Chronic Low-Dose Methylmercury Administration Decreases Mitochondrial Enzyme Activities and Induces Myopathic Changes in Rats

Fusako Usuki,* A Akira Yasutake, Miyuki Matsumoto, and Itsuro Higuchi

*Department of Clinical Medicine and Biochemistry Section, National Institute for Minamata Disease, 4058–18 Hama, Minamata 867–0008, Japan, and Third Department of Internal Medicine, Kagoshima University, 8–35–1 Sakuragaoka, Kagoshima 890–0075, Japan

(Received January 5, 2001; Accepted January 19, 2001)

Methylmercury (MeHg)-induced neurotoxicity includes skeletal muscle symptoms (muscle weakness and wasting, muscle cramp). In this study the effects of long-term low dose exposure to MeHg on skeletal muscle were investigated using rats which had received food containing MeHg (5 ppm Hg; the average intake: 200 µg Hg/kg/day). From six months after the first MeHg administration, total mercury levels in the skeletal muscle remained almost stable at 3.5–5.5 µg/g, about one tenth of the levels achieved in the acute MeHg-intoxicated model receiving 5 mg MeHgCl/kg/day for 12 days. However low-dose, long-term administration of MeHg induces histochemical changes similar to those in the acute model with decreases in mitochondrial electron transport system enzyme activities. The results indicate that even low dose MeHg administration causes disturbances in mitochondrial enzyme activities if the administration continues long term. In rats treated with MeHg for 21 months, mild myopathic changes appeared: variations in fiber size, increases in central nuclei and an increase of acid phosphatase activity. The declining function of the redox system in skeletal muscle during aging may accentuate the effects of chronic MeHg intoxication on skeletal muscle.

Key words — methylmercury, low-dose exposure, chronic toxicity, mitochondrial electron transport system enzyme, aging, myopathy

INTRODUCTION

Methylmercury (MeHg) is a well-established neurotoxicant known to be the cause of Minamata disease, a condition characterized by ataxia, visual and hearing disturbances, sensory disturbances, convulsions, memory disturbances, extremity weakness and wasting, and muscle cramp.1,2) The underlying mechanisms responsible for the skeletal muscle symptoms are still poorly understood, although the effects of MeHg on synaptic transmission and membrane excitability in the neuromuscular junction have been reported to result in myasthenia gravis-like muscular weakness.3,4)

We previously reported that MeHg exposure affects skeletal muscle itself in acute MeHg-intoxicated model rats receiving 5 mg MeHgCl/kg/day for 12 days.5) These rats showed significant decreases in cytochrome c oxidase (CCO) and succinate dehydrogenase (SDH) activities in skeletal muscle. The decrease in mitochondrial electron transport system enzyme activities would cause a back-up of the tricarboxylic acid cycle products. The defect in mitochondrial energy metabolism in MeHg-intoxicated skeletal muscle should explain why Minamata disease patients often complained of fatigue and weakness. However, myopathic changes were not recognized in this acute model. Long-term exposure might be needed for intoxicated rats to develop myopathic changes.

In this study, we investigated the effects of chronic low dose MeHg exposure on skeletal muscle. The dose utilized seemed to be relevant to environmental or potential occupation exposure for human beings. This dosage made long-term administration possible.

MATERIALS AND METHODS

Animals and Methylmercury Administration — Male Wistar strain rats (age 9 weeks; weight
300–325 g) were obtained from CLEA Japan. Methylmercury chloride (MeHgCl) (Tokyo Chemical Inc. Co. Ltd., Tokyo, Japan) was used without further purification because purity was estimated to be >99.9%. Rats were housed in TPX cages (3 to 4 rats/cage), fed daily γ-ray-sterilized CE-7 laboratory chow (18% protein; CLEA Japan) containing MeHgCl (5ppm Hg), and given free access to water. The average intake was 200 µg Hg/kg/day. At the indicated times (6, 9, 12, 15, 18, 21 months after the first MeHg administration) five rats were sacrificed under ether anesthesia. At each time, three age-matched rats not exposed to MeHgCl served as controls. The experimental protocol was in accordance with the Animal Committee on Animal Experimentation of the National Institute for Minamata Disease.

