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The Effects of Methylmercury on the Mitochondrial Energetics of Rat Skeletal Muscle: An *in vivo* ^{31}P-NMR Study

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Summary. **In this report it showed that methylmercury chrolide (MMC) affected the mitochondrial energetics of rat skeletal muscles in case of chronic intoxication. High energy phosphate compounds were measured by ^{31}P-NMR spectroscopy in the living rat hindleg skeletal muscle.**

Decreased value of phosphocreatine (PCr)/inorganic phosphate (Pi) ratio was observed in the resting muscle of the MMC intoxicated group, and suspend recovery of the ATP, PCr and intracellular pH after muscle contraction was found in the MMC intoxicated muscle. There was no difference in the ATP levels of the resting muscle between the control and MMC group.

These results suggested that the synthesis of ATP was disturbed by the inhibition of mitochondrial respiration below TCA cycle.

Methylmercury is well known as a chemical pollutant which caused "Minamata Disease" first in Kumamoto and then in Niigata in the mid 1950s in Japan. They were caused by the ingestion of large quantities of fish and/or shellfish that had been contaminated with methylmercury compounds through discharge of industrial effluent into local waters.[11,17]

Although the toxic actions of methylmercury compounds have been well described in both clinical and experimental studies, the biochemical mechanisms underlying the process of methylmercury intoxication are not fully understood.

Chronic exposure to methylmercury has been found to produce ultrastructural damage to mitochondria in the liver[8] and the central nervous system of animals,[14] but little is known about the mechanisms in which mitochondrial respiration participate to produce the pathological syndrome.

From the point of view that disturbed mitochondrial respiration may have a deep bearing on methylmercury poisoning, Cheung et al.[15] have reported the dose-dependent decline in ATP synthesis in the presence of methylmercury and suggested that a decline in available ATP might account for the observed mercurial inhibition of synaptosomal protein synthesis. They have suggested that mercurial interacts with the mitochondrial inner membrane leading to the uncoupling of oxidative phosphorylation and a drop in the ATP synthesis.

Ally et al.[3] in a study of mitochondrial respiration and glucose transport activity in yeast cells further demonstrated that direct damage to mitochondrial respiration was responsible for the toxic effect of methylmercury. However, the primary biochemical lesions have not been identified conclusively.

Nuclear magnetic resonance (NMR) studies have yielded a great deal of information on the metabolism in vivo.[7] The recent introduction of the surface coil[1] for the study of muscle energetics permits non-invasive measurements of pH and of high energy phosphates and metabolites. Recent studies have yielded more information on mitochondrial energetics and also described the mechanism of defective glycolysis in animals and humans.[4,12,15]

To confirm the presence of disturbed mitochondrial respiration, we have examined the changes in high energy phosphate compounds, such as phosphocreatine (PCr) and ATP, in methylmercury intoxicated rat muscles by the *in vivo* ^{31}P-NMR method.

MATERIALS AND METHOD

Animal preparation

Male Wister rats weighing 200g were maintained in a room at constant temperature and they were allowed free access to water and food.

Methylmercury chloride (MMC) was administered according to the dosage schedule of Klein et al.[10]

Rats were given daily subcutaneous injections of MMC, 10 mg/kg in 10 mM sodium carbonate solution (Na_2CO_3). Controls received injections of 200 μl of Na_2CO_3 (10mM). Injections were continued 7 consecutive days at a different location each day under the skin of the back. Na_2CO_3 solution was adjusted to pH 8.6. Day 1 was the day on which the first injection was given.

By day 21, 10 rats whose hindlegs were paralyzed and showed strong flexion (crossed-hindleg phenomenon), were chosen for ^{31}P-NMR studies, and 9 were chosen for controls.

All the animals were anesthetized with intraperitoneal pentobarbital (35 mg/kg), and the right hindleg was exercised isometrically by electricalstimulation through copper electrodes placed at the poples and ankle. The stimulation, 20 volts in strength, 0.2 msec in duration and 5 Hz in frequency, was administered for 40 seconds.

