Brain metabolism is abnormal in the \textit{mdx} model of Duchenne muscular dystrophy

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Summary

Duchenne muscular dystrophy (DMD) is an X-linked genetic disorder primarily affecting young boys, often causing mental retardation in addition to the well-known progressive muscular weakness. Normal dystrophin expression is lacking in skeletal muscle and the CNS of both DMD children and the \textit{mdx} mouse model. To date, \textsuperscript{31}P-magnetic resonance spectroscopy (MRS) has shown in vivo several abnormalities within skeletal muscle of \textit{mdx} mice and DMD boys. In this study, we determined whether similar abnormalities occur in \textit{mdx} brain in vivo by using \textsuperscript{31}P-MRS in addition to metabolite and enzyme analysis to study cerebral metabolism. An increased inorganic phosphate (Pi)/phosphocreatine (PCr) and pH was found in vivo for \textit{mdx} brain compared with controls, and biochemical analysis showed a reduction in total creatine, an increased extracellular and decreased intracellular volume in \textit{mdx} brain. No differences were found in any glycolytic or mitochondrial maximal enzyme activities. These changes are discussed with respect to the biochemical changes found in muscle from DMD patients and \textit{mdx} mice. It is proposed that these biochemical changes may be a factor in the reduced cognitive capacity of \textit{mdx} mice and some DMD children.

Keywords: \textit{mdx}; \textsuperscript{31}P-MRS; brain; Duchenne muscular dystrophy; retardation

Abbreviations: DMD = Duchenne muscular dystrophy; ECV = extracellular volume; ICV = intracellular volume; IPD = interpulse delay; MRS = magnetic resonance spectroscopy; PCr = phosphocreatine; Pi = inorganic phosphate

Introduction

Duchenne muscular dystrophy is an X-linked recessive disorder resulting in progressive degeneration of the muscle and affecting about one in 3500 male children (Emery, 1988). Although DMD mRNA is most abundant in skeletal and cardiac muscle (Monaco et al., 1986; Hoffman et al., 1987; Nudel et al., 1988) significant levels are found in brain tissue (Chamberlain et al., 1988; Nudel et al., 1988). Patients suffering from DMD fail to express a functional dystrophin protein either in muscle or brain (Hoffman et al., 1987) and their IQ distribution is 1 SD lower than that of control children (Karagan, 1979). It follows that the impaired mental function may be attributed to a lack of dystrophin in the CNS (Hoffman et al., 1987).

The \textit{mdx} mouse has a genetic defect in the homologous region of the genome to the human DMD gene (Bulfield et al., 1984) and similarly lacks the dystrophin protein both in muscle and brain (Hoffman et al., 1988). In addition, similarities in metabolic changes which occur in the muscles of DMD boys and the \textit{mdx} mouse are well documented (Newman et al., 1982; Younkin et al., 1987; Dunn et al., 1991; Kemp et al., 1993). These genetic and biochemical criteria make the \textit{mdx} mouse a good model for DMD. There is evidence that \textit{mdx} mice also show a mental abnormality consistent with retardation (Muntoni et al., 1991), which may make the \textit{mdx} mouse a good model for studying neuronal changes related to the cognitive dysfunction seen in DMD.

Recent work from our laboratory shows that DMD brain has \textsuperscript{31}P-MRS abnormalities \textit{in vivo} similar to those found in DMD muscle (Tracey et al., 1995), which suggests that the cognitive dysfunction may be related to an altered metabolism. It has, therefore, been shown clearly that dystrophic muscle and brain, whether \textit{mdx} or DMD, have similar abnormalities, as determined by \textsuperscript{31}P-MRS. To confirm the suitability of the \textit{mdx} mouse as a model for the brain abnormalities associated with DMD, we hypothesize that similar changes to those already documented in tissues lacking dystrophin (an increased Pi, pH and reduced total creatine), will be found in \textit{mdx} brain.
Metabolites and enzymes

After MRS analysis, the mice were cervically dislocated and the brains rapidly frozen in liquid nitrogen. Half the brain was extracted into ice-cold buffer (100 mM triethanolamine, 8 mM MgCl₂, 1 mM EDTA, pH 7.4) using a ground glass homogenizer on ice and sonicated for 10×2 s. Mitochondrial enzymes cytochrome oxidase, citrate synthase and succinate cytochrome reductase were assayed as described (Hayasaka et al., 1989). The remaining enzymes were assayed as in Bergmeyer (1974).

