the tested PARP inhibitors (Fig. 5). These results are congruent with those reported by Czapski et al. (2004) that DPQ has no effect on free radical reactions, i.e., protein oxidation or lipid peroxidation, while DHIQ and 3-AB possess antioxidative properties. In light of accruing evidence, a plausible explanation is that antioxidant activity of DPQ is not directly involved in its protective effect against methylmercury-induced cell death. Therefore, the data from the present study indicate that DPQ should be an efficient substance to protect cells from methylmercury and GSH-depletion neurotoxicity, and the results further suggest that DPQ's activity as a PARP inhibitor is not involved in the protection mechanisms of DPQ treatment. DPQ may reduce neuronal degradation via some activity other than inhibiting PARP and scavenging ROS. Thus, activities of DPQ other than PARP inhibition should be considered when evaluating the protective effect of DPQ, although there are some reports that DPQ as a PARP inhibitor prevents PARP activation and rescues the cells from neuronal degradation conditions (Eliasson et al., 1997; Takahashi et al., 1999; Meli et al., 2003; Ying et al., 2005).

It was noteworthy that coincubation with DPQ for more than 24 hr before cells normally start to die was needed for DPQ to exert a protective effect against the

methylmercury-induced cell death (data not shown). DPQ treatment may bring about some gene expression of antioxidative or neuroprotective genes that contribute to the protection against cell death through methylmercury, BSO or DEM treatment—e.g., bcl-2, manganese–superoxide dismutase, and metallothionein from increases of the relevant gene expressions prevent methylmercury-induced cell death (Kane et al., 1993; Aschner, 1997; Naganuma et al., 1998). Clarifying the target factor and the mechanism of the protective effect of DPQ against methylmercury-induced cell death will provide novel information about the mechanism of methylmercury cytotoxicity.

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Research Report

Acceleration of Methylmercury-induced Cell Death of Rat Cerebellar Neurons by Brain-Derived Neurotrophic Factor In Vitro

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The number of text pages: 21 pages
The number of figures: 3 figures

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Abstract

Brain-derived neurotrophic factor (BDNF) is a member of the nerve growth factor (NGF) family and has been shown to promote neuronal survival and contribute to neural development. Although methylmercury, a neurotoxin, induces the cell death of neurons in vitro, there is little information regarding the effects of neurotrophins on the methylmercury-induced cell death of neurons. In the present study, we investigated the effect of BDNF on methylmercury-induced cell death in a primary culture of rat cerebellar granular cells. BDNF increased the viability of the cultured cells when treated alone, but unexpectedly accelerated the cell death induced by administration of methylmercury. Among other growth factors tested, only neurotrophin-4 (NT-4) demonstrated a similar acceleration of methylmercury-induced cell death. The cell death-accelerating effect of BDNF was inhibited by a BDNF-neutralizing antibody or a MAPK inhibitor. To determine whether the effect of BDNF occurs via TrkB, a receptor of BDNF and NT-4, we investigated the effects of BDNF and methylmercury in a TrkB transformant of rat neuroblastoma B35 cells. The methylmercury-induced cell death of the TrkB transformant was accelerated by BDNF, while that of the mock transformant was not. These results indicate that BDNF accelerates methylmercury-induced cell death via TrkB, at least in vitro, and suggest that BDNF and TrkB may also contribute to the sensitivity of neurons to methylmercury toxicity.

Section: Disease-related Neuroscience

Key Words: TrkB; BDNF; NT-4; B35 cell line; Methylmercury; Primary culture

1. Introduction

The neurotrophin family, which includes nerve growth factor (NGF), brain-

derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4/5 (NT-4/5), regulates the survival and functions of central and peripheral neurons (Snider, 1994). BDNF has been well investigated with regard to these functions and regulations in the central nervous system, and has been shown to confer protection against various types of neuronal cell death, including serum-deprived, excitotoxic, and oxidative stress-mediated death. Neural cells secrete BDNF to serve as a paracrine or endocrine factor for the survival of neurons and for maintenance of neural normal function. The effects of BDNF are mediated by its binding to tropomyosin-related kinase (Trk) B, a neurotrophin receptor, with high affinity, after which TrkB processes signal transduction through primarily three signaling pathways—the mitogen-activated protein kinase (MAPK), the phospholipase C (PLC) γ, and the phosphoinositide 3-kinase (PI3K) pathway. TrkB binds not only BDNF, but also NT-4/5 with high affinity and NT-3 with lesser affinity. In contrast, TrkA binds NGF and TrkC binds NT-3 specifically, after which both these Trks process signal transduction in the same manner as TrkB (for review, see Bibel and Barde, 2000; Reichardt, 2006) in order to realize the neurotrophic effects. Phenotypic analysis in BDNF- or TrkB-null mice has revealed that BDNF and TrkB are essential for normal brain development (Snider, 1994). Furthermore, even in adults, BDNF plays an important role as a modulator in synaptic development and plasticity in learning and memory.