NMR measurement

^{31}P-NMR spectra were recorded at 109.14 MHz on a JNM GX270 NMRspectrometer (JEOL Ltd. Tokyo, Japan). A detailed description of the condition used to operate the NMR spectrometer and to obtain the ^{31}P-NMR spectra has been described previously.[12]

A capillary containing a small amount of hexamethyl phosphoric acid (HMPA), the chemical sift of which was about 28.5 ppm away from the position of PCr, was placed on the opposite side of the surface coil. This allowed us to calculate the changing values of the peak height of each signal compared to that of HMPA.[21]

The surface coil was placed on the sural surface of the right hindleg, and the animal was held in the center of a magnet.

The intracellular pH was determined by measuring the chemical shift of the inorganic phosphate (Pi) resonance and by determining from the standard titration curve the pH to which this chemical shift corresponded.[13] The standard titration curve was made from phosphate-buffered saline containing 10mM PCr, which was adjusted to various pH levels by addition of HCl and NaOH at 37℃.

RESULTS

A^{31}P-NMR spectrum from a normal anesthetized rat hindleg is shown in Fig 1. The chemical shift assignments of six resonance bands are (1) β-phosphate of ATP, (2) α-ATP, (3) γ-ATP, (4) PCr, (5) Pi and (6) Phosphomonoesters (PMEs).[21] The peak area was chosen to observe the changing value of each compound, and β-ATP peak was chosen to observe the change of ATP.

No difference was found in the resting ATP levels in the control and MMC group; ratio of β-ATP to HMPA of the control was 0.55 ± 0.01 and that of the MMC group was 0.54 ± 0.07 (mean$\pm$SD).

A significant difference was found in the PCr/Pi ratio of the resting muscle of the control and MMC groups; the mean$\pm$SD of the control was 10.78 $\pm$1.68, whereas that of the MMC group was 7.77 ± 9.45. The results were significantly different at the 1% level by the Student t-test.

A sequential change of ^{31}P-NMR signals from a normal rat hindleg muscle before and after exercise is shown in Fig. 2, and the percentile changes of each signal is shown in Fig. 30-A. Each spectrum was a 60-times accumulation of a spectrum repeating every 2 seconds. Following 40 seconds of muscle contrac-

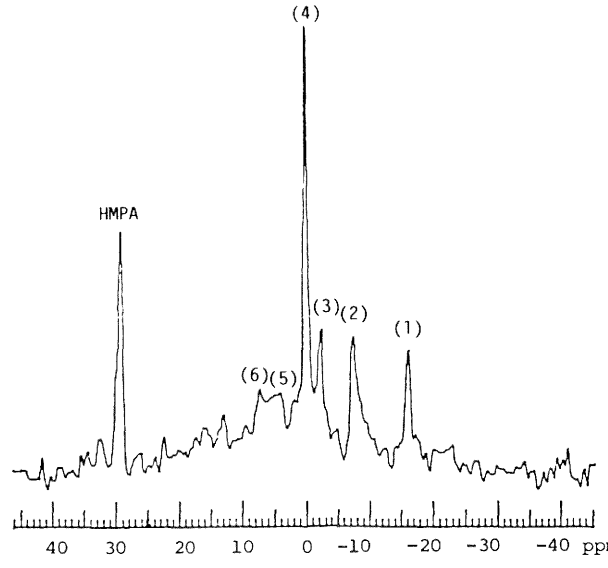

Fig. 1. ^{31}P-NMR spectrum obtained at 109.14 MHz from a normal anesthetized rat hindleg muscle. Spectral width was set to 5400 Hz. Hexamethyl phosphoric acid (HMPA), the chemical sift of which was about 28.5 ppm away from position of PCr, was used as an external reference.

