Effect of propylthiouracil-induced hypothyroidism on the onset of skeletal muscle necrosis in dystrophin-deficient mdx mice

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ABSTRACT

1. Duchenne and Becker muscular dystrophies are X-linked disorders caused by defects in muscle dystrophin. The mdx mouse is an animal model for Duchenne muscular dystrophy which has a point mutation in the dystrophin gene, resulting in little (<3%) or no expression of dystrophin in muscle. Mdx mice show a characteristic pattern of muscle necrosis and regeneration. Muscles are normal until the third postnatal week when widespread necrosis commences. This is followed by muscle regeneration, with the persistence of centrally nucleated fibres.

2. This work has examined the hypothesis that the onset of this muscle necrosis is associated with postnatal maturation of the thyroid endocrine system and that pharmacological inhibition of thyroid hormone synthesis delays the onset of muscle necrosis.

3. Serum T4 and T3 concentrations of mice were found to rise immediately before the onset of muscle necrosis in the mdx mouse, and induction of hypothyroidism by treatment of animals with propylthiouracil was found to delay the onset of muscle necrosis.

4. The results provide the first demonstration of experimental delay of muscle necrosis by manipulation of the endocrine system in muscle lacking dystrophin, and provide a novel insight into the way in which a lack of dystrophin interacts with postnatal development to precipitate muscle necrosis in the mdx mouse.

INTRODUCTION

The mdx mouse is an animal model for Duchenne muscular dystrophy. The mouse has a point mutation in the dystrophin gene, resulting in little (<3%) or no expression of dystrophin in muscle [1]. However, although the protein is absent from birth, mdx mice show a characteristic pattern of muscle necrosis and regeneration [2]. Muscles are normal until the third postnatal week when a phase of widespread necrosis commences. This is followed by muscle fibre regeneration, with the persistence of centrally nucleated fibres indicating that fibres have undergone a cycle of necrosis and regeneration. Necrosis and regeneration continue at a low level for up to 6 months [2]. Previous data from our group indicate that serum creatine kinase (CK) activities in the mdx mouse are normal at 14 days of age but rise rapidly in the third postnatal week of life [3], resulting in an 8-10-fold elevation of serum CK in mdx mice at 21 days of age compared with control mice. This is associated with the

Key words: creatine kinase, development, Duchenne muscular dystrophy, thyroid.
Abbreviations: CK, creatine kinase; PTU, propylthiouracil.
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necrosis of fibres which becomes apparent from 21 days of age [2].

This study has examined the possibility that the acute onset of muscle degeneration in the mdx mouse is associated with postnatal development of the thyroid endocrine system. Thyroid development in mice has not been extensively studied but development in rats occurs after birth with an acute rise in both circulating \( T_3 \) and \( T_4 \) concentrations over the first 3 weeks of life [4]. This increase in thyroid hormone concentrations mediates many maturational changes in developing muscle [5,6].

The purpose of this study was to determine total serum \( 3,3',5\)-tri-iodo-L-thyronine (\( T_3 \)) and \( 3,5,3',5\)-tetra-iodo-L-thyroxine (L-thyroxine, \( T_4 \)) concentrations in both control C57Bl/10 and mutant mdx mice of differing ages to examine the possibility of a temporal relationship between the onset of skeletal muscle necrosis in the mdx mouse and maturation of the thyroid endocrine system. In order to determine whether the onset of muscle necrosis was precipitated by the postnatal development of thyroid hormone production, animals were made hypothyroid by treatment with propylthiouracil (PTU) from birth.

**METHODS**

**Postnatal changes in serum \( T_4 \) and \( T_3 \) concentrations**

Control and mutant mdx mice who were 6, 9, 12, 15, 21, 28 and 40 days old were given an overdose of sodium pentobarbitone. Blood was removed from the abdominal aorta and centrifuged at +4 °C. Serum was analysed for total \( T_3 \) and \( T_4 \) concentration by RIA (Amerlex M, Ortho Clinical Diagnostics, A mersham, Bucks, U.K.).