Methylmercury is well known as a neurotoxicant. Methylmercury toxicity involves disruption of intracellular homeostasis with, for example, increases in intracellular calcium (Ca^{2+}) concentrations, reactive oxygen species production and extracellular concentration of glutamate via inhibition of glutamate uptake of astrocytes (Sarafian and Verity, 1991; Aschner et al., 1993; Marty and Atchison, 1997; Sakaue et

al., 2005). Cerebellar granule cells, which are particularly vulnerable to methylmercury-induced damage in vivo, have provided a good model for analysis of methylmercury-induced neuronal cell death in vitro (Dare et al., 2000; Castoldi et al., 2000). We have previously investigated the mechanisms of methylmercury toxicity and screened growth factors and chemicals in order to identify those that confer protection against methylmercury (Sakaue et al., 2005; 2006; 2008). In the present study, we continued this investigation by examining whether BDNF, which has been shown to protect against some types of cell death, would also protect against methylmercury-induced cell death in a primary culture of rat cerebellar granular cells. Our results unexpectedly demonstrated that BDNF accelerates methylmercury-induced cell death. To investigate the possible mechanism of this effect, we used a stable transformant of TrkB in rat neuroblastoma B35 cells, and showed that TrkB was involved in the acceleration of methylmercury-induced cell death by BDNF.

2. Results

2.1. BDNF increases methylmercury-induced cell death of rat cerebellar granule cells

To examine the effects of BDNF on the neurotoxicity of methylmercury, we measured the viability of rat cerebellar granule cells after treatment of BDNF, methylmercury, or both, expecting that BDNF would exhibit a neurotrophic action. Treatment with methylmercury alone significantly decreased the viability of the cells to approximately 70% of that of non-treated cells ($p = 0.002$; Fig. 1B and E). Treatment with BDNF alone significantly increased the viability of the cells to approximately 115% of that of non-treated cells ($p < 0.001$). However, cotreatment with BDNF and methylmercury further decreased the viability of the cells to 50.4%, which was

significantly lower than the viability of the methylmercury-treated cells ($p = 0.009$). Both the increase and decrease of the cell viability occurred in a BDNF concentration-dependent manner (Fig. 1D and E). In addition, cotreatment with BDNF and methylmercury had different effects on the cell viability depending on whether the incubation time was 24 h or 48 h. At 24 h of incubation, the viability of cells co-treated with BDNF and methylmercury was 103%, significantly greater than the 96% viability of cells treated with methylmercury alone ($p = 0.009$). Conversely, at 48 h of incubation, the viability of co-treated cells was 47.8%, significantly smaller than the 69% viability of methylmercury-treated cells ($p < 0.001$). Thus the accelerating effect of BDNF on the methylmercury-induced cell death appeared only after incubation for 24 h, increased between 24 h and 48 h of incubation, and was slightly but not significantly apparent after incubation for 36 h ($p = 0.065$; methylmercury-treated versus co-treated cells) (Fig. 1F). Therefore these results indicate that BDNF functions as an accelerator of cell death after methylmercury cytotoxicity begins to appear in primary cultured cells, but not before.

NT-4 is another ligand of TrkB (Bibel and Barde, 2000; Reichardt, 2006). Accordingly, we next examined whether NT-4 also accelerates methylmercury-induced cell death. When administered singly, NT-4 at 100 ng/ml significantly increased cell viability to 115% compared with control cells ($p = 0.01$). However, the viability of cells co-treated with methylmercury and NT-4 was significantly decreased compared to the viability of treated with methylmercury alone (Fig. 1G; $p = 0.004$). This effect of NT-4 on methylmercury-induced cell death occurred in a concentration-dependent manner. The percentages of viable NT-4-treated, methylmercury-treated, and NT-4/methylmercury co-treated cells were 115%, 69%, and 55%, respectively. This result

indicates that NT-4 accelerated methylmercury-induced cell death in a manner similar to BDNF, and suggests that TrkB is involved in the mechanism of the BDNF effect. Finally, because NGFβ also has the ability to bind to TrkA, we examined whether NGFβ had any effect on methylmercury-induced cell death. However, treatment of cerebellar neurons with NGFβ and methylmercury did not significantly alter the viability of cells compared with treatment by methylmercury alone ($p = 0.441$), and in fact, treatment with NGFβ alone did not induce a significant change in viability compared with the non-treated control cells (Fig. 1H; $p = 0.753$).

