Successful treatment of murine muscular dystrophy with the proteinase inhibitor leupeptin

(muscle degeneration/protein degradation/inhibition of proteolysis)

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ABSTRACT Mice with genetic muscular dystrophy were treated with intraperitoneal injections of the proteinase inhibitor leupeptin, beginning before the onset of weakness. A significant number of the treated animals failed to develop histological evidence of dystrophy, compared with controls. Leupeptin treatment prevented (or delayed) the onset of muscular dystrophy in this experiment.

In muscular dystrophy, there is biochemical and morphological evidence of abnormality of the muscle cell plasma membrane (1–3). Such defective membranes may allow calcium influx, leading to activation of proteinases and initiating muscle fiber necrosis (4–8). The eventual severe loss of sarcoplasmic and contractile proteins in dystrophy is associated with increased activity of acidic and neutral proteinases (7–10).

The proteinase inhibitor leupeptin (11) is known to inhibit cathepsin B and calcium-activated neutral endopeptidase, proteinases thought to play an important role in muscle catabolism in dystrophy. Leupeptin and pepstatin (a similar proteinase inhibitor that acts on cathepsin D) (11, 12), delay degeneration of dystrophic chicken muscle in vitro and in vivo (13–15). Likewise, normal and dystrophic mouse muscles show decreased protein degradation in vitro when incubated with leupeptin (16).

These findings suggest that leupeptin may be able to limit proteolysis in early dystrophic lesions, minimizing muscle fiber damage. Therefore, we studied the effects of leupeptin treatment of genetically dystrophic mice.

We used C57BL/6J dy<sup>31</sup>/dy<sup>31</sup> mice (17, 18), the progeny of matings between dystrophic homozygous dy<sup>31</sup> males and brown females with transplanted ovaries from homozygous dy<sup>31</sup> females, or of matings between the homozygous littersmates that resulted from the first breeding pairs. Sixteen animals received intraperitoneal injections of leupeptin dissolved in saline (12 mg/kg of body weight) three times a week beginning at age 3 weeks, before signs of clinical weakness occur in this strain. Each treated animal had 1 or 2 littermate controls. Ten control animals were injected with saline and 14 received no treatment. The mice were sacrificed at 12, 16, and 24 weeks of age. The soleus and gastrocnemius muscles were examined by light and electron microscopy. Frozen sections were stained with hematoxylin and eosin, the modified Gomori trichrome stain, and for succinate dehydrogenase and NADH–tetrozolium reductase activity.

The animals were observed for evidence of weakness as determined by their ability to climb a 45° incline and by their ability to pull themselves up on and balance on the edge of a plastic cage. Although the weakness was not quantitated, cinematographic records of the animals' performance were made. None of the treated animals showed weakness, but a majority of the untreated animals were clinically weak.

One of us (J. H. S.) examined the histological sections with no knowledge of the treatment history of the animals. Although a spectrum of abnormalities was apparent in the dystrophic muscles, the sections could be divided into two groups: those showing no or minimal histological change and those showing evidence of a necrotizing myopathy, as defined by the presence of necrotic fibers plus at least 5% other abnormal fibers in the soleus (Fig. 1 A and B). These abnormal fibers were mainly smaller than normal, with internal nuclei. In succinate dehydrogenase preparations some fibers that appeared normal in hematoxylin and eosin stains showed patchy or lost mitochondrial staining. Fifteen of the 16 treated animals had no significant histological changes in their muscle, whereas 19 of the 24 untreated animals' muscle sections showed a necrotizing myopathy (P < 0.001; Table 1).

Cross-sectional smallest diameters were measured on 200 soleus muscle fibers from each of 10 randomly selected animals, 5 treated and 5 controls. Mean fiber diameter (±SD) in the treated group was 31.5 ± 6.8 μm, and in the untreated group, 28.8 ± 10.9 μm. The difference is statistically significant (P < 0.001). The muscle from untreated dystrophic animals contained many extremely small fibers as well as some hypertrophied fibers, resulting in the significantly smaller mean fiber diameter. What is more important, 27% of the fibers had diameters outside the normal range (21–35 μm) in untreated muscle, as opposed to 8% in treated muscle (Fig. 2). This emphasizes the marked variability of fiber size in untreated muscle. Measurements of 300 soleus fiber diameters from two normal age-matched C57BL mice yielded a mean of 35.6 ± 6.5 μm, which is significantly different from the mean fiber diameter in both treated and untreated groups (P < 0.001). Leupeptin treatment therefore did not prevent some decrease in muscle fiber size, although it inhibited the clinical and histological manifestations of dystrophy.

The degree of severity of observed myopathic change varied from mild to extremely severe, and the most marked changes

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were seen in animals of all ages, indicating considerable variability in severity and rate of progression of the disease. The lack of muscle change in five control animals may have occurred because of variability in the time of onset of disease in these mice.

Ultrastructural studies of the soleus muscles of the control dystrophic animals showed marked swelling of the mitochondria with reduced numbers of cristae, compared to the normal appearance of electron micrographs of muscle from treated animals (Fig. 1 E and F).

Although swollen mitochondria are a common fixation artifact, in our samples they appeared to be characteristic of cells in the early stage of degeneration. Normal and treated muscles prepared in an identical manner did not show this change, discounting the possibility of artifact. An early effect on mitochondria in this strain of mice has been described (18). Succinate dehydrogenase preparations of untreated muscle showed fibers with absent or focally decreased staining compared with treated muscle (Fig. 1 C and D). This finding correlates with the ultrastructural evidence of mitochondrial abnormalities. There was increased sarcoplasmic reticulum at the I band level in many fibers. Collagen was increased focally, surrounding small groups of fibers and apparently originating by formation of fibroblast processes circling individual muscle fibers.

This experiment demonstrates that leupeptin treatment of homozygous $dy^{13}$ mice begun prior to the onset of weakness prevents or delays appearance of the myopathy for at least 24 weeks.

A previous study of combined leupeptin and pepstatin treatment of dystrophic mice showed no effect (19). The animals used
were homozygous dy mice and treatment via the subcutaneous route was begun at 35 days, at a time when the disease is well advanced in this strain. For successful treatment with a proteinase inhibitor, it may be necessary to begin before the disease process is established. The authors' suggested explanation of the treatment failure was that these antiproteinases are unable to enter muscle cell. However, tissue culture experiments have since suggested that these inhibitors can cross the sarcolemma to exert an antiproteolytic effect (20). In addition, measurement of the $\text{Ca}^{2+}\text{-activated protease activity in muscle after intraperitoneal injection of leupeptin leads to abolishment (}=90\%$) of this activity within 24-48 hr and a return to the original after 5-6 days, again suggesting that leupeptin has entered the muscle cell (unpublished observations). Another study of the effect of intraperitoneal pepstatin on dy/dy mice, begun at 3 weeks of age, showed some beneficial effect of treatment (21).

Leupeptin inhibits cathepsin B, an enzyme increased early in human dystrophic muscle (10) that can degrade myosin. It is also a potent inhibitor of a calcium-activated proteinase present in muscle that degrades troponin T and I as well as tropomyosin, and causes dissolution of the $Z$ lines (22-24). The results presented here indicate that increased activity of these and possibly other leupeptin-inhibitable proteinases play an important role in the increased muscle catabolism associated with dystrophy. They also support our previous suggestion (13, 14) of a potential therapeutic role for proteinase inhibitors in muscular dystrophy.

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