Abstract

Objectives: The present study investigated the involvement of oxidative stress in the degeneration of the cerebellum during methylmercury (MeHg) intoxication and the protective effect of α-tocopherol (Vit E) against MeHg toxicity.

Methods: After 5 mg/kg of MeHg was administered to Wistar rats for 12 consecutive days, the cerebellum were examined histopathologically. In addition, the same amount of MeHg was administered to 3 different groups of Wistar rats: rats with a Vit E-deficient diet, rats fed 150 mg/kg of Vit E for 20 consecutive days after initial MeHg administration, and rats with an ordinary diet.

Results: Positive immunoreactivity against anti-hydroxynonenal (HNE), a marker of lipid peroxidation, was observed in the cerebellum after MeHg administration. Levels of thiobarbituric acid reactive substance (TBARS), another marker of lipid peroxidation, and those of protein carbonyl, a biomarker for protein oxidation, increased after MeHg administration. In the rats with MeHg and a Vit E-deficient diet, mortality and prevalence of piloerection significantly increased, and in the rats with MeHg and Vit E, mortality, piloerection, retracted and crossed hind leg, and ataxic gait significantly decreased, compared with the rats with MeHg alone. The levels of NO$_2^-$ and NO$_3^-$ in the serum significantly increased in the rats with MeHg alone 14 days after the initial MeHg administration, but were significantly suppressed by Vit E administration.

Conclusions: Oxidative stress, especially lipid peroxidation, may play an important role in the cerebellar degeneration process during MeHg intoxication and Vit E may play a protective role against MeHg toxicity as an effective antioxidant.

Key words: methylmercury, oxidative stress, vitamin E, cerebellar degeneration, lipid peroxidation

Introduction

Methylmercury (MeHg) intoxication is noted for Hunter-Russell syndrome (Minamata disease), the clinical manifestations of which are cerebellar ataxia, concentric constriction of visual fields, and sensory and auditory disturbances (1, 2). Among these disturbances, cerebellar ataxia is one of the most important clinical signs for diagnosis of this disease, and cerebellar degeneration is one of the most outstanding histopathological findings upon autopsy (3, 4). The disease occurs world wide because of industrial pollution, and many animal models of the disease have been developed (5). However, the mechanism of cerebellar degeneration during MeHg intoxication is not well known, and an effective therapy for MeHg intoxication has not been established.

Growing evidence suggests the involvement of oxidative stress in the brain and the cerebellum in neurodegenerative diseases such as Alzheimer disease and spinocerebellar degeneration (6, 7). The cerebellum is a nitric oxide synthase (NOS)-rich organ in which nitric oxide (NO) is produced. NO reacts with superoxide to form peroxynitrite and hydroxyl radicals, which are highly reactive free radical species inducing oxidative stress (8). It has been suggested that NO production in the...
cerebellum may be strongly correlated with cerebellar degeneration during MeHg intoxication (9–11). Recently, a major target molecule of MeHg toxicity was reported in yeast cells (12). However, in mammalian cells, the target molecules causing MeHg toxicity have not yet been identified. Therefore, we hypothesize that oxidative stress induced by NO production may play an important role in the mechanism of MeHg toxicity.

α-tocopherol (Vit E) has been reported to prevent oxidation of lipid and protein, scavenging free radicals on the cell membrane and protecting unsaturated fatty acids from lipid peroxidation (13–15). Vit E is transported in association with lipoproteins in the blood and taken up by the central nervous system through the blood-brain barrier (16). The aim of our investigation was to confirm the presence of oxidative stress in the cerebellum during MeHg intoxication and to assess the protective effect of Vit E as an antioxidant against MeHg toxicity.

