Effect of pentobarbitone sodium on serum creatine kinase of normal and dystrophic hamsters

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Summary

1. The dose of pentobarbitone required for anaesthesia was significantly greater for dystrophic hamsters than for normal animals.

2. Serum creatine kinase activity was significantly higher in dystrophic than in normal hamsters.

3. Brain, heart and tibialis anterior muscle from dystrophic animals contained significantly less creatine kinase than the normal tissues.

4. Creatine kinase in normal and dystrophic sera, as in skeletal muscles, consisted of MM isoenzyme. Heart creatine kinase consisted of both MM and MB types and brain contained only the BB isoenzyme.

5. Pentobarbitone raised serum creatine kinase activity of normal and dystrophic hamsters to the same extent, elevation of enzyme activity being dependent on the amount of pentobarbitone injected.

6. The sera of pentobarbitone-treated normal and dystrophic hamsters contained only the MM isoenzyme.

Key words: creatine kinase, muscular dystrophy, pentobarbitone, tibialis anterior muscle.

Introduction

Creatine kinase (ATP-creatine N-phosphotransferase, EC 2.7.3.2) catalyses the reaction of adenosine diphosphate (ADP) with creatine phosphate to produce adenosine triphosphate (ATP) and creatine. The enzyme is a dimer of molecular weight of about 84,000 and is composed of M and B type subunits. Brain creatine kinase consists of BB isoenzyme. Skeletal muscle creatine kinase consists of MM isoenzyme and heart tissue contains MM and some MB hybrid enzyme (Burger, Richterich & Abei, 1964; Eppenberger, Dawson & Kaplan, 1967; Dawson, Eppenberger & Kaplan, 1965, 1967). An additional isoenzyme from mitochondria was reported by Jacobs, Heldt & Klingenberg (1964) and by Somer, Uotila, Konttinen & Saris (1974).

Elevated serum creatine kinase activity has been observed in muscular dystrophies of man (Okinaka, Kumagai, Ebashi, Sugita, Momoi, Toyokura & Fujie, 1961; Munsat, Baloh, Pearson & Fowler, 1973), hamster (Eppenberger, Nixon, Baker & Homburger, 1964) and chicken (Holliday, Asmundson & Julian, 1965; Wilson, Linkhart, Walker & Nieberg, 1973). Because skeletal muscle is rich in creatine kinase, it has been suggested that the increase in the enzyme activity in dystrophic serum is due to 'leakage' of this enzyme through defective muscle membranes. However, elevated serum creatine kinase has been observed under several other conditions, including administration of certain general anaesthetic agents (Innes & Strømme, 1973; Phornruptkul, Anuras, Koff, Seeff, Mahler & Zimmerman, 1974).

Although there is some evidence of a defective muscle membrane in hamster dystrophy (Sulakhe, Fedeleisova, McNamara & Dhall, 1971; Dhall, McNamara, Balasubramanian, Green-
law & Tucker, 1973; Wrogemann, Jacobson & Blanchaer, 1974; Dhalla, Singh, Lee, Anand, Bernatsky & Jasmin, 1975), it is not known if the dystrophic muscle membrane can control leakage of muscle creatine kinase into the serum. In the present study we have attempted to assess the 'leakiness' of the dystrophic muscle membrane by comparing the efflux of muscle creatine kinase in normal and dystrophic hamsters after the administration of pentobarbitone, a fast and centrally acting barbiturate commonly used for anaesthetizing laboratory animals.

Materials and methods

Normal (Coombehurst Golden Brown strain) and dystrophic (B10 14.6 strain) hamsters used in the present study were obtained from litters born and bred in the animal house at the Royal Postgraduate Medical School. Both sexes were used and all hamsters were 10 months old and weighed 100-120 g.