**Morphological Examination** — Rats were perfused through the heart with ice-cold saline under ether anesthesia. The soleus and extensor digitorum longus (EDL) muscles were dissected. The reported fiber-type distribution in 3-month-old rats is that 95% of fibers in soleus muscles are mitochondria-rich type 1 (red muscle) and 90% of fibers in EDL type 2 (white muscle). A portion of the muscle specimen was immediately frozen in isopentane cooled with liquid nitrogen and then stored in liquid nitrogen for later histochemical examination. Serial frozen sections were stained with hematoxylin and eosin (H&E), modified Gomori trichrome, periodic acid-Schiff, oil red O, NADH-tetrazolium reductase, and ATPase, according to the method of Dubowitz and Brooke. Acid phosphatase was stained by the method of Barka and Anderson, SDH by the method of Nachlas et al., and cytochrome c oxidase by the method of Seligman et al.

**Mercury Concentration** — Specimens of muscle, kidney, liver, cerebellum, and cerebrum were prepared for the measurement of mercury concentration. The tissues were homogenized in ice-cold distilled water (10% w/v), and the total mercury levels of the homogenates were determined by atomic absorption spectrometry following the oxygen combustion-gold amalgamation method. The equipment comprised a mercury analysis vaporizer MV-250R (Sugiyamagen Environmental Science Co. Ltd., Japan) and a mercury detector MD-A (Nippon Instrument Co., Osaka, Japan). Samples for the analysis of inorganic mercury were prepared by extracting the MeHg with toluene-petroleum ether from the tissue homogenates. A standard was prepared from a commercially available standard solution (Wako Pure Chemical Ind., Osaka, Japan).

**Estimation of Antioxidant Enzyme Activities** — A portion of the soleus muscle was immediately frozen in liquid nitrogen for biochemical studies and then stored at −80°C. Catalase activity was determined by the rate of sodium perborate decomposition. Glutathione peroxidase (GSH-Px) activity was determined by the rate of NADPH oxidation. Enzyme activity was expressed as units per mg of total protein as determined by the method of Lowry et al. using bovine serum albumin as the standard.

**Statistical Analysis** — Data were expressed as mean ± S.D. Two-way analysis of variance (ANOVA) tests were used to assess differences between groups. A difference was considered statistically significant when $p < 0.05$.

**RESULTS**

**Clinical Manifestations**

No differences in body weight were observed...
between MeHg-treated and control rats throughout the experiment. MeHg-treated rats did not exhibit hind limb crossing when suspended by their tails, a typical sign of MeHg-intoxication in rats. Little difference in movement between MeHg-treated rats and controls could be detected. Mild anemia in this model recognized from six months after the first MeHg administration has been reported previously.16)

Mercury Concentration

The time courses of total mercury concentrations in skeletal muscle (soleus, EDL), liver, kidney, and brain (cerebellum, cerebrum) are shown in Fig. 1a), b). No differences in the levels between soleus and EDL muscles were recognized. From six months after the first MeHg administration, total mercury levels in the skeletal muscle remained almost stable at 3.5–5.5 µg/g. This level was almost twice that in brain and much lower than in liver or kidney. Inorganic mercury levels in skeletal muscle were very low (<2% of total mercury: 0.11 ± 0.02 µg/g even after 21 months of treatment), whereas 30–50% of the total mercury was inorganic in liver (8.8 ± 4.5 µg/g) and kidney (33.7 ± 7.1 µg/g).

Histopathological Findings

The only obvious histopathological findings in skeletal muscle were decreases in mitochondrial electron transport system enzyme activities up until 18 months of treatment. The early finding of decreases in these enzyme activities were detected even six months after the first MeHg administration. However, in rats treated for 21 months, mild myopathic changes appeared in addition to decreases in mitochondrial electron transport system enzyme activities. Staining for CCO activity indicated decreased activity in MeHg-treated rats compared to age-matched controls (Fig. 2A, B). SDH also showed decreased activity in MeHg-treated rats (Fig. 2C, D). Mild myopathic changes were observed including

Fig. 2. Histochemistry of Soleus Muscle from a Rat Administered MeHg for 21 Months (A, C) and an Age-Matched Control Rat (B, D). Cytochrome c oxidase activity is decreased in many fibers in the MeHg-treated sample (A) compared to normal (B). Succinate dehydrogenase (SDH) activity is also decreased (C) compared to normal (D). A, B: Cytochrome c oxidase activity staining, × 94; C, D: Succinate dehydrogenase activity staining, × 94.
variations in fiber size, an increase in the number of central nuclei and an increase of acid phosphatase activity (Fig. 3A–D). These histochemical findings were present in all MeHg-treated rats and more apparent in the mitochondria-rich soleus muscle than in EDL muscle. Ragged-red fibers indicating mitochondrial proliferation and abnormal mitochondrial aggregation were not recognized. No vacuolated fibers were detected. Neither neurogenic atrophied-fibers nor cellular infiltration was observed.