Materials and Methods

1. Materials

Methylmercury (II) chloride (CH₃HgCl) was purchased from Katayama Chemical Industries (Osaka, Japan). Vit E and thiobarbituric acid were obtained from Sigma (St. Louis Missouri, USA). The Vit E-deficient diet was obtained from Kuroda Laboratory Animal Center (Kumamoto, Japan). Affinity-purified rabbit anti-hydroxynonenal (HNE) antibody was provided by Uchida K (17). The medium solutions and the standard for NO₂⁻/NO₃⁻ analysis were purchased from Toyokasei (Tokyo, Japan). Other chemical agents were of the highest analytical grade commercially available.

2. Animals

The male Wistar rats (250–300 g) used for all the in vivo experiments were obtained from Japan SLC (Shizuoka, Japan). All efforts were made to minimize animal suffering, to reduce the number of animals used, and to utilize alternatives to in vivo techniques. MeHgCl was dissolved in water (1.25 mg/ml) and orally administered (5 mg/kg) once a day to the rats for 12 consecutive days.

3. Assessment of oxidative stress in the cerebellum during MeHg intoxication

At various times after MeHg administration, the rats were sacrificed after 5 min of pentobarbital anesthesia. The cerebellum samples were excised and frozen rapidly in liquid nitrogen.

3.1. Immunohistochemical analysis with anti-HNE antibody

The frozen cerebellum samples were cut into 6-μm sections. For inhibition of endogenous peroxidase, the sections were incubated with 0.3% H₂O₂ in methanol for 30 min (18, 19). After rinsing in phosphate buffer with saline (PBS), the sections were incubated with normal goat serum (Dako, diluted 1:10) for 20 min to inhibit nonspecific binding. For immunohistochemical demonstration of HNE, the avidin-biotin complex (ABC) method was used. The sections were incubated overnight at room temperature with an affinity-purified rabbit anti-HNE antibody (17, 20) (diluted 1:500). After rinsing with PBS, sections were incubated with biotin-labeled goat anti-rabbit IgG antiserum (Dako, diluted 1:100) for 60 min and then with avidin-biotin complex (Vectastain ABC kit, Vector Laboratories, diluted 1:100) for 60 min. After rinsing, sections were finally incubated with 0.02% 3,3-diaminobenzidine and 0.03% H₂O₂ in deionized water for 9 min. Nuclear counterstaining was performed with Harris’ hematoxylin solution. As a control staining, sections were incubated with normal rabbit serum as well.

3.2. Measurement of thiobarbituric acid reactive substance (TBARS) and protein carbonyl in the cerebellum

TBARS content in the cerebellum samples was determined by the method of Buege and Aust with a slight modification (21). To prevent additional chromophore formation during the assay, 0.1% 2,6-di-tert-butyl-p-cresol was added to the reaction mixture.

Protein carbonyl levels were measured by the method described by Levine et al. (22). The cerebellum samples were homogenized (10% wt/vol) in 50 mM phosphate buffer, pH 7.5. Streptomycin sulfate (10% wt/vol in 50 mM HEPES buffer, pH 7.5) was added to a final concentration of 1% (wt/vol) to precipitate nucleic acids. The samples were allowed to stand at room temperature for 15 min and then centrifuged at 11,000 g for 15 min. The pellet was discarded and the supernatant retained. The protein content of the supernatant was determined by the method of Lowry et al. For each sample, the supernatant was divided into aliquots, an equal volume of 20% TCA was added to each aliquot, and the samples were centrifuged at 3,000 g for 15 min. The supernatant was discarded and the pellet retained. For each sample, the pellet from one aliquot was reconstituted in 0.5 ml of 10 mM 2,4-dinitrophenylhydrazine in 2 M hydrochloric acid (HCl), and the pellet from the other aliquot in 0.5 ml of 2 M HCl (for reagent blank assay). After 1 hr at room temperature, 0.5 ml of 20% TCA were added, the samples were centrifuged at 3,000 g for 5 min, and thereafter the supernatant was discarded. The pellet was washed 3 times with 1 ml of ethanol: ethyl acetate (1:1 vol/vol) to remove unbound reagent. The samples were left for 10 min before centrifugation and the supernatant was discharged. The protein pellet was redissolved in 1 ml of 6 M guanidine HCl in 20 mM potassium phosphate adjusted to pH 2.3 with trifluoroacetic acid and allowed to stand at 37°C for 15 min. For each sample, the absorption spectrum between 360 and 400 nm was obtained in a spectrophotometer. The carbonyl content was calculated from the maximum absorption (relative to reagent blank), using a value of 22,000/M/cm for the extinction coefficient ε, and expressed as nmol/mg/protein.