Hamsters were anaesthetized by intraperitoneal injection of sodium pentobarbitone (Veterinary Nembutal, Abbot Laboratories). The criteria used to define anaesthesia were loss of (a) consciousness, (b) eyelash reflex (when the eyelashes were stroked), and (c) limb-withdrawal reflex (when the toes were pinched). The following procedure was employed to anaesthetize hamsters for 1, 3 and 6 h. For any one period of anaesthesia, forty-eight normal or dystrophic hamsters were divided into six groups each consisting of eight animals. Hamsters destined to undergo 1, 3 and 6 h anaesthesia were injected with 0.03-0.08 ml (2.4-4.8 mg), 0.06-0.11 ml (3.6-6.6 mg) and 0.08-0.13 ml (4.8-7.8 mg) of pentobarbitone respectively. All animals in any one group were injected with the same amount of pentobarbitone. Animals recovering from anaesthesia within the periods 50-70, 160-190 and 340-370 min were picked out and were taken to have undergone 1, 3 and 6 h anaesthesia respectively. Serum creatine kinase activity was monitored after 1, 3, 7 and 11 days after a single dose of pentobarbitone given on day 0.

Creatine kinase was determined in serum from blood obtained by cardiac puncture. On day 0, this was done within 2-3 min after pentobarbitone injection. Subsequently, all hamsters were lightly anaesthetized with ether before cardiac puncture. The latter method was employed because blood samples could be obtained within 1–2 min and the animals were awake and walking around without undue stress by 3–4 min. Moreover, preliminary studies showed that this period of ether anaesthesia had no effect on serum creatine kinase activity. For tissue creatine kinase activity of normal and dystrophic hamsters, the brain, heart and tibialis anterior muscle were quickly removed, frozen in isopentane cooled in liquid nitrogen, freeze-dried and powdered with a pestle and mortar. Portions (5 mg) of the freeze-dried powder were extracted at 4°C with 1.0 ml of Tris/HCl buffer, pH 7.4, containing sucrose (1 mol/l) and dithiothreitol (500 μmol/l). Extraction was carried out for 1 h with continuous mechanical shaking. The homogenate was centrifuged at 4°C for 30 min at 15 000 g. As these tissues contained high activity of creatine kinase, each volume of the tissue extracts had to be diluted 50 or 100 times with Tris/HCl buffer containing sucrose and dithiothreitol before the activity could be measured. Serum and tissue creatine kinase activity was determined by using the Boehringer ultraviolet test 'optimized standard method'. Creatine kinase isoenzyme pattern was determined in serum and extracts of tibialis anterior muscle, heart and brain by the method of Somer & Konttinen (1972) after electrophoresis on cellulose acetate strips with glycine/NaOH buffer, pH 8.6, with glycine (50 mmol/l) and lithium chloride (30 mmol/l). Control strips were incubated with a reaction mixture containing all reagents except creatine phosphate. Electrophoresis was carried out in a Shandon apparatus at 4°C for 30 min and the applied voltage and current were 300 V and 19 mA respectively.

Results

The data given in Table 1 indicate that dystrophic hamsters required significantly more pentobarbitone than normal hamsters to be anaesthetized for 1, 3 and 6 h.

Table 2 shows creatine kinase activity in the serum, brain, heart and tibialis anterior muscle of normal and dystrophic hamsters. Whereas the serum enzyme activity was significantly elevated in dystrophic hamsters, their brain, heart and muscle enzyme activity was significantly less than those of normal animals. Electrophoretic separation of creatine kinase isoenzymes
Anaesthesia in dystrophic hamsters

Table 1. Doses of pentobarbitone required to induce anaesthesia for 1, 3 and 6 h in normal and dystrophic hamsters

Results shown are mean values ± SEM. Numbers in parentheses represent the number of animals used. *P values were calculated by Student’s *t*-test.

<table>
<thead>
<tr>
<th>Duration of anaesthesia (h)</th>
<th>Dose of pentobarbitone (mg)</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal hamster</td>
<td>Dystrophic hamster</td>
<td><em>P</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>2.61 ± 0.07 (20)</td>
<td>3.60 ± 0.18 (20)</td>
<td>&lt;0.001</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>3.90 ± 0.07 (20)</td>
<td>6.42 ± 0.06 (20)</td>
<td>&lt;0.001</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>5.70 ± 0.08 (20)</td>
<td>7.53 ± 0.07 (20)</td>
<td>&lt;0.001</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

Table 2. Creatine kinase activity in serum, brain, heart and tibialis anterior muscle of normal and dystrophic hamsters

Results shown are mean values ± SEM. Numbers in parentheses represent the number of animals used.

