Higher Activity of Oxidative Drug Demethylation in the Liver Microsomes from Dystrophic Mouse

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The activities of NADPH-dependent oxidative demethylation of aminopyrine and other methyl compounds in the liver microsomes from dystrophic mice were found to be about 30% higher than those of the normal mice. Consumption of reduced pyridine nucleotides during the demethylation reactions was also significantly larger in the dystrophic mouse system than in the normal mouse system. The synergistic effect of further addition of NADH on the oxidative demethylation in the reaction system with NADPH, however, was not significant in either the normal or the dystrophic mouse system. The activities of NADPH-cytochrome c reductase and lipid peroxidation were also higher by about 30% in the dystrophic mouse than in the normal mouse, but the contents of cytochrome P-450 and phospholipids in the liver microsomes from normal and dystrophic mice were not appreciably different. The results suggest the possibility that the progressive muscular dystrophy may involve abnormal features in not only muscle but also liver and other tissues.

dystrophic mouse; drug demethylation

Numerous papers have appeared describing various biochemical abnormalities in the muscle of dystrophic patients as well as dystrophic animals (Bourne and Golarz 1963). Among them, hypercreatinuria, hypocreatininuria, and elevation of some enzymes in blood of patients with muscular dystrophy are well known. Also the rate of protein turnover has been reported to be higher in dystrophic muscle than in the normal control muscle (Kruh et al. 1960; Kitchin and Watts 1973). These biochemical abnormalities have been ascribed to metabolic disturbances in muscle due to either myogenic or neurogenic lesion, and so far, poor information is available with respect to biochemical changes in other tissues of the dystrophic patients or animals (Coleman and Ashworth 1959; Bester and Gevers 1973). Previously we have observed that the microsomal arylhydrocarbon hydroxylase activity of the dystrophic mouse liver was considerably higher than that of the normal mouse liver (Abe 1974). The present paper describes that the liver microsomes from dystrophic mouse exhibit about 30% higher activity of oxidative demethylation of aminopyrine and other drugs than that of the normal mouse. The activities of NADPH-cytochrome c reductase and lipid peroxidation were also higher in the dystrophic mouse than in the normal mouse.

Received for publication, February 14, 1976.

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MATERIALS AND METHODS

Animals

Male normal and dystrophic mice, strain C57BL/6J, were obtained from the Central Research Institute of Experimental Animals, Tokyo, and were maintained at 23–28°C in our laboratory, fed on pellets of mouse chow (Oriental Yeast Company, Osaka).

Chemicals

Tris, cytochrome c (Type XII), alcohol dehydrogenase, phosphatidyicholine, phosphatidylethanolamine and sphingomyelin were purchased from Sigma Chemical Company, St. Louis; NADPH and NADH were obtained from Oriental Yeast Company, Tokyo; glutamate dehydrogenase, NADP+, glucose-6-phosphate and glucose-6-phosphate dehydrogenase were purchased from Boeringer, Mannheim. Other reagents, analytical grade, were obtained commercially.

Preparation of microsomes

Normal and dystrophic mice of the same age were used in each series of the experiment. Mice, aged 3 to 4 months, were fasted for 24 hr prior to sacrifice by decapitation. Livers were immediately removed and washed twice with 0.25 M sucrose containing 0.05 M Tris-HCl buffer (pH 7.5) unless otherwise described. Washed livers were homogenized with a Potter-type glass homogenizer in 9 volumes of the same sucrose-Tris solution, followed by centrifugation at 20,000 × g for 10 min, and the supernatant fluid was further centrifuged for 1 hr at 105,000 × g. The microsomal pellet obtained was washed twice with the same solution and finally suspended in the same solution of the same volume of the original liver and stored at -20°C until the next day, when they were used for enzymic or chemical examinations. Protein was determined by the method of Lowry et al. (1951).

Assay of demethylation activity

The activity of oxidative demethylation was determined by following the formation of formaldehyde by the spectrophotometric method of Nash (1953). The reaction mixtures contained in a final volume of 1.5 ml: 75 μmoles of Tris-HCl buffer (pH 7.5), 7.5 μmoles of magnesium acetate, 12 μmoles of aminopyrine or other methyl compounds, 0.15 μmole of NADPH, and 0.4 to 0.5 mg of microsomal protein unless otherwise described. The reaction was started by the addition of enzyme after a 3-min preincubation at 25°C and was carried out for 8 min with shaking. The reaction was terminated by adding trichloroacetic acid (final, 5%), followed by centrifugation, and 1 ml of the supernatant fluid was mixed with 1 ml of Nash’s reagent and left at 37°C for 40 min to develop the color.

