Effects of the Protease Inhibitor Leupeptin on Proteolytic Activities and Regeneration of Mouse Skeletal Muscles After Exercise Injuries

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Leupeptin, a nontoxic thiol protease inhibitor, has been proposed to have therapeutic use in hereditary muscular dystrophies. The purpose of this study was to characterize the *in vivo* changes in proteolytic activity of skeletal muscles induced by the repeated administration of leupeptin. Further, whether the modulation of proteolytic capacity by leupeptin affects the repair process of muscle injuries caused by heavy exercise was studied. Leupeptin was administered in mice intraperitoneally at a dose level of 15.5 mg/kg twice a day for 9 days. Leupeptin, known to be an inhibitor of cathepsin B both *in vitro* and after a single injection *in vivo*, paradoxically induced an increase of cathepsin B activity in mouse skeletal muscles after repeated administration. In addition, leupeptin administration for 9 days increased the activities of cathepsins C and D, as well as the rate of acid autolysis. The activity of β-glucuronidase also increased, while those of arylsulfatase, ribonuclease, and alkaline protease were unaffected. No histopathologic changes were observed. At the low dosage used, leupeptin had no effect on the repair process of skeletal muscle after exercise injuries, although several proteolytic processes occur during the regeneration. It is suggested that the increase of acid protease activities in skeletal muscles is an adaptive response to the administration of the proteolytic inhibitor leupeptin and that leupeptin can be administered without prevention or delay of regenerative processes after the onset of myopathic changes. (Am J Pathol 1984, 117:64–70)

LEUPEPTIN, a tripeptide containing an aldehyde group, is produced by certain *Streptomyces* species.[1] Leupeptin inhibits several extra- and intracellular thiol proteases.[1–6] For instance, leupeptin is a potent inhibitor of lysosomal cathepsin B both *in vitro*[3,4] and *in vivo*.[5,6] Perhaps because of enzyme inhibition, lysosomal function is reduced in rat liver after leupeptin administration.[5] Similarly, protein degradation decreases in rat cardiac and skeletal muscles incubated *in vitro*.[7] Therapeutically, leupeptin has been used with good results in animal experiments to inhibit increased protein degradation and muscle wasting in progressive hereditary muscular dystrophies *in vivo*[8,9] and *in vitro*.[10] The mechanism of the prevention or delay in the onset of muscular dystrophy by leupeptin therapy is not known.

Our studies have shown that heavy physical exercise causes necrotic lesions in mouse skeletal muscles and stimulates the lysosomal system of muscle fibers adjacent to necrotic foci.[11,12] Necrotic lesions appear the day after exertion and the first regenerating fibers appear on the fifth day after exercise. An increase in the number of autophagic vacuoles and an intensive histochemical pattern of lysosomal acid hydrolases occur in surviving muscle fibers between 3 and 7 days after exertion.[11,12] Exercise injuries are repaired by the regeneration during 2 weeks.[11]

It was considered interesting to study whether the decrease in proteolytic capacity affects the repair process of muscle injuries. A hypothesis was presented[11] that the lysosomal function would be essential for the repair of muscle injuries. Degenerative and regenerative processes also prevail in hereditary muscular dystrophies, and hence decreased recovery due to leupeptin administration could be injurious after the onset of dystrophy. A further consideration is that the studies on leupeptin inhibition of thiol proteases *in vivo* are

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based on a single injection of leupeptin. Only a few studies on the proteolytic responses to repeated administration of leupeptin have been made. Interestingly, the administration of leupeptin in culture medium has been observed to stimulate the activity of cathepsin B in skin fibroblasts[13] and in fetal mouse heart.[14] Furthermore, the activity of cathepsin D is increased by leupeptin administration in cultured hepatocytes.[15] In skeletal muscle, the administration of a high dose of leupeptin inhibited the activity of cathepsin B during the first 2 hours, but thereafter the activity began to increase, being twofold 24 hours after the injection.[6] It is probable that cells respond by adaptation to the prolonged inhibition of thiol proteases.