**Effect of hypothyroidism on the onset of muscle necrosis**

PTU (0.5 mg/ml) was added to the drinking water of cages containing breeding pairs of mdx and control C57Bl/10 mice and newborn mdx and control mice from the time of birth of the litter. Control animals were not given PTU. At either 21 or 28 days old, mice were given an overdose of sodium pentobarbitone, blood was removed from the abdominal aorta and centrifuged at +4 °C. Serum was analysed for CK activity as previously described [3] and for total \( T_3 \) and \( T_4 \) concentration by RIA (Amerlex M, Ortho Clinical Diagnostics, Amersham, Bucks, U.K.). The prevalence of necrotic fibres, active regeneration (myotubes) and centrally nucleated fibres (indicative of previous regeneration) was determined by morphometric techniques on 6-\( \mu \)m cryostat sections of gastrocnemius/soleus or quadriceps muscles (21-day-old mice) and 6-\( \mu \)m paraffin sections of limb and lumbar muscles (28-day-old mice), stained with haematoxylin and eosin [7]. The proportion of necrotic, actively regenerating and centrally nucleated fibres in sections stained with haematoxylin and eosin was quantified by point counting at \( \times 100 \) magnification using a square lattice grid with 100 intersections used as points. A necrotic fibre was defined as an increase or loss of normal cytoplasmic eosinophilia, usually accompanied by swelling of the fibre, an inflammatory infiltrate and sometimes dystrophic calcification. Small regenerating fibres were defined as basophilic fibres in which the nucleus occupied more than one-third of the fibre diameter. Centrally nucleated fibres showed nuclei occupying less than one-third of the diameter. A total of 175-500 points were counted per cryostat section and 600-2485 points per group of paraffin sections, where the smaller number of points counted corresponded to the smaller muscles. Muscle fibre diameter was measured on cryostat sections of quadriceps using a Seescan semi-automatic image analysis system at a magnification of approximately \( \times 100 \). Small, basophilic (regenerating) fibres were not included in these data. Muscle sections were assessed 'blind', without knowledge of the experimental group allocated.

In a separate experiment, breeding pairs of mdx mice were treated with PTU from 3 weeks before mating, allowed to breed and litters obtained in which the offspring had been exposed to PTU in utero. These litters were killed at 28 days old and blood and muscle were removed. Serum was analysed for total \( T_3 \) and \( T_4 \) concentrations and CK activity and muscles were analysed by morphometric techniques as described above.

**RESULTS**

**Postnatal changes in serum \( T_3 \) and \( T_4 \) concentrations**

No significant differences were seen in either serum total \( T_3 \) or \( T_4 \) levels between mdx and control mice and hence data were pooled at each age studied. Serum concentrations of both \( T_3 \) and \( T_4 \) were low at birth but rose rapidly with increasing age. Serum \( T_3 \) concentrations peaked at 12 days of age. \( T_4 \) concentrations followed a similar pattern although they appeared to peak later than \( T_3 \). Both \( T_3 \) and \( T_4 \) serum concentrations fell by 40 days of age (Figure 1).

**Effect of hypothyroidism on onset of muscle necrosis**

PTU treatment had a dramatic effect on morphological features of muscle from mdx mice. Treatment with PTU effectively prevented necrosis in the muscles of 21-day-old mdx mice (Table 1 and Figure 2). At this age, a mean of 7.4% of fibres in the untreated mdx mice were either
Regulation of muscle necrosis in mdx mice

Figure 1 Age-related changes in total serum concentration of (a) T₃ and (b) T₄ in C57Bl/10 mice
Results are expressed as means ± S.E.M. n = 8–30 mice per group. No significant differences were seen in either T₃ or T₄ levels between mdx and control mice and hence data were pooled.

Table 1 Effect of PTU treatment of mdx mice on the proportion of the muscle cross-sectional area showing normal fibres, necrosis, active regeneration (myotubes) or centrally nucleated fibres
Results are expressed as means ± S.E.M. *P < 0.05, **P < 0.01, values significantly different to untreated mice using Student’s unpaired t-test. n = 5–8 mice per group. PTU-treated mice (1), animals treated with PTU from birth; PTU-treated mice (2), animals exposed to PTU in utero and from birth; see Methods section for details of treatment protocols.