Assay of lipid peroxidation

Lipid peroxidation was assayed by measuring the formation of malondialdehyde with the method of Wills (1966). Reaction mixtures contained in a final volume of 1.5 ml: 75 μmoles of Tris-HCl buffer (pH 7.5), 7.5 μmoles of magnesium acetate, 0.06 μmole of ferrous ammonium sulfate, 0.3 μmole of NADPH, and 0.4–0.5 mg protein of the microsomal preparations. The microsomes used in this experiment were prepared by using 0.05 M Tris-HCl buffer containing 0.15 M KCl instead of sucrose, since sucrose interfered with the development of color after the addition of 2-thiobarbiturate. Reactions were carried out at 25°C, and at desired time of the reaction, 0.5 ml of the reaction mixture was pipetted into a centrifuge tube in which 1 ml of 20% trichloroacetic acid had previously been placed, then the mixture was immediately added with 2 ml of 0.67% aqueous solution of sodium 2-thiobarbiturate. The mixture in the centrifuge tube was heated at 100°C for 10 min, and after cooling, it was centrifuged for 10 min at 3,000 rpm. Absorbance at 532 nm of the supernatant fraction was measured.
**Determination of NADPH and NADH**

NADPH and NADH remaining in the reaction mixture after the demethylation reaction were determined according to the method of Estabrook and Mairta (1962). Namely, 0.6 ml of the reaction mixture was added with 0.2 ml of 3 N KOH which had been placed in a boiling water bath, then the mixture was rapidly cooled in an ice bath and was further added successively with 0.2 ml of 1 M triethanolamine buffer (pH 7.0) and 0.1 ml of 3 N HCl to bring pH of the mixture to 8.4, and centrifuged for 10 min at 3,000 rpm. For the determination of NADPH, 0.5 ml of the supernatant fluid was taken in a cuvette and diluted to 1.5 ml with a solution containing 0.05 M phosphate buffer (pH 7.5), 15 mM acetaldehyde, 2 mM α-ketoglutarate (neutralized with KOH) and 2 mM ammonium chloride, then glutamate dehydrogenase 40 µg as protein were added to the mixture and the decrease in absorbance at 340 nm was monitored by a Hitachi spectrophotometer, type 139. Glutamate dehydrogenase was also added to the reference side cuvette. The amount of the remaining NADPH was calculated from the difference of absorbance before and after the addition of the enzyme. NADH was determined in a similar manner but using 8.3 µg protein of alcohol dehydrogenase. When both NADPH and NADH were present in the reaction mixture, NADH was first assayed by alcohol dehydrogenase, then NADPH was determined by employing glutamate dehydrogenase.

**Assay of NADPH- and NADH-cytochrome c reductase activities**

The activities of NADPH- and NADH-cytochrome c reductases in the liver microsomes were determined spectrophotometrically by the reduction of cytochrome c as described by Jones and Wakil (1967).

**Assay of cytochrome P-450**

The amount of cytochrome P-450 in the liver microsomes were determined by the method of Omura and Sato (1964) on the basis of the CO difference spectra of the dithionite-reduced samples employing an extinction coefficient of 91 mM⁻¹cm⁻¹ between 450 nm and 490 nm. For the determination of the content of cytochrome P-450 the microsomes were prepared with 0.05 M Tris-HCl buffer (pH 7.5) containing 0.15 M KCl instead of sucrose so as to minimize the contamination by hemoglobin. The spectra were recorded by a Hitachi 323 recording spectrophotometer.

**Assay of total heme**

The amount of total heme in the liver microsomes was determined spectrophotometrically by converting the heme into pyridine hemochrome as described by Omura and Sato (1964), using an extinction coefficient of 32.4 mM⁻¹ cm⁻¹ between 557 and 575 nm.

**Extraction of microsomal lipids and detection of phospholipids**

The method of Radin (1969) was used to extract total lipids from the microsomal suspension. Usually 1 ml of the microsomal suspension was homogenized with 20 ml of a chloroform-methanol mixture (2:1) in a Potter-type glass homogenizer at 4°C for 3 min. Then the homogenate was filtered by a glass filter. The filtrate was shaken with 4 ml of water to remove sucrose or Tris contained in the filtrate and was centrifuged at 3,000 rpm. The resulting upper layer was removed by suction and the lower layer was evaporated to dryness under a reduced pressure in N₂ gas and left on phosphoric anhydride in a vacuum desiccator for several hours. The lipid residue was weighed and dissolved in a fixed volume of chloroform-methanol (2:1) and stored at -20°C in N₂ gas until used. For the analysis of phosphatidylethanolamine, phosphatidylcholine and sphingomyelin, the lipid sample was subjected to a silica gel thin layer chromatography using a mixture of chloroform: methanol: water (65:25:4) or chloroform: methanol: acetic acid: water (25:15:4: 2) as solvent. The color for the phospholipids was developed by spraying 50% sulfuric acid followed by a few min heating at 150°C.
RESULTS