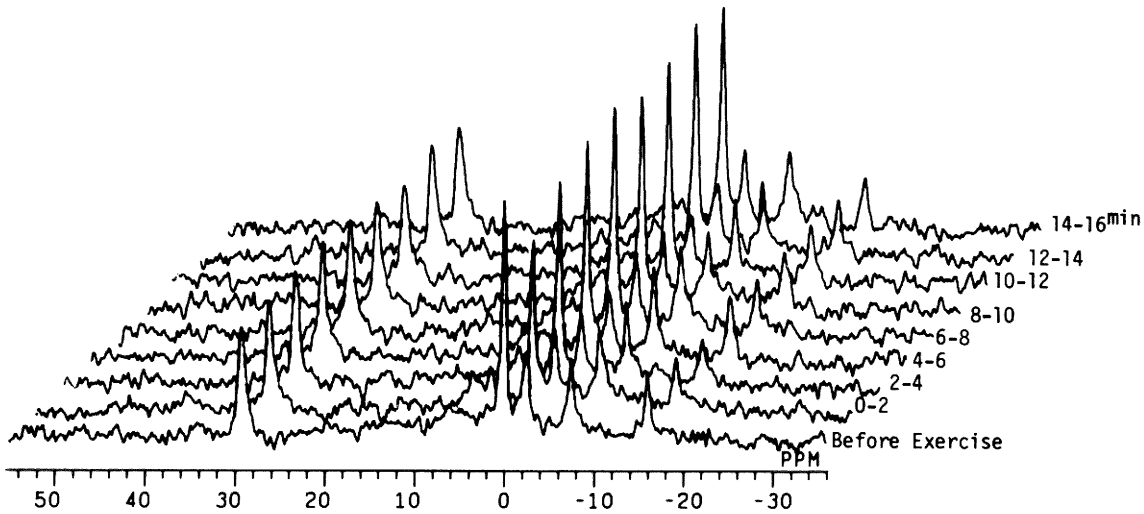

Fig. 2. A sequence of ^{31}P-NMR spectra from normal rat hindleg muscle. After 40 seconds of muscle contraction, PCr and ATP were hydrolyzed, and they were rebuilt succesively.