<table>
<thead>
<tr>
<th></th>
<th>Normal (%)</th>
<th>Necrosis (%)</th>
<th>Active regeneration (%)</th>
<th>Central nucleation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>21-day-old mice</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untreated mice</td>
<td>92.5 ± 7.1</td>
<td>5.4 ± 7.2</td>
<td>2.0 ± 3.1</td>
<td>0</td>
</tr>
<tr>
<td>PTU-treated mice</td>
<td>99.4 ± 0.7*</td>
<td>0.5 ± 0.7*</td>
<td>0.02 ± 0.06</td>
<td>0</td>
</tr>
<tr>
<td>28-day-old mice</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untreated mice</td>
<td>40.3 ± 10.8</td>
<td>5.3 ± 1.1</td>
<td>2.0 ± 0.3</td>
<td>52.4 ± 10.1</td>
</tr>
<tr>
<td>PTU-treated mice (1)</td>
<td>79.6 ± 12.3**</td>
<td>3.4 ± 1.9</td>
<td>1.2 ± 0.8**</td>
<td>15.9 ± 10.5**</td>
</tr>
<tr>
<td>PTU-treated mice (2)</td>
<td>91.6 ± 6.0**</td>
<td>4.3 ± 3.9</td>
<td>1.2 ± 1.2**</td>
<td>2.8 ± 3.6**</td>
</tr>
</tbody>
</table>

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Figure 2 Haematoxylin/eosin-stained cryostat sections demonstrating the histological appearance of quadriceps muscles from 21-day-old mdx mice without (a) and with (b) PTU treatment
(Magnification × 150.) Focal necrotic changes occurred in a mean of 5.4% of the fibres in untreated mice and 0.5% in the PTU-treated animals (Table 1). The figure illustrates the nature of the degenerative changes rather than the extent of the necrosis.
necrotic or actively regenerating (i.e. had previously undergone degeneration), compared with 0.5% in the PTU-treated mdx mouse. By 28 days, consider able necrosis and regeneration had occurred in muscles of untreated mdx mice, so that only approximately 40% of fibres retained normal morphology, with 7.3% showing necrosis or active regeneration, and 52.4% showing central nucleation, indicative of previous degeneration (Table 1). PTU treatment of mdx mice for 28 days from birth significantly reduced the number of fibres which were either necrotic or in the process of active regeneration to 4.6% (P < 0.05), and substantially decreased the number of centrally nucleated fibres (P < 0.01). This effect was greater in the animals which had been treated both in utero and postnatally with PTU (Table 1, Figure 2), where only a mean of 8.3% of fibres showed necrosis or evidence of previous regeneration (i.e. 4.3% necrotic, 1.2% actively regenerating and 2.8% centrally nucleated). There was no evidence of necrosis, active regeneration or centrally nucleated fibres in muscles of control mice treated with PTU for either 21 or 28 days. At 21 days old, untreated mdx mice showed the characteristic 10-fold elevation in serum CK activities. This was not seen in the PTU-treated mdx mice (Figure 3). There was no significant reduction in serum CK activity in mdx mice treated with PTU for 28 days compared with untreated mdx mice (Figure 3).

Serum T₃ concentrations were significantly decreased by PTU (Table 2). No decrease in serum T₄ concentrations was observed (Table 2). PTU treatment significantly reduced the body weight of both control and mdx mice at 21 days of age, although there was no significant reduction in muscle fibre diameters at that age (Table 3). By 28 days, PTU-treated control and mdx mice showed greater relative deficits in body weight than mice treated for 21 days, accompanied by significant reductions in muscle fibre diameters (Table 3).

TABLE 2  Effect of PTU treatment on serum total T₃ and T₄ concentrations in mdx and control mice

<table>
<thead>
<tr>
<th></th>
<th>T₃ concn. (nM)</th>
<th>T₄ concn. (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>21-day-old mice</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control mice - PTU</td>
<td>0.98 ± 0.13</td>
<td>58.8 ± 7.6</td>
</tr>
<tr>
<td>Control mice + PTU</td>
<td>0.86 ± 0.08</td>
<td>113.1 ± 1.2**</td>
</tr>
<tr>
<td>mdx mice - PTU</td>
<td>0.76 ± 0.08</td>
<td>49.6 ± 2.5</td>
</tr>
<tr>
<td>mdx mice + PTU</td>
<td>1.25 ± 0.21</td>
<td>12.9 ± 1.8**</td>
</tr>
<tr>
<td>28-day-old mice</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control mice - PTU</td>
<td>1.41 ± 0.24</td>
<td>55.7 ± 6.6</td>
</tr>
<tr>
<td>Control mice + PTU</td>
<td>1.14 ± 0.23</td>
<td>21.8 ± 3.6**</td>
</tr>
<tr>
<td>mdx mice - PTU</td>
<td>0.87 ± 0.16</td>
<td>48.6 ± 3.4</td>
</tr>
<tr>
<td>mdx mice + PTU</td>
<td>2.2 ± 0.4</td>
<td>33.6 ± 5.7*</td>
</tr>
<tr>
<td>mdx mice + PTU (2)</td>
<td>1.7 ± 0.07</td>
<td>13.0 ± 4.9**</td>
</tr>
</tbody>
</table>