4. Assessment of a protective effect of Vit E against MeHg toxicity

4.1. Administration of Vit E-deficient diet, Vit E, and MeHg

Five mg/kg/day of MeHgCl were orally administered to the 3 groups of rats for 12 consecutive days. The rats administered MeHg alone: The normal diet was given to the rats for 3 months before, during, and after MeHg administration. One ml of olive oil (vehicle) was orally administered to the rats
30 min before MeHg administration once a day from the initial day of MeHg administration for 20 consecutive days. The rats administered MeHg and the Vit E-deficient diet: The Vit E-deficient diet was given to the rats for 3 months before, during, and after the MeHg administration. The rats administered MeHg and Vit E: The normal diet was given to the rats for 3 months before, during, and after the MeHg administration. One hundred fifty mg/kg/day of Vit E mixed in 1 ml of olive oil were orally administered once a day to the rats from the initial day of MeHg administration 30 min before the MeHg administration. Since the Vit E-deficient diet or normal diet was given to the 3 groups of rats for 3 months before MeHg administration, the body weight of the rats reached approximately 400 g at the beginning of MeHg administration in all 3 groups.

4.2. Mortality, body weight loss, and signs of MeHg-administered rats

Toxicity of MeHg was assessed by mortality, body weight loss, piloerection, and neurological disorders, such as retracted or crossed hind legs when suspended by the tail, and ataxic gait.

4.3. Changes in \( NO_2^- \) and \( NO_3^- \) levels in the serum

Blood samples were taken from the tail vein, and \( NO_2^- \) and levels in the serum were measured by the modified method of Green et al. using a TCI-NOX 1000 m (Tokyokasei, Tokyo, Japan) (23, 24). \( NO_3^- \) levels in the serum was also measured after reduction by cadmium column.

5. Statistics

Statistical significance of the data was determined by one-factor ANOVA and Scheffe's F. The statistical analysis of mortality and the appearance of signs were tested by Kaplan-Meier method. \( P \) value of 0.05 or less was considered statistically significant. Statistical data were expressed as mean±S.D.

Results

1. Oxidative stress in the cerebellum during MeHg intoxication

1.1. Immunohistochemical analysis using anti-HNE antibody

To detect oxidative injury in the cerebellum after MeHg administration once a day from the initial day of MeHg administration.
administration, immunohistochemical examination was performed using anti-HNE antibody. While HNE immunoreactivity was not observed in the cerebellum before MeHg administration (Fig. 1-A), the immunoreactivity was observed in the Purkinje cells (arrows) in the cerebellum 84 days after the initial MeHg administration (Fig. 1-B).

2.2. Changes in NO $^\text{−}$ levels in the serum
To investigate the changes in free radical formation after MeHg administration, the levels of NO $^\text{−}$ and NO $^3$ in the serum were measured. The levels of NO $^\text{−}$ and NO $^3$ increased in the cerebellum 84 days after the initial MeHg administration compared to those before MeHg administration (Fig. 1-D).