The liver microsomes from the dystrophic mouse showed about 30% higher activity of oxidative demethylation than the activity in the microsomes from the normal mouse, as shown in Fig. 1. Sometimes, the difference in the activity was more than 30%. The activity of oxidative demethylation of the dystrophic microsomes was higher at any NADPH concentrations tested and also under conditions where both NADPH and NADH were added (Fig. 2). The time courses of the demethylation in the reaction systems with NADPH, NADH, or NADPH plus NADH are shown in Fig. 3. The data in Figs. 2 and 3 also indicate that so-called synergistic effect by NADH upon the NADPH-dependent oxidative demethylation of aminopyrine, as described by Cohen and Estabrook (1971), was not particularly significant in either the normal or the dystrophic microsome system. This was further confirmed by the experiment shown in Fig. 4, where varying amounts of NADH were added to the reaction systems with a fixed amount of NADPH. Fig. 4 also indicates that the activity of the NADH-supported demethylation was not appreciably different in the dystrophic and normal mice. In an independent experiment, we measured the consumption of reduced pyridine nucleotides during the reaction and found that the consumption was significantly larger in the reaction with the microsomes from dystrophic mouse regardless of whether NADPH or NADH alone or both NADPH and NADH were employed, as shown in Table 1. This finding is consistent with the observed higher activity of oxidative demethylation in the liver microsomes from dystrophic mouse. In Table 2 are shown the results of experiments with various methyl compounds as substrate. In all cases the dystrophic microsomes exhibited definitely higher demethylation activities than those of the normal microsomes.

Then the activity of lipid peroxidation was examined. Since the extent of

Fig. 1. Relationship between the rate of oxidative demethylation of aminopyrine and the amount of microsomal protein used. Reaction conditions were as described in Methods except that the amount of microsomal protein was varied. ○—○, normal; ●—●, dystrophic.
Fig. 2. Effect of addition of varying amounts of reduced pyridine nucleotides on the rate of oxidative demethylation of aminopyrine. Reaction conditions were similar to those described in Methods. Solid lines show the activity in the presence of indicated amounts of NADPH alone, and the broken lines indicate the activity in the presence of a fixed amount (0.2 mM) of NADH and varying amounts of NADPH. ○, normal; ●, dystrophic.

peroxidation of microsomal lipids was found to be quite small in the reaction of the oxidative demethylation described above, the reaction for the lipid peroxidation was carried out in the presence of 40 μM ferrous ammonium sulfate and without
Fig. 4. Effect of addition of NADH on the rate of oxidative demethylation of aminopyrine. Reaction conditions were similar to those described in Methods except for pyridine nucleotides. Solid lines show the activities in the presence of indicated amounts of NADH alone; broken lines indicate the activities in the presence of 0.1 mM NADPH and varying amounts of NADH. ○, normal; ●, dystrophic.

Table 1. Consumption of NADPH and NADH in the reaction of oxidative demethylation of aminopyrine

<table>
<thead>
<tr>
<th>Addition (nmoles/1.5 ml)</th>
<th>Consumption of reduced pyridine nucleotide (nmoles/mg protein/8 min/1.5 ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal mouse</td>
</tr>
<tr>
<td>NADPH, 150</td>
<td>144.5</td>
</tr>
<tr>
<td>NADPH, 300</td>
<td>188.9</td>
</tr>
<tr>
<td>NADPH, 600</td>
<td>197.8</td>
</tr>
<tr>
<td>NADH, 150</td>
<td>92.7</td>
</tr>
<tr>
<td>NADH, 300</td>
<td>90.6</td>
</tr>
<tr>
<td>NADPH, 150</td>
<td>120.0</td>
</tr>
<tr>
<td>+ NADH, 300</td>
<td>127.3</td>
</tr>
<tr>
<td>NADPH, 300</td>
<td>131.1</td>
</tr>
<tr>
<td>+ NADH, 300</td>
<td>88.7</td>
</tr>
<tr>
<td>NADPH, 600</td>
<td>120.0</td>
</tr>
<tr>
<td>+ NADH, 300</td>
<td>52.1</td>
</tr>
</tbody>
</table>

Reaction conditions and the experimental procedures were as described in Methods except that various combinations of pyridine nucleotides were employed, and 0.81 mg protein of normal microsomes and 0.68 mg protein of dystrophic microsomes were used.