DISCUSSION

Our data provide the first demonstration of an experimental manipulation of endocrine function altering the onset of necrosis in muscle lacking dystrophin. Data indicate that the developmental postnatal rise in thyroid hormones may precipitate muscle necrosis in the dystro-
Regulation of muscle necrosis in mdx mice

Dystrophin-deficient mdx mouse, and that PTU-induced hypothyroidism substantially delayed the onset of muscle necrosis in the dystrophic animals.

Serum concentration of \( T_3 \) and \( T_4 \) were low at birth and then increased acutely after 6 days of age. There was no further significant increase in serum \( T_4 \) after 12 days old, i.e. 2 to 3 days before the onset of necrosis in dystrophic muscle. Serum \( T_3 \) levels followed a similar pattern although peak values were achieved later than \( T_4 \). These data are in agreement with studies of the maturation of the thyroid system in normal rats [4] and indicate that the onset of muscle necrosis in mdx mice is temporally associated with a postnatal rise in circulating thyroid hormones.

Chemical induction of hypothyroidism by treatment with PTU had a dramatic effect on development of muscle necrosis in the mdx mouse. Treatment with PTU effectively prevented the onset of necrosis and the rise in circulating CK activities which are apparent in mdx mice at 21 days of age, and significantly reduced the number of fibres which were either necrotic, in the process of regeneration or contained central nuclei (indicative of earlier necrosis) in 28-day-old mdx mice. No effect of PTU treatment on the elevated circulating CK activity of mdx mice at 28 days of age was seen. These elevated CK activities in the 28-day-old PTU-treated mdx mice are compatible with the observation of similar numbers of necrotic fibres in these animals compared with untreated animals although the proportion of fibres which had previously undergone necrosis and regeneration (centrally nucleated fibres) was greatly reduced (Table 1).

PTU has marked effects on thyroid hormone production in that it inhibits both thyroid synthesis of \( T_3 \) and also conversion of \( T_4 \) to \( T_3 \) by the type I iodothyronine deiodinase, although it has little effect on the activity of type II iodothyronine deiodinase. Evidence that PTU treatment influenced thyroid metabolism in the mice can be obtained from the observation that serum \( T_3 \) concentrations were significantly decreased by PTU (Table 2). However, no decrease in serum \( T_3 \) concentrations was observed. This lack of decrease in serum \( T_3 \) concentration is compatible with data indicating that type II iodothyronine deiodinase is the predominant pathway of deiodination of \( T_3 \) in neonatal rodents [8]. \( T_3 \) exerts its action in the nucleus through binding to receptors to modify the expression of thyroid hormone responsive genes. Nuclear \( T_3 \) can arise from either local intracellular production via deiodination of \( T_4 \) or from plasma \( T_3 \) and there are marked differences between various tissues regarding which is the preferred source. The relative contribution of each source of nuclear \( T_3 \) determines the vulnerability of the tissue to changes in the plasma concentration of \( T_3 \) and \( T_4 \). Tissues which derive most of their nuclear \( T_3 \) from local intracellular production via \( T_4 \) deiodination will be more affected by changes in plasma \( T_3 \) than \( T_4 \). Alternatively, tissues which derive most of their nuclear \( T_3 \) from plasma will be most affected by changes in plasma \( T_4 \) [9].

Our observation that muscle degeneration in the mdx mouse can be prevented by PTU treatment which modifies only plasma \( T_4 \) levels suggests that mouse skeletal muscle utilizes intracellular \( T_3 \) production via type II iodothyronine deiodinase.