Materials and Methods

Animals and Physical Exertion

Male NMRI-mice, 5 months old, were made to run for 8 hours on a motor-driven treadmill with 6-degree uphill tracks at a speed of 14 m/min. The exertion was divided into two 4-hour phases interrupted by a 20-minute pause, during which the mice could freely eat and drink. All the mice were able to perform this program. The mice were housed under standard cage conditions with free access to solid food pellets (R_3, Astra Ewos, Sweden) and tap water. Temperature (20–22 C) and daylight rhythm (12 hours light/12 hours dark) were kept constant. The mean weight (±SE) of control mice was 37.2 ± 0.4 g and varied from 36.8 ± 1.6 to 39.0 ± 1.3 g in the different groups without significant differences between the groups.

Leupeptin Administration

Leupeptin (acetyl-L-leucyl-L-leucyl-L-argininal, Sigma Chemical Company) was dissolved in 0.9% saline and administered intraperitoneally at a dose of 15.5 mg/kg body weight. This dose was injected twice a day at 12-hour intervals. The first dose was administered 24 hours after the termination of exertion, at which time the first signs of necrotic lesions appear.[12] Thereafter the injections were repeated daily until the mice were killed on the third or ninth day after exercise. The mice were always killed 6 hours after the last injection. Leupeptin injections were administered at the same time to a group of exercised or control (leupeptin control) mice. Likewise, only saline was injected into a group of exercised (exercise control) or control mice.

Muscle Samples

The mice were killed by cervical dislocation. Two muscle samples were excised for biochemical measurements: one sample from the quadriceps femoris muscle comprising the red proximal parts of this muscle[16] and one from the lower leg comprising all the muscles. The samples were quickly prepared, weighed, frozen and kept at −80 C until analyzed. The muscle samples of the control and experimental groups were equal in weight.

Muscle samples were cut into small pieces after thawing and homogenized in ice-cold distilled water (quadriceps femoris muscle, 3% wt/vol) or in 0.25 M sucrose[17] containing 0.1% Triton X-100, 1.0 mM EDTA, and 0.5 mM dithiothreitol (muscles of the lower leg, 5% wt/vol) with the use of an all-glass Potter-Elvehjem homogenizer (670 rpm).

Histopathologic changes were traced from the proximal part of the quadriceps femoris muscle of the opposite leg to that from which the sample for biochemical measurements was taken. Transverse cryostate sections were stained with hematoxylin and eosin (H&E). The histologic study was undertaken from all the mice.

Assays

The enzyme substrates (N-α-benzoyl-DL-arginine *p*-nitroanilide for cathepsin B, glycyl-L-phenylalanine 2-naphthylamide for cathepsin C, bovine hemoglobin for cathepsin D, *p*-nitrocatechol sulfate for arylsulfatase, and *p*-nitrophenyl-β-D-glucuronide for β-glucuronidase) were purchased from Sigma Chemical Company. Casein (for alkaline protease) and ribonucleic acid (for ribonuclease) were obtained from BDH Biochemicals and Fluka Co., respectively.

The activity of cathepsin B (EC 3.4.22.1) was measured by the method of Nakagawa et al.[17] The activities of cathepsin C (EC 3.4.14.1), cathepsin D (EC 3.4.23.5), arylsulfatase (EC 3.1.6.1), β-glucuronidase (EC 3.2.1.31), ribonuclease (EC 2.7.7.16), and alkaline protease, as well as the rate of acid autolysis and protein concentrations were assayed as described in detail in our earlier studies.[16,18]

Standard statistical procedures were used for calculating means and standard errors. The significance of the differences between means was determined by the Student *t*-test.

Results

Effects of Leupeptin Administration

The repeated administration of leupeptin, a potent inhibitor of cathepsin B *in vitro*,[4] surprisingly increased the activity of cathepsin B in mouse skeletal muscle (Figure 1). The increase was almost linear and the activity was doubled during 9 days. Simultaneously, the

activities of cathepsin C, cathepsin D, and β-glucuronidase, as well as the rate of acid autolysis, also increased, but these changes were not statistically significant until after 9 days (Figures 1 and 2). Leupeptin had no effect on the activities of alkaline protease, arylsulfatase or ribonuclease.

Effects of Exertion

Exhaustive exertion of untreated control mice increased the activities of cathepsin C, cathepsin D, and β-glucuronidase as well as the rate of acid autolysis in the skeletal muscles studied (Figures 1 and 2). The highest activities were observed on the third day after the exertion and thereafter partially decreased until the ninth day. The degree of increase was higher in the samples of the red part of the quadriceps femoris muscle than in the muscles of the lower leg (Figures 1 and 2).