2.2. The acceleration of the cell death by BDNF involves its binding to TrkB and MAP signal transduction

BDNF and NT-4 bind to TrkB and activate it, which initiates signal transductions. To clarify whether BDNF binding to TrkB and activation of the signal transduction of MAP are involved in the effect of BDNF, we elucidated the effects of a BDNF-neutralizing antibody and MAPK inhibitor on the acceleration of methylmercury-induced cell death by BDNF. As shown in Fig. 2A, the viability of the cells cotreated with methylmercury and BDND was 46%, and that of the cells cotreated with plus BDNF-neutralizing antibody was 64%, which indicates that the treatment with the BDNF-neutralizing antibody significantly suppressed the BDNF-induced acceleration of cell death. This suppression was in a concentration-dependent manner of the antibody ($p < 0.001$, methylmercury/BDNF co-treated versus plus the neutralizing antibody co-treated cells).

A MAPK inhibitor, U0126, suppressed the cell death induced by co-treatment with methylmercury and BDNF or by treatment with only methylmercury (Fig. 2B). These results indicate that the MAPK inhibitor inhibited not only the accelerating effect

of BDNF on methylmercury-induced cell death, but also the cell death induced by methylmercury alone. The cell viability of the cells co-treated with methylmercury, BDNF, and the MAPK inhibitor tended to be lower than the cell viability of BDNF-treated cells, which suggests that U0126 prevented BDNF from acting as an accelerator and a neurotrophic factor by inhibiting the MAPK pathway. Furthermore, to examine the mRNA expression of the neurotrophin receptors, TrkA, TrkB, and TrkC, total RNAs from the primary cultured cerebellar cells and from the cerebrum as a positive control were subjected to RT-PCR. mRNA expression of all Trks was detected in the cerebrum. However, only TrkB mRNA was intensively expressed among receptors in the primarily cultured cerebellar cells, while mRNA expression of TrkA and TrkC was not observed (Fig. 2C).

To examine whether TrkB is involved in the effect of BDNF on methylmercury cytotoxicity, we performed experiments using a stable transformant for TrkB (Fig. 3). BDNF significantly increased the viability of the TrkB transformant cells, to 135%, but not that of the mock-transformant cells. These effects indicate that the over-expressed TrkB protein can function normally in TrkB-transformant cells, and that BDNF has a neurotrophic effect on the TrkB-transformant cells through TrkB. In the group treated with 150 nM methylmercury, the viability was similar between the both transformants. In contrast, in the cells treated with 600 nM methylmercury, the cell viabilities of both transformants significantly decreased to approximately 80%. Only the viability of the TrkB-transformant cells treated with BDNF fell further, to 38% (Fig. 3I; $p < 0.001$; 600 nM methylmercury-treated versus plus BDNF-treated cells of TrkB-transformant). This effect in the TrkB-transformant cells was dependent on the dose of BDNF (Fig. 3J). Western blot analysis showed that two signal-transduction factors in a TrkB down-

stream, extracellular signal-regulated kinase (ERK) and MAPK/ERK kinase (MEK), were phosphorylated by BDNF treatment in the TrkB transformant, but not in the mock transformant cells (Fig. 3K), which demonstrates that the TrkB transformant has a normally functioning TrkB protein, while the mock transformant does not. These results indicate that TrkB is necessary for the acceleration of methylmercury-induced cell death or for the increase of cell viability through BDNF treatment.

