Introduction
Muscular dystrophy is now known to result from mutations in the gene that encodes for the cytoskeletal protein called dystrophin. It is also well known that there is an increased calcium accumulation in human dystrophy, as well as, in the murine mdx model. Increased calcium levels may trigger activation of calcium dependent proteases, now known as calpains. Therefore, the calpain inhibitor leupeptin may have merit as a potential therapeutic agent in this severe muscle wasting disorder. The present study was undertaken to test the ability of the calpain inhibitor, leupeptin, to delay and/or inhibit features of muscle degeneration in the murine mdx dystrophy model.

Materials and Methods
Fourteen day old male mdx mice were given IM injections of leupeptin dissolved in physiologic saline, twice daily for 30 days at doses of 12mg/kg (n=5) and 18mg/kg (n=5). Injections were at varied sites in the hindlimbs to avoid site specific muscle necrosis. Age and gender matched mdx mice (n=5) and age and gender matched normal C57BL/10SNJ mice (n=5) served as controls and were injected similarly with physiologic saline. The mdx model shows signs of muscle pathology at approximately 14-28 days post partum. After 30 days, the right and left gastrocnemius, soleus, anterior tibialis and the diaphragm were harvested, snap frozen, and sectioned in a cryostat at 6µm in a serial manner. Slides were stained for routine histologic analysis using hematoxylin and eosin for histochemical muscle fiber typing and using the calcium myosin ATPase at pH 4.3. Mean myofiber diameters were quantified in each muscle and then by similar muscle within an animal group. Digitizing and computer assisted image analysis followed. Fifty myofibers per muscle on five constant magnification fields were measured and the diameters of similar muscles between the groups tested for significance using a paired student’s t test and by one way analysis of variance (ANOVA). A portion of all muscle samples was placed in 50% glycerol and frozen at -70° for calpain activity assay. Muscle was homogenized in a sodium potassium EGTA buffer at pH 7.4 and the homogenates centrifuged to recover the supernatant, after which the protein concentration was determined. Supernatants were assayed in a buffer containing 14C-labeled casein as a substrate. All muscle samples were assayed in triplicate and the units obtained were in counts per minute per microgram of muscle homogenerate.

Results

Myofiber Histology/Histochemistry
The histologic appearance of untreated mdx myofibers was characterized by evidence of myofiber degeneration/regeneration. However, at both doses of leupeptin, myofiber diameters in the mdx groups were significantly larger when compared with saline treated control mdx muscles. Leupeptin treated myofibers consistently showed significant increases in myofiber diameter when compared to untreated mdx myofibers. In the gastrocnemius, when compared to the untreated mdx, leupeptin (12mg/kg and 18mg/kg) induced a 37% and 39% increase in myofiber diameter, respectively (p<0.01). In the soleus muscle, when compared to the untreated mdx, 12mg/kg leupeptin induced a 22% increase and 18mg/kg a 25% increase in diameter, p<0.05. In the diaphragm, leupeptin treatment at both doses induced a 25% increase in myofiber diameter, p<0.01. The anterior tibialis, in particular, showed large increases in myofiber diameter (Graph 1).

Calcium, myosin ATPase fiber typing at pH 4.3 showed no difference in fiber types of the various muscles from those reported in the literature.

Muscle Calpain Activities
Muscle calpain activities decreased significantly and substantially in response to leupeptin treatment.

Conclusion
The results of this study show that inhibition of muscle degeneration by the tripeptide calpain inhibitor leupeptin was significant when tested in vivo in the dystrophin deficient murine mdx model. Histologic analysis of treated muscles, in comparison to untreated mdx myofibers, consistently showed increases of myofiber diameter. In addition, there was a clear correlation between increased myofiber size and decreased calpain activities in leupeptin treated muscles. The results of this study have shown that leupeptin may have potential merit as an inhibitor of muscle degeneration in the human form of Duchenne muscular dystrophy.

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