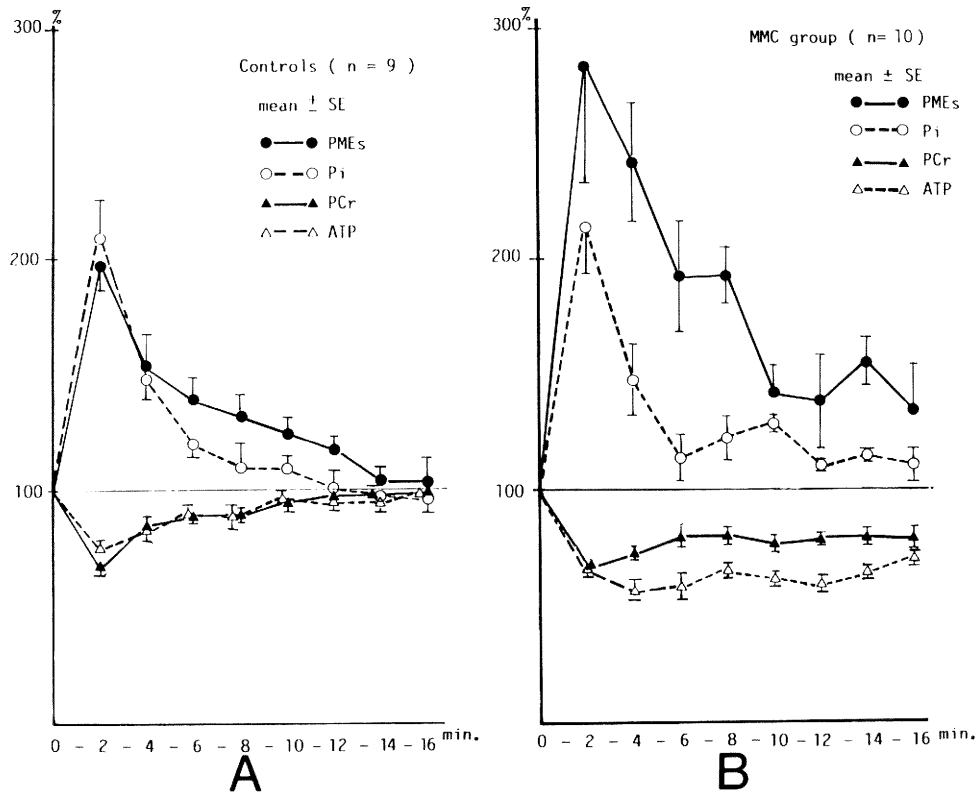

Fig. 3. Percentile changes of PCr (▲), ATP (△), Pi (○) and PMEs (●) after muscle exercise. Control (A) and MMC group (B), Values are mean values±S. E.

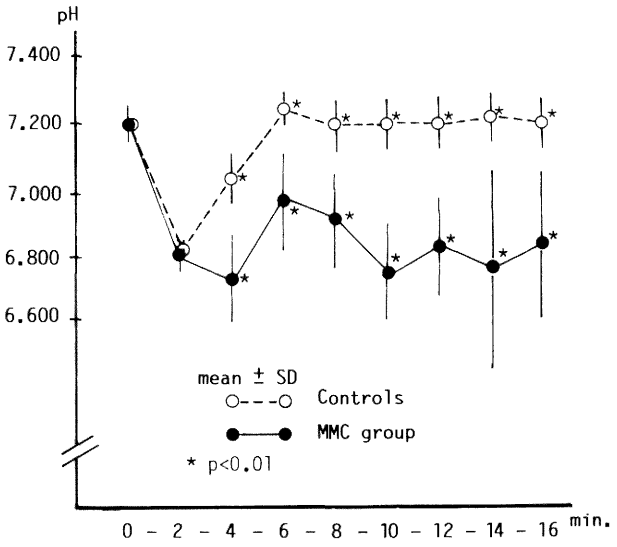

Fig. 4. Sequential changes of muscle pH of controls (○, n=9) and MMC group (●, n=10) before and after muscle contraction. There is no difference of pH at the resting condition. After exercise, the pH of the MMC group remained around 6.7 throughout the experiment, whereas that of the control animals returned to the pre-exercise level within 6 minutes of the end of muscle contraction. The difference of pH values between control and MMC group is statistically significant in 1% level as assessed by a Student t-test. Values are mean±S. D.

tion, PCr and ATP had been hydrolysed, and they were rebuilt exponentially. The increase in Pi and PMEs immediately after the exercise and the time course of their disappearance corresponded to the changes in PCr and ATP.

The position of the Pi resonance was observed to move towards the highfrequency direction (acidotic condition) by a few ppm and to return to its pre-exercise value immediately.

Fig. 3-B shows the percentile change of each peak of ^{31}P-NMR spectra of a MMC administered group. Following the tetanic contraction for 40 seconds, transient increase and prompt disappearance of Pi and PMEs took place in the same way in the control group. There was also consumption of both ATP and PCr, although there was no recovery during the resting period. Their levels remained unchanged during the recovery period.

The time course of changing value of muscle pH before and after muscle contraction is shown in Fig. 4. The muscle pH of the MMC group remained around 6.7 throughout the experiment, whereas that of the control animals returned to the pre-exercise level within 6 minutes after muscle contraction. The difference of pH values between the control and MMC group is statistically significant ($p<0.01$).