3. Discussion

This is the first study documenting that BDNF aggravates the neuronal death induced by an environmental toxin, methylmercury, although BDNF is included in the NGF family. We demonstrated that BDNF and NT-4 accelerate the methylmercury-induced cell death of rat cerebellar granule cells in primary cultures in a dose-dependent manner, but that NGFβ does not, which is supported by the evidence that primary cultured cells expressed a high level of TrkB but not TrkA mRNA, because the neurotrophins need to bind to specific receptors in order to achieve their cell death-accelerating effects (Bibel and Barde, 2000; Reichardt, 2006). The experiment using a neutralizing antibody against BDNF also proved the necessity of BDNF binding to TrkB for the effect of BDNF as an accelerator of methylmercury-induced cell death. Furthermore, the methylmercury-induced death in the TrkB transformant of rat neuroblastoma cell line B35 was accelerated by BDNF treatment, but not that in the mock-transformant. These results indicate that TrkB activation by BDNF is responsible for the accelerating effect on methylmercury-induced cell death. Among Trks, the cells of the primary culture expressed only TrkB in the present study. Therefore, as TrkB is a receptor of BDNF, only BDNF should have been an accelerating factor against methylmercury-induced cell death among neurotrophins. Had TrkA or TrkC been

expressed in the primary culture, NGF or NT-3 might have induced an acceleration of methylmercury-induced cell death, because TrkA and TrkC can process signal transduction through at least three signaling pathways, MAPK, PLC γ, and PI3K, that TrkB also processes (Bibel and Barde, 2000; Reichardt, 2006). However, the results of the present study did not sufficiently clarify whether TrkA and TrkC are involved in the accelerating effect on methylmercury-induced cell death in the manner of TrkB. To resolve these questions, more experiments will be needed to observe the effects of NGFβ on methylmercury-induced death in a TrkA-expressing cells, such as rat adrenal pheochromocytoma PC-12 cells.

When the cytotoxicity of methylmercury did not appear, BDNF functioned as a neurotrophin for both the primary cultured neurons of the cerebellum and the TrkB transformant of B35 cells cotreated with methylmercury and BDNF. However, when the cell viability was decreased by methylmercury toxicity, BDNF functioned as an accelerator of the methylmercury-induce cell death (Fig. 1 and 3). These results indicate that BDNF exhibits not only trophic activity, but also death acceleration activity, and thus that the role of BDNF is altered by the appearance of methylmercury cytotoxicity.

There may be cross-talk mechanisms between the BDNF and methylmercury pathways. In primary cultured neurons from mice cerebella, methylmercury shows its neurotoxicity by increases in intracellular Ca^{2+} concentrations through NMDA receptor activation, and the toxicity is suppressed with NMDA receptor antagonists (Park et al., 1996). The disappearance of methylmercury neurotoxicity by treatment with NMDA receptor antagonists has also been observed in an in vivo study (Miyamoto et al., 2001). Using the same culture system used in the present study, we previously showed that methylmercury induces an increase in intracellular Ca^{2+} concentrations, which in turn

activates calpain, a Ca^{2+} concentration-dependent protease, to play its role in methylmercury-induced cell death (Sakaue et al., 2005). BDNF phosphorylates NMDA receptors via TrkB, resulting in a slight increase in intracellular Ca^{2+} concentrations, but the levels of intracellular Ca^{2+} concentrations should still be low compared to the levels of intracellular Ca^{2+} concentrations increased by methylmercury. This BDNF effect on the intracellular Ca^{2+} concentrations is suppressed by treatment with a kinase inhibitor against Trks, K-252a, or an NMDA receptor antagonist, MK-801 (Xu et al., 2006; Sanchez-Perez et al., 2006). As shown above, U0126 also inhibited the effects of methylmercury and BDNF (Fig. 2B). Moreover, we determined that a subtype of the NMDA receptor, NR1, is phosphorylated by BDNF or NT-4 treatment, but not by NT-3 in our primary culture system (data not shown). Thus, BDNF might realize its acceleration of methylmercury-induced cell death by stimulating a methylmercury-induced increase of intracellular Ca^{2+} concentration. However, confirmation of this proposed mechanism is beyond the scope of the present study. Further studies using Ca^{2+} indicators or endogenous markers for increases in Ca^{2+} concentrations will be needed in order to detect any augmentation of intracellular Ca^{2+} concentrations by BDNF treatment.

In conclusion, the present results indicate that BDNF exacerbates methylmercury-induced cell death via TrkB, indicating that BDNF and TrkB are factors regulating the sensitivity to methylmercury cytotoxicity. The neurotoxicity of methylmercury has at least two toxicological characteristics, as follows. First, fetuses and infants show a higher sensitivity to methylmercury than adults (Takeuchi, 1968; Bakir, 1973; Harada, 1968; Marsh, 1980). BDNF is indispensable for the development of normal structures, as well as for cell survival and function, in the nerve tissue of developing animals such