Methods

31P-MRS

Mice (mdx and the control strain C57Bl/10ScSn) were housed on a 12 h light/dark cycle and fed ad libitum. The mice were aged 180–240 days and were of mixed sex. Mice were anaesthetized with an intraperitoneal injection of midazolam (0.0167 mg kg⁻¹), fentanyl citrate (0.53 mg kg⁻¹) and fluanisone (0.0167 mg kg⁻¹) diluted with water for injection. This maintained anaesthesia for 2–2.5 h. Scaps were removed to prevent signal contamination from scalp muscle and a piece of insulating Teflon tape was placed over the exposed skull.

A single tuned 31P surface coil with a single turn of 0.5 mm copper wire of 1 cm diameter was positioned over the skull and the mouse secured to a Plexiglass plate. Optimum magnetic field homogeneity was established by maximizing the free induction decay arising from protons. The line-widths at half height were in the range 30–45 Hz. Spectra were obtained at the 31P frequency of 162 MHz using a 9.4 T Oxford magnet with a Bruker AM400 spectrometer, a 2-s interpulse delay (IPD) in blocks of 1024 scans, pulse width of 6 μs (which approximates the Ernst angle (Ernst and Anderson, 1966) and sweep width of 15 000. Saturation factors were obtained by comparing spectra at 2 s IPD with those collected with an IPD of 20 s. The longitudinal relaxation times (T₁) were determined using a 2x2x2 composite 180° exorcyle inversion recovery method (Vold et al., 1968) followed by fitting the data to a single exponential. The spectra were profiled to remove the broad bone peak and the Bruker Gliffitting program was used to calculate peak areas representing the following metabolites: PCr, ATP, phosphomonoesters and Pᵢ. All ratios are reported after correcting for saturation. The pH is determined using Equation 1 where S is the chemical shift between Pᵢ and PCr (Gadian et al., 1982).

\[
pH = 6.75 + \log[(S-3.27)/(5.69-S)]
\]

Free adenosine diphosphate determination

The free ADP can be calculated using Equation 3.

\[
ADP_{free} = (Cr_{internal} \times ATP_{internal})/(PCr_{internal} \times [H^+] \times K_{eq}) \quad (3)
\]

where

\[
K_{eq} = 1.66e^9(M) \quad (Veech et al., 1979), [H^+] = 10^{-pH}M, \quad ATP_{internal} = (ATP_{total}/ICV), \quad PCr_{internal} = (ATP_{total}/ICV) \times (PCr/ATP)_{MRS} \quad \text{and} \quad Cr_{internal} = (Cr_{total}/ICV) \times \text{[minus]} PCr_{internal}.
\]

Significant differences between mdx and control means for all measures taken were determined using standard t tests. Results were considered significant if P < 0.05*.

Results

Typical 31P-MRS brain spectra from control and mdx mice are shown in Fig. 1. The saturation factors for each MRS visible metabolite between controls and mdx (data not shown)
Abnormalities in dystrophic brain

Table 2 \(^{31}\)P-MRS metabolite ratios from mdx and control brain

<table>
<thead>
<tr>
<th>Ratio</th>
<th>Control</th>
<th>mdx</th>
</tr>
</thead>
<tbody>
<tr>
<td>(P/\text{PCr} )</td>
<td>0.73±0.07</td>
<td>0.93±0.12*</td>
</tr>
<tr>
<td>(P_\text{Pi}/\text{ATP} )</td>
<td>1.78±0.34</td>
<td>2.17±0.29</td>
</tr>
<tr>
<td>(\text{PCr}/\text{ATP} )</td>
<td>2.43±0.28</td>
<td>2.33±0.13</td>
</tr>
<tr>
<td>(\text{PME}/\text{ATP} )</td>
<td>2.57±0.48</td>
<td>2.30±0.31</td>
</tr>
<tr>
<td>pH</td>
<td>7.02±0.07</td>
<td>7.08±0.06*</td>
</tr>
</tbody>
</table>

Values are presented as mean±SD; \(n = 6,10\) (pH only). Collection parameters were described in Methods. IPD = 2 s, number of scans = 1024, spectral width = 15 K, pulse width = 6 \(\mu\)s using a 9.4 T magnet. Spectra were corrected for saturation. PME = phosphomonoesters. \(P < 0.05\).