Our findings do not allow any definitive conclusions to be drawn on the mechanisms by which thyroid hormone regulates this process. Treatment with PTU resulted in a significant reduction in body weight of the mice (Table 3). Karpati et al. [10] have proposed that in dystrophin-deficient muscle, the larger diameter fibres are more susceptible to degeneration, but our findings cannot be fully explained by this. Thus, although PTU effectively prevented the onset of necrosis in mdx muscle at 21 days of age, the mean fibre diameter of PTU-treated mdx mice was not significantly reduced compared with untreated animals. Furthermore, no correlation was found between the percentage of necrotic fibres and muscle fibre diameter for mdx mice at either 21 and 28 days of age (results not shown in detail).

Other workers [11,12] have provided evidence that the dystrophin-related protein, utrophin, may replace dystrophin functionally in dystrophic skeletal muscle. Utrophin is present in regenerating muscle fibres [13] and in mouse muscle at birth but the levels gradually decrease, becoming undetectable by approximately 21 days of age [14]. It may be that utrophin reaches a critical level in mdx mouse muscle at approximately 2 weeks of age and that the effects of dystrophin deficiency are only unmasked once utrophin reaches this low level. One possible explanation for our findings would be that thyroid hormones are involved in the postnatal down-regulation of utrophin expression. PTU treatment might therefore prolong utrophin expression. To test this hypothesis, cryostat sections of muscle were immunohistochemically labelled for utrophin [13]. Urophin was not detectable at the plasma membrane of fibres at 21 days of age in either PTU-treated or untreated mdx mice, although utrophin was expressed in actively regenerating fibres in untreated mdx mice at 28 days of age (results not shown).

In related studies, Anderson and workers have examined the effects of hyperthyroidism and hypothyroidism on the mdx mouse [15–17]. \( T_3 \) supplementation for 2 weeks was found to increase the prevalence of necrosis and central nucleation in soleus and cardiac muscle of 5- or 10-week-old mdx mice [15]. The authors suggested that these changes might be related to \( T_3 \)-induced modification of myosin heavy chain expression. This appears not to be a major effect in our 21-day-old animals, as the patterns of immunohistochemical labelling for fast and slow myosin heavy chain isoforms in cryostat sections of gastrocnemius, soleus and quadriceps were
unaffected by PTU treatment (results not shown). McIntosh et al. [16] reported that the treatment of mdx mice with 0.05% PTU from 3 to 11 weeks of age increased the prevalence of necrosis. The experimental protocol for these experiments differs substantially from ours with regard to the ages of the animals, the number of muscles studied and the timing of the PTU treatment. The phase of skeletal muscle necrosis in mdx mice between 2 and 4 weeks old is followed by a compensatory phase of active regeneration [2], and studies where X-irradiation was used to inhibit this regenerative phase have shown that recovery from necrosis is dependent upon this regeneration [18]. McIntosh et al. [16] also demonstrated that PTU treatment reduced the rate of regeneration of normal muscle after crush injury. The detrimental effects of PTU observed by McIntosh et al. [16] on mdx muscle at an age when muscle is normally actively regenerating can be attributed to a reduction in regeneration of the mdx muscle. In our study, we treated mdx mice with PTU from birth to 21/28 days to attempt to specifically prevent the onset of the necrotic phase.

PTU treatment has also been reported to reduce the rate of muscle degeneration in avian muscular dystrophy [19] (which does not result from dystrophin deficiency), supporting the possibility that the effects of thyroid hormone are indirect. Thyroid hormone mediates many maturational changes in developing muscle including the activation of expression of myosin heavy chain, MyoD and the sarcoplasmic reticulum ATPase [5,6]. Similar postnatal changes in thyroid hormone levels are not seen in man in comparison with rodents. In man, development of the thyroid gland occurs primarily in utero [20]. Such species differences may be relevant to our understanding of the onset of degenerative features in muscle of fetuses affected with Duchenne muscular dystrophy [21].

In summary, the postnatal increase in thyroid hormone production appears to be responsible for a critical maturational change in muscle when mice are 2-3 weeks old, which precipitates necrosis in dystrophin-deficient muscle. The mechanism by which this muscle necrosis is triggered remains unknown although various hypotheses have been suggested (see McArdle et al. [22] for review). These data provide the first demonstration that manipulation of an endocrine system can lead to a delay in necrosis in muscle lacking dystrophin, and a unique insight into potential ways of studying those developmental factors which interact with the dystrophin deficiency in mdx mice and Duchenne muscular dystrophy to lead to fibre necrosis.

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