<table>
<thead>
<tr>
<th>Creatine kinase activity</th>
<th>Normal hamster</th>
<th>Dystrophic hamster</th>
<th><em>P</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum (i.u./l)</td>
<td>105 ± 3 (14)</td>
<td>1114 ± 18 (14)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Brain (i.u./g dry wt.)</td>
<td>3262 ± 40 (10)</td>
<td>2580 ± 78 (10)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Heart (i.u./g dry wt.)</td>
<td>3179 ± 91 (10)</td>
<td>1967 ± 78 (10)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Tibialis anterior muscle</td>
<td>15659 ± 1146 (10)</td>
<td>11924 ± 738 (10)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

showed the presence of only the MM isoenzyme in both the muscle and serum of normal and dystrophic hamsters. Heart extracts of normal and dystrophic hamsters contained both MM and MB isoenzymes whereas brain extracts showed only the BB type (Fig. 1a, b, c, d and e).

The effect of pentobarbitone on serum creatine kinase activity of normal and dystrophic hamsters is shown in Table 3. All pentobarbitone-treated hamsters showed significantly elevated serum creatine kinase activity on days 1 and 3. Moreover, the greater the dose of pentobarbitone given, the greater was the rise.

Table 3. Changes in serum creatine kinase activity of normal and dystrophic hamsters after a single dose of pentobarbitone given on day 0 to induce anaesthesia for 1, 3 and 6 h

Results shown are mean values ± SEM. Numbers in parentheses indicate number of animals in which creatine kinase activity was determined.

<table>
<thead>
<tr>
<th>Days on which enzyme activity was determined</th>
<th>Normal hamsters</th>
<th>Dystrophic hamsters</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 h</td>
<td>3 h</td>
</tr>
<tr>
<td>0</td>
<td>121 ± 7 (6)</td>
<td>124 ± 6 (6)</td>
</tr>
<tr>
<td>1</td>
<td>472 ± 18 (6)</td>
<td>662 ± 10 (6)</td>
</tr>
<tr>
<td>3</td>
<td>235 ± 16 (6)</td>
<td>379 ± 9 (6)</td>
</tr>
<tr>
<td>7</td>
<td>142 ± 8 (6)</td>
<td>163 ± 7 (6)</td>
</tr>
<tr>
<td>11</td>
<td>124 ± 6 (6)</td>
<td>128 ± 7 (6)</td>
</tr>
</tbody>
</table>
FIG. 1. Electrophoretic separation of MM, MB and BB isoenzymes of creatine kinase from extracts of tibialis anterior muscle, heart and brain of normal hamsters, and from sera of untreated and pentobarbitone-treated normal and dystrophic hamsters. (a) Normal tibialis anterior muscle; (b) normal heart; (c) normal brain; (d) normal serum; (e) normal serum 24 h after pentobarbitone treatment; (f) dystrophic serum; (g) dystrophic serum 24 h after pentobarbitone treatment.

FIG. 2. Changes from resting serum creatine kinase activity of normal (■) and dystrophic (□) hamsters over a period of 11 days after anaesthesia for 1, 3 and 6 h.

in the serum enzyme activity (Table 3). Consequently, enzyme activity was most elevated in hamsters which had undergone anaesthesia for 6 h. Although resting creatine kinase activity was higher in dystrophic serum, the increase in the serum enzyme observed after pentobarbitone treatment was not significantly different in normal and dystrophic hamsters (Fig. 2). Creatine kinase activity returned to resting values by day 11.

Serum samples from normal and dystrophic hamsters treated with pentobarbitone, like those from untreated animals, consisted of only the MM isoenzyme after electrophoresis on cellulose strip (Fig. 1e and g).

Discussion

The results of this study indicate that (a) for anaesthesia, dystrophic hamsters require significantly more pentobarbitone than normal hamsters, (b) creatine kinase activity in the brain, heart, tibialis anterior muscle and serum of normal and dystrophic hamsters is significantly different, (c) treatment with pentobarbitone raised serum creatine kinase activity of normal and dystrophic hamsters to the same extent, and (d) serum creatine kinase of pentobarbitone-treated and control hamsters consisted of the MM isoenzyme.