**Estimation of Antioxidant Enzyme Activities**

Catalase and GSH-Px activities in the skeletal muscle of rats of different ages are shown in Fig. 4a), b). MeHg induced an increase in catalase activity, but the level was not significantly different from that of age-matched controls. In contrast, the activity of GSH-Px in rat skeletal muscle decreased with age \((p < 0.01)\). All of the MeHg-treated rats showed significantly lower activities of GSH-Px than age-matched control \((p < 0.001)\).

**DISCUSSION**

In this study we clarified the effects of chronic long-term low dose MeHg exposure on rat skeletal muscle, including decreases in mitochondrial electron transport system enzyme activities and myopathic changes. It is important to know chronic low dose MeHg toxicity in order to assess the environmental risk of MeHg. We adopted in this study the low dose of MeHg that seemed to be relevant to potential environmental or occupation exposure for human beings.

We used rats fed MeHg-contaminated food (5 ppm Hg; the average intake: 200 µg Hg/kg/day) as a chronic MeHg-exposed model. The body weight of this model was similar to that of controls even after 21 months of treatment, and a typical sign of MeHg-intoxication in rats exhibiting hind limb crossing when suspended by their tails was not recognized. The content of total mercury in the skeletal muscle was about one tenth of the levels in the acute model showing a typical MeHg-intoxicated...
However, histochemical examination of skeletal muscle revealed decreases in mitochondrial electron transport system enzyme activities, CCO and SDH, which were similar to those seen in the acute model. The early finding of these decreases were detected six months after the first MeHg administration. These findings indicate that even low dose MeHg administration causes disturbances in mitochondrial electron transport system enzyme activities if the exposure is continuous.

A long-term process was needed to develop myopathic changes. Apparent myopathic changes, including variations in fiber size, an increase in the number of central nuclei, and an increase in acid phosphatase activity were first recognized in rats treated for 21 months. Aging causes disturbances in mitochondria even in normal animals. Age-related mitochondrial defects should increase the vulnerability to MeHg toxicity. Recently we indicated the role of MeHg-induced oxidative stress in MeHg-intoxication in vivo.\textsuperscript{17,18} So in this study we estimated the changes with age of two antioxidant enzymes, catalase and GSH-Px. The activity of catalase, which exists in peroxisomes, remains essentially constant with age. However, the activity of GSH-Px, which exists in mitochondria and cytosol, decreases with age, and MeHg causes a further decrease in activity. Changes in the redox system in skeletal muscle during aging may be important in terms of increasing the vulnerability to oxidative stress caused by MeHg.

These results suggest that aging accentuates the effects of chronic MeHg toxicity on skeletal muscle. In this study, a low protein diet was adopted in order to expand the life span of the rats and to know the long-term effect of MeHg on skeletal muscle. It is known that a low protein diet produces less oxidative stress in vivo than a usual diet. One of the reasons that a very long-term process is needed in order for intoxicated rats to develop myopathic changes might be that the low protein diet had some protective effect against MeHg-induced oxidative stress.

The increase in acid phosphatase activity indicates active lysosomal proliferation. However, no mitochondrial proliferation to compensate for the decreases in mitochondrial enzyme activities occurred in these MeHg-treated rats, although abnormal mitochondrial aggregation and mitochondrial proliferation are often recognized in mitochondrial myopathy.\textsuperscript{19} This might be due to the fact that one of the target organelles for MeHg-intoxication is mitochondrion itself. It is known that mitochondrial enzymes are synthesized not only from nuclear DNA but also from mitochondrial DNA, as is the case for CCO. It remains to be determined whether MeHg induces mutations in mitochondrial DNA or not.

Recently we reported that antioxidants protect the cells against MeHg-induced apoptosis in vitro.\textsuperscript{17,18} In vivo protection study of antioxidants is underway to determine whether antioxidants can protect against MeHg-induced decreases in mitochondrial enzyme activities and myopathic changes.

\textbf{Acknowledgments} The authors wish to thank Ms. Noriko Hirata and Ms. Miyoko Furusawa for their...
excellent technical assistance.

REFERENCES