The exercise response was selective, because the activities of alkaline protease, cathepsin B, arylsulfatase, and ribonuclease were statistically unaffected after exertion (Figures 1 and 2).

Effects of Leupeptin Administration on Exercise Response

Administration of leupeptin to exercised mice induced an exercise response in the activity of cathepsin B, which was unaffected in untreated exercised mice (Figure 1). A slight exercise response also occurred in the activities of arylsulfatase and ribonuclease 3 days after exertion in leupeptin-treated mice. Otherwise, the exercise responses were similar 3 days after exertion in leupeptin-treated and untreated groups.

Nine days after exertion the highest activities of lysosomal hydrolases were observed in leupeptin-treated exercised mice (Figures 1 and 2). This was due to the cumulative effect of leupeptin administration and exercise response. The cumulative effect was observable in the rate of acid autolysis and in the activities of cathepsin C, cathepsin D, and β-glucuronidase. A potentiated effect was observed in the activities of cathepsin B and cathepsin C. In general, leupeptin effects were similar in the muscles of control and exercised mice.

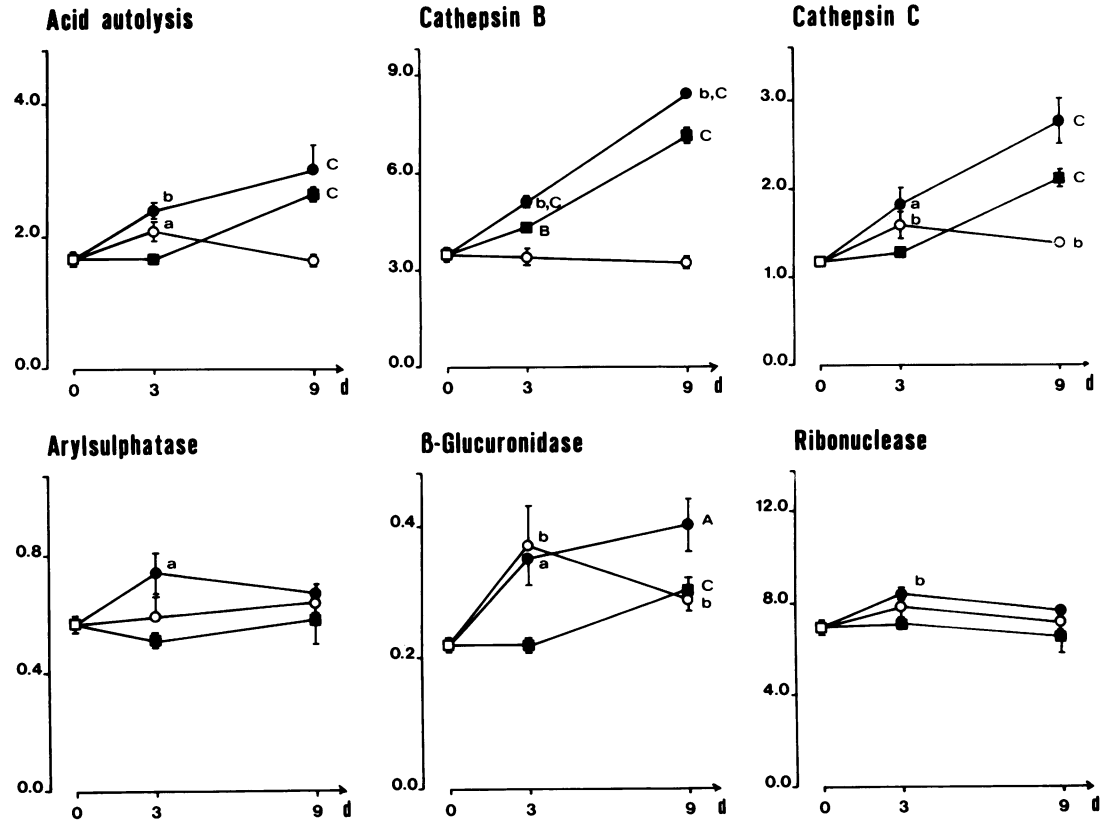

Figure 1 — The effects of leupeptin administration and physical exertion on the activities of hydrolytic enzymes and the rate of acid autolysis in the muscles of the lower leg of mice. Symbols: □, untreated and unexercised mice (n = 9); ■, leupeptin-treated mice (n = 5 and 3 after 3 and 9 days); ○, exercised mice (n = 5 and 8, respectively); ●, leupeptin-treated exercised