Table 3 Total ATP, creatine, free ADP, dry/wet ratios, ICV and ECV in mdx and control brain

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Control</th>
<th>mdx</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\text{ATP} (\mu\text{mol g wet wt}^{-1}))</td>
<td>2.24±0.64 (12)</td>
<td>1.86±0.79 (12)</td>
</tr>
<tr>
<td>(\text{Creatine_{total}} (\mu\text{mol g wet wt}^{-1}))</td>
<td>13.14±2.94 (12)</td>
<td>8.79±2.52* (12)</td>
</tr>
<tr>
<td>Dry/wet weight</td>
<td>0.22±0.004 (6)</td>
<td>0.22±0.004 (6)</td>
</tr>
<tr>
<td>ICV (ml g wet wt(^{-1}))</td>
<td>0.64±0.02 (6)</td>
<td>0.59±0.03* (6)</td>
</tr>
<tr>
<td>ECV (ml g wet wt(^{-1}))</td>
<td>0.15±0.02 (6)</td>
<td>0.20±0.03* (6)</td>
</tr>
<tr>
<td>ADP free (mM)</td>
<td>0.030±0.010 (6)</td>
<td>0.026±0.006 (5)</td>
</tr>
</tbody>
</table>

Values are presented as mean±SD \((n)\). Metabolites, ICV and ECV were determined as described in Methods. \(^*P < 0.05\).

The \(P/\text{PCr} \) ratio and pH were significantly elevated in mdx mice compared with controls (Table 2). Application of a Bonferroni correction to the \(^{31}\)P-MRS data to correct for multiple tests removes the significance of the pH result \((P = 0.10 \text{ after correction})\), but maintains the significance of the \(P/\text{PCr} \) finding \((P = 0.0045 \text{ after correction}, P = 0.0009 \text{ before correction})\). As we had prior expectations of pH being increased in mdx brain (because of the data from dystrophic muscle), then application of the Bonferroni correction is not necessary and standard \(t\) tests can be used. We are, therefore, confident in our finding that pH is significantly elevated in mdx brain compared with control. Control values for total creatine and ATP (Table 3) agree well with literature values reported for rat brain (Ponten et al., 1973). There was no significant difference in the ATP content between control and mdx brain, but the mdx brain had a significantly decreased total creatine (Table 3). There was no difference in the calculated free ADP concentrations (Table 3).

The ECV of mdx brain was significantly increased compared with the controls (and correspondingly the ICV was significantly decreased in mdx brain), although the dry weight/wet weight ratio was unchanged (Table 3). The ECV measurements agree well with literature values (Fenstermacher et al., 1970).

Table 4 contains the results from the enzyme analysis in control and mdx brain. No significant differences were found.
be a suitable model for the retardation seen in human DMD. This suggests that the dysfunction with respect to learning and memory skills might be expected that neurons will be primarily affected from different promoters in healthy muscle, neural and glial cells, with a high expression in neural cells and little to no in astro-glial cells derived from adult mouse brain (Chamberlain et al., 1988; Chelly et al., 1990). Therefore, only neurons express dystrophin, and in dystrophic brain it is similar to the findings in both DMD brain, muscle and mdx resting muscle (Newman et al., 1982; Younkin et al., 1987; Dunn et al., 1991; Kemp et al., 1993). This suggests, therefore, that the absence of dystrophin causes similar changes and the work reported here does not distinguish between neural and glial cells, which means that if only neurons are affected, then the overall metabolic affect on the brain will be smaller and more difficult to detect compared with muscle. This was found to be the case.

One possible explanation for the increased P_i/PCr could be that the mitochondria are impaired, which has been suggested to occur in DMD muscle (Wrogeman and Pena, 1976). It is known that patients suffering mitochondrial encephalopathies often have mental abnormalities associated with an increased P_i/ATP (Hayes et al., 1985; Barbiroli et al., 1993). However, it is not known how a lack of dystrophin could affect the mitochondria, and based upon other results from our study, we do not believe this explains our observations. The ATP content for mdx brain is normal, indicative of healthy mitochondrial function, and contrasts mdx muscle which has a decreased ATP content (Dunn et al., 1991). Further evidence against a mitochondrial impairment in dystrophic brain is that there is no difference between [ADP]_free in mdx brain and control brain (Table 3). As [ADP]_free is thought to be a regulator of mitochondrial activity we suggest that the mitochondrial activity in mdx brain is normal despite low total creatine. This is supported by the enzyme data (Table 4), which show no differences in the maximal activities of mitochondrial or glycolytic enzymes studied.