As with dystrophic hamsters, a larger dose of
pentobarbitone is also required to anaesthetize dystrophic mice of 129 ReJ and C57BL dy² strains (unpublished personal observations). The cause for this difference between normal and dystrophic animals is not known. It is quite likely that dystrophic animals with their extensive fat deposits bind and metabolize the highly lipid-soluble pentobarbitone in fat-cells faster than in brain cells. Consequently, because an optimum concentration is needed to be established in the brain cells for anaesthesia, a larger dose of pentobarbitone is required by dystrophic animals. Another possibility is that pentobarbitone stimulates the dystrophic liver microsomal enzyme to a greater extent, thus enhancing its own rapid breakdown.

Although elevated serum creatine kinase activity is a common clinical finding in humans and animals with muscular dystrophy (Okinaka et al., 1961; Eppenberger et al., 1964; Holliday et al., 1965; Munsat et al., 1973; Wilson et al., 1973), there are relatively few studies on dystrophic tissue creatine kinase (Hooton & Watts, 1965, 1966; Roy, 1974). Our results indicate that both normal and dystrophic muscle contain about five times more creatine kinase than heart and brain tissue. Moreover, dystrophic brain, heart and muscle contain less enzyme than the normal tissues. In the light of only MM isoenzyme being present in the dystrophic serum, our results support the hypothesis that increased serum creatine kinase activity in dystrophy is due to leakage of this enzyme from the muscle fibres. Because there is a cardiomyopathy in hamster dystrophy (Bajusz, Homburger, Baker & Opie, 1966) it is possible that some heart enzyme also leaks out into the serum. However, no MB isoenzyme was observed in the dystrophic serum.

Raised creatine kinase activities have been observed in dystrophic hamsters after starvation and hydrocortisone administration (Solymoss & Jasmin, 1975), and in normal humans after administration of general anaesthetic agents such as halothane, thiopentone and succinylcholine (Innes & Strømme, 1973; Porthophutkul et al., 1974) or muscle relaxant such as diazepam (Küster, 1972). The cause(s) of elevated serum creatine kinase in pentobarbitone-treated hamsters is not known. It is possible that this effect is due to depressed oxidative metabolism in all tissues as a result of rapid redistribution of pentobarbitone from the brain to other tissues, particularly during the termination of its central depressant action (Goodman & Gilman, 1975). However, in the present study, electrophoretic separation of creatine kinase isoenzymes clearly showed that the elevated serum creatine kinase is derived from skeletal muscle. Consequently, in view of the large amount of creatine kinase in skeletal muscle and the serum isoenzyme being of the MM type, a more likely event is the leakage of muscle enzyme into the serum through either a direct or indirect effect of pentobarbitone on the muscle membrane.

The mechanism by which pentobarbitone induces release of creatine kinase from skeletal muscle is not known. According to Seeman (1972, 1974) and Edström & Larsson (1974) pentobarbitone, like other fast-acting barbiturates, acts by blocking the depolarization of nerve cells and by inhibiting fast axoplasmic transport. Because the functions of a skeletal muscle depend on its nerve supply, such effects on the motor nervous system could induce increased permeability of the muscle membrane. Moreover, there is some evidence that pentobarbitone may also render the post-synaptic muscle membrane insensitive to the depolarizing effect of acetylcholine (Thesleff, 1956). Another property of pentobarbitone is its high solubility in lipids of plasma membranes. Apparently, this high affinity of pentobarbitone for membrane lipids causes an increase in permeability to K⁺ (Goodman & Gilman, 1975). Changes in the ionic fluxes across the muscle membrane could affect the Na⁺-K⁺-dependent adenosine triphosphatase activities in such a way that there is greater transport of creatine kinase to the outside of the muscle fibre.

In spite of increased Na⁺-K⁺-adenosine triphosphatase activities in the sarcolemma of dystrophic hamster muscle (Wrogemann et al., 1974) the ‘leakage’ of creatine kinase after pentobarbitone anaesthesia was comparable in normal and dystrophic animals. This could indicate that pentobarbitone is acting either directly or indirectly at regions of the sarcolemma which are not affected by dystrophy, in which case it could be argued that dystrophic alterations occur only at restricted sites of the sarcolemma. On the other hand, if the entire muscle membrane is affected it would appear that in dystrophy and after pentobarbitone treatment creatine kinase ‘leaks’ out of the muscle fibre by different mechanisms.
Acknowledgments

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References