2.1. Mortality, body weight loss, and signs of MeHg intoxication
To assess the protective effect of Vit E against MeHg toxicity, mortality, body weight loss, and prevalence of signs of MeHg intoxication were examined. No significant differences in weight loss were observed among the 3 groups (Fig. 2-A). However, the mortality and prevalence of piloerection were significantly higher in the rats administered MeHg and Vit E-deficient diet compared to the rats administered MeHg alone, and were significantly lower in the rats administered MeHg and Vit E compared to the rats administered MeHg alone (Fig. 2-B, C). The rats administered MeHg and the Vit E-deficient diet began to die on day 6 after initial MeHg administration and, all had died by day 12. The rats administered MeHg alone and the rats administered MeHg and Vit E began to die on days 21 and 22, respectively, and all had died by days 24 and 31, respectively, after initial MeHg administration. Due to early death of the rats administered MeHg and the Vit E-deficient diet, prevalence of retracted or crossed hind leg and ataxic gait could not be assessed in those rats. However, the occurrence of these signs significantly improved in the rats administered MeHg and Vit E compared to that in the rats administered MeHg alone (Fig. 2-D, E, F).

2. Protective effect of Vit E against MeHg toxicity

2.1. Mortality, body weight loss, and signs of MeHg-administered rats
To assess the protective effect of Vit E against MeHg toxicity, mortality, body weight loss, and prevalence of signs of MeHg intoxication were examined. No significant differences in weight loss were observed among the 3 groups (Fig. 2-A). However, the mortality and prevalence of piloerection were significantly higher in the rats administered MeHg and the Vit E-deficient diet compared to the rats administered MeHg alone, and were significantly lower in the rats administered MeHg and Vit E compared to the rats administered MeHg alone (Fig. 2-B, C). The rats administered MeHg and the Vit E-deficient diet began to die on day 6 after initial MeHg administration and, all had died by day 12. The rats administered MeHg alone and the rats administered MeHg and Vit E began to die on days 21 and 22, respectively, and all had died by days 24 and 31, respectively, after initial MeHg administration. Due to early death of the rats administered MeHg and the Vit E-deficient diet, prevalence of retracted or crossed hind leg and ataxic gait could not be assessed in those rats. However, the occurrence of these signs significantly improved in the rats administered MeHg and Vit E compared to that in the rats administered MeHg alone (Fig. 2-D, E, F).

2.2. Changes in NO $^\text{−}$ and NO $^3$ levels in the serum
To investigate the changes in free radical formation after MeHg administration, the levels of NO $^\text{−}$ and NO $^3$ in the serum were measured. The levels of NO $^\text{−}$ and NO $^3$ in the serum significantly increased in the rats administered MeHg alone 14 days after the initial MeHg administration compared to those before MeHg administration. The levels were significantly suppressed in the rats administered MeHg and Vit E (Fig. 3-A, B).

Discussion
We have demonstrated in this study that oxidative stress plays an important role in the degenerating process of cerebellar neurons during MeHg intoxication, and that Vit E deficiency increased and Vit E administration decreased MeHg intoxication. HNE is a toxic aldehydic metabolite produced during oxidation of membrane lipid polyunsaturated fatty acids (25). The membrane-peroxidation product exhibits several toxic properties such as cell stimulation and enzyme inactivation and modification (26). This toxic metabolite can be detected in tissues by immunohistochemical examination using affinity-purified polyclonal and monoclonal HNE antibody (17, 20, 27). HNE: adduct should be present in the cerebellum if free radical species induce cerebellar degeneration during MeHg intoxication. Since production of HNE in an H$_2$O$_2$ incubation during immunohistochemical procedures was not seen in our previous studies, it is speculated that positive immunoreactivity of HNE antibody indicates lipid peroxidation in tissue samples (7, 27). As shown in Fig. 1-A, B, HNE immunoreactivity was found predominantly in the Purkinje cells (arrows) in the cerebellum, and these findings increased the likelihood that lipid peroxidation is involved in the degeneration process of MeHg intoxication. Elevated TBARS levels and protein oxidation as evaluated by protein carbonyl formation in the cerebellum after MeHg administration also indicate that lipid peroxidation and protein degeneration by oxidative stress are produced in the cerebellum during MeHg intoxication. It has been previously reported that nitric oxide synthase activities in the cerebellum were elevated from day 13 to day 23 after oral administration of 5 mg/kg of MeHg to rats for 12 days (9). Since the elevation of TBARS and protein carbonyl levels after MeHg administration was in accordance with the NOS activation period, it is considered that the elevation did not reflect the aging of the rats but the oxidative injury by free radicals.