Support for the in vivo finding that P_i/PCr is increased comes from the in vitro determination of total creatine. This is decreased in mdx brain compared with control, and despite a small but significant rise in pH within mdx brain, which would itself affect the creatine kinase equilibrium (Equation 3), the lower total creatine could partly account for the increased P_i/PCr (Table 3) found in vivo. A decreased creatine level has also been found in older mdx mice (Dunn et al., 1991). A possible explanation for the in vivo and in vitro observed changes in mdx brain is that normal development might be affected in dystrophic brain. Total creatine is thought to increase in developing brain (Cerdan et al., 1985; Toft et al., 1994). In younger mdx mice (aged 60–150 days) we found no significant differences in the creatine level compared with control (33.7±4.9 and 37.6±8.1 μmol g dry wt⁻¹, respectively) (Tracey et al., 1994). However, as the mice mature to the age group used for this study (180–240 days), brain creatine content increases in control mice, as expected for normal development, whereas in the mdx mice it does not.

### Table 4 Maximal enzyme activities from control and mdx brains

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Activity in controls (μmol min⁻¹ g wet wt⁻¹)±SD</th>
<th>Activity in mdx (μmol min⁻¹ g wet wt⁻¹)±SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Succinate dehydrogenase</td>
<td>1.95±0.44</td>
<td>1.94±0.57</td>
</tr>
<tr>
<td>G6P dehydrogenase</td>
<td>1.48±0.34</td>
<td>1.42±0.31</td>
</tr>
<tr>
<td>Citrate synthase</td>
<td>25.4±1.37</td>
<td>26.23±3.85</td>
</tr>
<tr>
<td>Lactate dehydrogenase</td>
<td>80.85±8.76</td>
<td>95.28±4.4</td>
</tr>
<tr>
<td>Hexokinase</td>
<td>5.21±0.69</td>
<td>5.09±1.10</td>
</tr>
<tr>
<td>Pyruvate kinase</td>
<td>175.08±14.6</td>
<td>184.08±27.9</td>
</tr>
<tr>
<td>Creatine kinase</td>
<td>403.46±131</td>
<td>395.40±123</td>
</tr>
<tr>
<td>Cytochrome c oxidase</td>
<td>5.35±1.98</td>
<td>4.10±2.37</td>
</tr>
<tr>
<td>Succinate cytochrome c reductase</td>
<td>1.28±0.15</td>
<td>1.06±0.35</td>
</tr>
</tbody>
</table>

Values are presented as mean±SD; n = 10. Brain tissue was extracted into ice-cold buffer by homogenization (100 mM triethanolamine, 8 mM MgCl₂, 1 mM EDTA, pH 7.4) and enzyme activities determined (see Methods). P ≥ 0.05 for all data—not significant.

### Discussion

Boys with DMD have varied degrees of intellectual impairment; their IQ distribution is 1 SD lower than that of control children (Karagan, 1979). Despite great efforts, the underlying cause of the mental impairment is unknown and there is no conclusive evidence of selective impairments that are developmentally consistent and not secondary to overall impairment in DMD. More importantly, no correlation has been found between the extent or location of DNA deletions and IQ (Hodgson et al., 1992), which makes interpretation of metabolic abnormalities associated with a loss of dystrophin difficult. There is increasing evidence that the lack of dystrophin in the brains of DMD children may contribute to the mental retardation often seen in this condition (Hoffman et al., 1988; Nudel et al., 1988; Chelly et al., 1990). Lower yet significant levels of a near identical muscle type dystrophin (differing only in the first few N-terminal amino acids) is normally expressed in brain tissue (Nudel et al., 1989). However, this dystrophin is not found in either DMD or mdx mouse brain (Chamberlain et al., 1988; Hoffman et al., 1988). Results have shown that the gene is transcribed from different promoters in healthy muscle, neural and glial cells, with a high expression in neural cells and little to none in astro-glial cells derived from adult mouse brain (Chamberlain et al., 1988; Chelly et al., 1990). Therefore, only neurons express dystrophin, and in dystrophic brain it might be expected that neurons will be primarily affected due to a lack of dystrophin. This suggests a possible biochemical basis for the mental retardation seen in this condition (Karagan, 1979). Indeed, recent evidence also suggests that the mdx mouse suffers from cognitive dysfunction with respect to learning and memory skills (Muntoni et al., 1991). This suggests that the mdx mouse may be a suitable model for the retardation seen in human DMD.