DISCUSSION

Ultrastrtuctural changes in mitochondria are reported as the resultant toxic action of methylmercury, and the damage to mitochondrial biogenesis is responsible for the structural changes of rat mitochondria.[8,14]

ATP depletion in an isolated brain synaptosome[5] and decreased coronary fluid ATP in an isolated perfused rat heart[2] have been observed in the presence of MMC. But the primary lesions responsible for the toxic effect induced by MMC have not been clarified conclusively, although the study using yeast cells strongly suggests that mitochondrial electron transport is inhibited by MMC.[3]

There has been no report on the ultrastractural changes of muscle mitochondria, and residual radioactivity after intraperitoneal injection of ^{203}Hg-MMC has been rather low in skeletal muscle when compared with the central nervous system[16]; however, the biochemical effect of MMC on mitocondrial energetics should not be negrected.

Although no decrease in the resting ATP levels in MMC intoxicated rat hindleg muscle was found, a lowered PCr/Pi ratio in theresting state and suspended recovery of ATP and PCr after muscle contraction were observed.

The results indicate that synthesis of ATP in depressed in the MMC intoxicated muscles. There are two possible causes which may interfere with the synthesis of ATP:

1. Inhibition of aerobic glycolysis/glycogenolysis.
2. Inhibition of mitochodrial respiration below the TCA cycle.

We have previously reported on a ^{31}P-NMR study of an animal model of defective muscle glycolysis and showed that an increase in the PMEs peak after muscle contraction was characteristic of the condition, where aerobic glycolysisi/glycogenolysis was inhibited at the level of glyceraldehyde 3-phosphate dehydrogenase.[12]

Hayes et al.[9] demonstrated ^{31}P-NMR spectra of an animal model of mitochondrial myopathy, which was produced by using an NADH-coenzyme Q reductase inhibitor, diphenyleneiodonium. According to their exeriment, after following the muscle contraction, the PMEs resonance showed a transient increase and subsequent fall, and there was no recovery of ATP and pH during the resting period. The sharp increase

in the PMEs was ascribed to the probable reflection of a transient inhibition of glycolysis/glycogenolysis by diphenyleneiodium. Aside from the poor recovery of the PCr signal, the movements of the phosphorus compounds in our experiment on MMC intoxicated rat skeletal muscle can be interpreted as resembling the results of the experiment using a mitochondrial electron transport system blocking agent. The poor recovery of PCr in our experiment may be ascribed to a insufficient synthesis of ATP to reproduce PCr during the period of obserbation.

We showed that the muscle pH values in the MMC group after muscle contraction remained acidotic. This is explained by the lactic acidsis due to the inhibition of mitochondrial respiration below the TCA cycle.

We should also mention the possibility of limited purine substrate availability as a cause for the insufficient return of ATP following muscle contraction. According to the experiment by Dudley et al.,[6] the acidotic condition following muscle contraction stimulates AMP deaminase activity, and enhanced deaminase activity can lead to increased substrate cycling through the purine nucleotide pathway. A continuing acidotic condition can result in lowered substrate availability for ATP synthesis from inosin monophosphate (IMP), although it may be appro-proate to suppose that the inhibi tion of mitochondrial respiration by MMC plays a primary role in insufficient reproduction of ATP and lactic acid accumulation following muscle contraction.

Biochemical studies in the adult rat have demonstrated altered mitochondrial respiration in synaptosomes isolated from the brains of animals exhibiting clinical manifestations of methylmercury intoxication.[18] A reduction in oxygen consumption has been shown in brain slices prepared from adult rats exhibiting methylmercury induced neurological disorders.[20] Moreover, in brain slices prepared from normal rats, an inhibition of oxygen consumption occurs after MMC is added to the incubation medium.[19]

In our *in vivo* study, there were no differences in resting ATP levels in control and MMC intoxicated rat hindleg musule. But following tetanic contraction, PCr as well as ATP showed no recovery. We have not examined the rate constants for the creatine kinase raction, so we cannot explain the delayed recovery of PCr. Nevertheless, we conclude that defective mitochondrial respiration is the proximate consequence of methylmercury intoxication.

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