addition of demethylation substrates. Fig. 5 shows the time courses of lipid peroxidation in the normal and dystrophic microsomal systems. In the early stage of the reaction the rate of lipid peroxidation was higher in the dystrophic microsome system than in the normal, but in the later stage the relation was reversed for some unknown reason.
Table 2. Comparison of the activities of oxidative demethylation of liver microsomes with various substrates

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Formaldehyde formed (nmoles/mg protein/8 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal mouse</td>
</tr>
<tr>
<td>Aminopyrine (8 mM)</td>
<td>48.6</td>
</tr>
<tr>
<td>Codeine (4 mM)</td>
<td>20.3</td>
</tr>
<tr>
<td>Ethylmorphine (12 mM)</td>
<td>36.3</td>
</tr>
<tr>
<td>N-Methylaniline (8 mM)</td>
<td>8.7</td>
</tr>
</tbody>
</table>

Reaction conditions were the same as described in Methods except for the substrates. Codein phosphate and ethylmorphine phosphate were neutralized to pH 7.0 before use.

In an attempt at accounting for the observed higher activity of oxidative demethylation in the dystrophic microsomes, further study was made of the microsomal components required for the drug hydroxylation. It is generally accepted that cytochrome P-450, NADPH-cytochrome c reductase, and phosphatidylcholine are required for the NADPH-dependent drug hydroxylation (Iau and Levin 1974). The content of cytochrome P-450 in the dystrophic microsomes, however, was not much different from that in the normal microsomes; namely, the values of 1.45 and 1.60 nmoles were obtained per mg protein of the normal and dystrophic mouse liver microsomes, respectively (Fig. 6). Furthermore, the amounts of total heme contained in the liver microsomes from the normal and dystrophic mice were not appreciably different; 1.80 nmoles of heme were found per mg protein of the normal microsomes, whereas the value of 1.76 nmoles was obtained for the dystrophic microsome. No appreciable difference could be found with respect to the amount of total lipids or the composition of phospholipids in those microsomes (data not shown). However, the activity of NADPH-cytochrome c reductase of the dystrophic microsome was about 30% higher than that of the normal microsome as
Fig. 6. Carbon monoxide difference spectra of liver microsomes. Both the sample and reference cuvettes contained a microsomal suspension that had been treated with CO gas. Then dithionite was added to the sample cuvette and after several min the difference spectra are recorded. Solid line, normal microsomes (0.74 mg protein/ml); broken line, dystrophic microsomes (0.89 mg protein/ml).

Fig. 7. Time courses of the NADPH-cytochrome c reductase reaction. 0.232 mg as protein of microsomes was employed. NADPH was not added to the control cuvette. Readings in the control system were subtracted from the values in the test system which contained all the necessary components. o—o, normal; •—•, dystrophic.

Recently, Kotake et al. (1975) reported that the activity of oxidative demethylation of ethylmorphine was increased by the addition of a 105,000 Xg supernatant fraction from rat liver homogenate and indicated that the function of
the supernatant fraction could be accounted for by its inhibitory effect on the NADPH-supported microsomal lipid peroxidation. Therefore, considering the possibility that our microsomal preparations might be contaminated with a soluble factor which inhibits the lipid peroxidation of microsomes, a series of experiments shown in Fig. 7 were performed. Namely, the microsomes were preincubated for 10 min with or without the supernatant fraction prepared from either the normal or the dystrophic mice and the effects of addition of those supernatant fractions on the subsequent oxidative demethylation of aminopyrine were compared. As shown in Fig. 7, in both the normal and the dystrophic microsome systems, the rate of oxidative demethylation was decreased by about 26% when the microsomes were preincubated for 10 min without the supernatant fraction. The protecting effects of the supernatant fractions from the normal or dystrophic mice were either not significantly different, when examined by employing various combinations of respective microsomes and supernatant fractions, although the demethylation activity of the dystrophic microsomes was always significantly higher under any reaction conditions tested. Thus, the supernatant fraction appears to have nothing to do with the observed higher activity of oxidative demethylation in dystrophic microsomes.