We have recently reported the first in vivo biochemical abnormality associated with DMD brain (Tracey et al., 1995) (increased P_i ratios) and in this study we extended our observations to the mdx mouse model of DMD. The in vivo 31P-MRS analysis shows a significantly increased P_i/PCr and pH for mdx brain compared with controls (Table 2), which is similar to the findings in both DMD brain, muscle and mdx resting muscle (Newman et al., 1982; Younkin et al., 1987; Dunn et al., 1991; Kemp et al., 1993). This suggests, therefore, that the acceptance of dystrophin causes similar energetic changes in both muscle and the CNS. It is not known how a lack of dystrophin in the CNS causes these changes and the work reported here does not distinguish between neural and glial cells, which means that if only neurons are affected, then the overall metabolic affect on the brain will be smaller and more difficult to detect compared with muscle. This was found to be the case.
not, and consequently they have a significantly decreased creatine compared with controls (Table 3). Similar age-related changes in the creatine content have been found in mdx muscle; no reduction in creatine relative to controls in 120–150 day old mice (Dunn et al., 1993a), but a significant decrease in muscle of 150–200-day-old mdx mice (Dunn et al., 1991).

The increased pH in mdx brain is also consistent with an abnormal developmental pattern because brain pH is lower in adults compared with infants, suggesting that the pH declines as the brain reaches maturity (Hope et al., 1984; Bottomley et al., 1986). Further work is needed to confirm whether developmental abnormalities do exist in mdx brain and if they could account for the retardation associated with this disease. However, it should be remembered that many of the abnormalities reported in this study, and in other studies on DMD brain, are not specific for DMD. In order to assess whether the changes found in this study are specific for a dystrophin gene defect, 31P-MRS studies of other mutations associated with retarded mental development should be considered. The results of this study simply point to a retarded development as one possible explanation for the abnormalities found in mdx mice, but further work is needed to relate these changes specifically to a lack of dystrophin.

The evidence on whether extensive neuronal necrosis occurs in DMD brain is contradictory (Dubowitz and Crome, 1969; Hovstad et al., 1976). Based upon our results of mdx brain, it seems unlikely that extensive necrosis is occurring in dystrophic brain because we find normal ATP levels and maximal enzyme activities (Tables 3 and 4) compared with controls. Other work reported from our laboratory, using 1H-MRS to measure the in vivo and in vitro N-acetyl aspartate content (a metabolic marker for neurons), shows that little to no neuronal necrosis occurs in dystrophic brain (Tracey et al., 1996). For this study, we determined ICV and ECV in mdx mouse brain. Necrosis should result in an increase in ECV as CSF replaces necrosed cells. We found a significant but small increase in ECV for mdx brain compared with control (with corresponding decreased ICV) (Table 3), suggesting that some necrosis may have occurred. In contrast, no differences in ICV or ECV occur in mdx muscle, despite the dry weight/wet weight ratio being significantly decreased (Dunn et al., 1993b). An alternative explanation for an increased ECV in mdx brain relates to abnormal development because in normal brain the extracellular space decreases during foetal development and postpartum maturation (Selzer et al., 1972; Rapoport, 1976). A high ECV was maintained in mdx brain compared with controls which is further evidence of abnormal development in dystrophic brain.

In conclusion, we have detected metabolic abnormalities in brain tissue of the mdx animal model of DMD in vivo using 31P-MRS and in vitro using biochemical measurements on wet tissue. The low IQ distribution of DMD boys and the cognitive deficits found in mdx mice may relate to this altered CNS metabolism. Dystrophic muscle displays some of these metabolic changes (but more severely), a more severe phenotype and lacks dystrophin. This raises the possibility that the cause of these metabolic changes is identical in both muscle and brain. It is not known how a lack of dystrophin contributes to these in vivo changes but further work on brain of dystrophic individuals may help elucidate the role of dystrophin in the CNS.

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References


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