It is well known that a 3-month administration of a Vit E-deficient diet causes complete depletion of Vit E in the brain (28). Vit E crosses through the blood-brain barrier, and is effective in various central nervous system diseases, i.e., Alzheimer disease (29–31). Taken these facts and our results together, it is suggested that oxidative injury, especially lipid peroxidation, via a powerful oxidant (e.g., hydroxyl radicals) may play an important role in cerebellar degeneration during MeHg intoxication, and that Vit E may be one of the most useful protective antioxidants against the cytotoxicity of MeHg in the cerebellum.

The rats, weight 250–300 g, used for the histochemical and biochemical examinations, did not die after 5 mg/kg of MeHg administration for 12 consecutive days. However, all the rats used in the studies for the effect of Vit E died by 31 days after the initial MeHg administration. Since the average body weight of the rats reached 400 g during the treatments, the absolute dose of MeHg increased to 2.0 mg, while in the rats weight 250–300 g it was 1.25–1.5 mg. This may be the main reason why all the rats weight 400 g died during the experiments.

We have reported that the numbers of granular cells in the cerebellum started to decrease at day 63 and decreased by 63% at day 84 after the initial MeHg administration (9). So, in this study, to assess the involvement of free radical intoxication, immunohistochemical examination was performed on day 84 after the initial MeHg administration. Unfortunately, histochemical examination could not be performed in the rats examined for the effect of Vit E because they all died during the examination.
**Fig. 2 Protective effect of Vit E against MeHg toxicity.** Changes in body weight (A); Open circles: the rats administered the Vit E-deficient diet and MeHg, black circles: the rats administered MeHg only, and black squares: the rats administered MeHg and Vit E. Mortality rate (B); Open circles: the rats administered the Vit E-deficient diet and MeHg, black circles: the rats administered MeHg only, and black squares: the rats administered MeHg and Vit E (150 mg/kg/day for 20 days). * P<0.05. Prevalence of piloerection (C), retracted hind leg (D), crossed hind leg (E), and ataxic gait (F). Open circles: the rats administered MeHg and the Vit E-deficient diet, black circles: the rats administered MeHg only, and black squares: the rats administered MeHg and Vit E (150 mg/kg/day for 20 days). * P<0.05.
The presence of free radical species including NO has been implicated in various forms of neurotoxicity (32). NO can also react with superoxide anions from the electron transfer system at the mitochondria to generate peroxynitrite and hydroxyl radical, which are extremely reactive and cytotoxic (8). Abnormally highly produced NO can be toxic to neurons as a free radical. After MeHg administration, bNOS activation via stimulation of N-methyl-D-aspartate (NMDA) receptor by glutamate was reported (9). The levels of NO$_2^-$ and NO$_3^-$ in the serum were elevated after MeHg administration, and Vit E administration decreased the levels in correlation with improvement of MeHg intoxication signs, suggesting that these metabolites may become a marker of free radical injury and that NO production may play an important role in MeHg intoxication in the cerebellum as, we have previously documented (9).

In conclusion, oxidative stress is present during MeHg intoxication. Vit E may be a useful drug for reducing acute MeHg intoxication. Administration of Vit E should be considered during acute MeHg intoxication in humans.

References

(3)  Takeuchi T. Pathology of Minamata disease. In Study Group of Minamata disease, Kumamoto University (ed. Kutsuna, M.), Shuhan Publisher, Tokyo, Japan. 1968, pp. 141–228.
(8)  Beckman JS, Beckman TW, Chen J, Marshall PA, Freeman BA. Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide. Proc. Natl. Acad. Sci. USA 1990; 87: 1620–1624.
(15) Usuki F, Yasutake A, Umehara F, Tokunaga H, Matsumoto