Fig. 8. Effects of preincubation and addition of the supernatant fraction on the activity of oxidative demethylation of aminopyrine. The preincubation mixture contained the following components in 4.5 ml of 0.05 M Tris-HCl buffer (pH 7.5): 0.3 mM NADP+, 3 mM glucose-6-phosphate, 5 mM magnesium acetate, 6.3 units of glucose-6-phosphate dehydrogenase, and 1.7 mg as protein of microsomes or 11.7 mg as protein of the supernatant fraction. For the supernatant fraction see text. The demethylation reaction was started by the addition of aminopyrine (final, 8 mM). Other conditions were as described in Methods. Solid lines, with preincubation; broken lines, without preincubation. ○, added with the supernatant fraction from normal mice; ●, added with supernatant fraction from dystrophic mice; △, without addition of the supernatant fraction.
DISCUSSIONS

The present study revealed that the activities of the oxidative demethylation of drugs as well as lipid peroxidation of the liver microsomes were significantly higher in the dystrophic mouse than in the normal mouse. However, the observed difference in the demethylation activity cannot satisfactorily be accounted for at present. In the drug demethylation as well as hydroxylation, cytochrome P-450 plays an essential role, but the content of cytochrome P-450 in the dystrophic mouse was not significantly higher than that in the normal mouse. In the present study we did not try to distinguish the relative contents of cytochrome P-450 and P-448 (Lu et al. 1972, 1974; Lu and Levin 1974) in the microsomes, although we have previously observed that the activity of arylhydrocarbon hydroxylase (assumed to be dependent upon the function of P-448 (Nebert and Gelboin 1968; Lu et al. 1972)) was about two times higher in the dystrophic microsomes than in the normal microsomes.

Cytochrome P-450 may also receive electron partly from cytochrome b_5 which is usually reduced by NADH through NADH-cytochrome b_5 reductase (Hildebrandt and Estabrook 1971). In fact, we have observed a slight activity of demethylation by employing NADH in place of NADPH (cf. Fig. 3), but in this case practically no difference was observed between the demethylation activities of the normal and dystrophic microsomes, indicating that the activity of the electron flow from the NADH-cytochrome b_5 reductase system to cytochrome P-450 may be quite similar in these microsomes. This possibility was further supported by the observation that the extents of so-called "synergistic effect", which is seen when NADH was further added to the reaction system containing NADPH, were also similar in the dystrophic microsome system and the normal microsome system (Fig. 4).

The function of cytochrome P-450 is greatly influenced by phospholipids. In fact, phospholipids, especially phosphatidylcholine, are the essential component in the reconstitution of the drug hydroxylation system from partially purified cytochrome P-450 and NADPH-cytochrome c reductase (Lu et al. 1972, 1974; Lu and Leven 1974). However, the total contents as well as the composition of phospholipids in either the dystrophic or the normal microsomes were not appreciably different, suggesting that phospholipids may not be responsible for the observed difference in the demethylation activity.

On the other hand, the activity of NADPH-cytochrome c reductase of the liver microsomes from dystrophic mouse was higher by about 30% or more as compared to the activity of the normal microsomes and the extents of difference in this activity were comparable to the extents of differences in the demethylation activity and lipid peroxidation. The higher activity of lipid peroxidation in the dystrophic microsome seems to be due to a higher activity of NADPH-cytochrome c reductase in view of the observation by Pederson et al. (1973) that a purified preparation of rat liver microsomal NADPH-cytochrome c reductase could catalyze the NADPH-dependent peroxidation of isolated microsomal lipid. These situations suggest that the higher demethylation activity in the dystrophic
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microsome may have resulted from the higher activity of NADPH-cytochrome c reductase. However, there still remains a question. For instance, when compared in terms of the specific activity, the activity of NADPH-cytochrome c reductase is much higher than that of the demethylation activity (cf. Figs. 3 and 7) and therefore it is doubtful whether the function of NADPH-cytochrome c reductase could be rate-limiting in the overall reaction of demethylation. In an independent experiment of the aminopyrine demethylation, we added a partially purified preparation of pig liver microsomal NADPH-cytochrome c reductase to the reaction system with the liver microsomes from the normal mouse, but the demethylation activity was not appreciably increased. Thus, the final conclusion must await further investigations.

At any rate, it is worth noting that the dystrophic mouse exhibits higher activities of the microsomal enzymes. We have also observed that the specific activities of NADH-cytochrome c reductase and glucose-6-phosphatase of liver microsomes from dystrophic mice were 13–17% higher than those of the normal microsomes (unpublished). These observations suggest that the progressive muscular dystrophy may involve abnormal features in not only muscle but also liver and other tissues. Further comparative studies on various metabolic activities in various tissues are desirable to obtain further insight into the biochemical disorder in the progressive muscular dystrophy.

Acknowledgment

I am indebted to Professor Goro Kikuchi, Department of Biochemistry, Tohoku University School of Medicine, for his valuable advice, and to Dr. Takeo Hosaka, Director of the Nishitaga National Sanatorium, for his encouragement and support throughout this work.

References