mice (n = 6 and 6, respectively). The activities are expressed as μmol hydrolyzed products × s^{-1} × kg^{-1} wet weight, except that of cathepsin B, which is given as nmol × s^{-1} × kg^{-1} wet weight. Values are means ± SE. Statistical significances: A ($P < 0.05$), B ($P < 0.01$), and C ($P < 0.001$) indicate the effect of leupeptin in control and exercised mice; a ($P < 0.05$), b ($P < 0.01$), and c ($P < 0.001$) indicate the effect of exertion in leupeptin-treated and untreated mice.

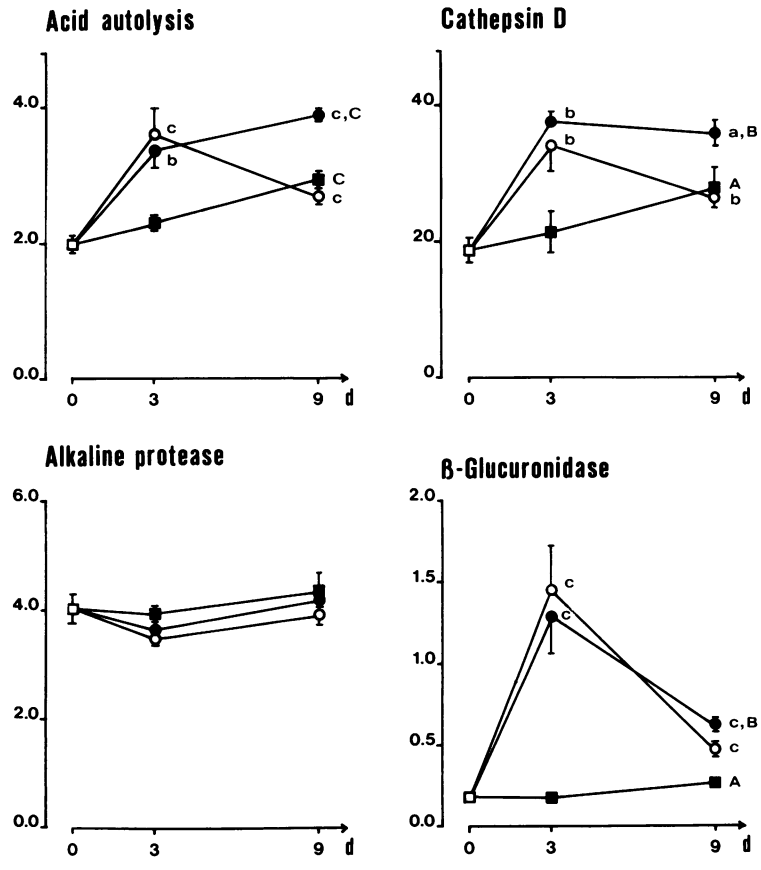

Figure 2—The effects of leupeptin administration and physical exertion on the activities of hydrolytic enzymes and the rate of acid autolysis in the red muscles of quadriceps femoris. Symbols, activities, and statistical significances are indicated as in Figure 1.

Histologic Findings

Leupeptin administration for up to 9 days did not cause any observable pathologic changes in the proximal part of mouse quadriceps femoris muscle (Figure 3B). Exhaustive exertion caused degenerative changes in mouse skeletal muscle (Figure 3). Three days after exertion there was either extensive or scattered necrosis of muscle fibers, together with focal inflammation. Exercise injuries in quadriceps femoris muscle were most frequent in vastus intermedius and in the red parts of vastus lateralis and medialis as well as of rectus femoris. Injurious responses to exertion were very similar in leupeptin-treated (Figure 3D) and untreated (Figure 3C) mice.

Nine days after exertion the regeneration of injured skeletal muscle fibers was far advanced both in untreated and in leupeptin-treated mice (Figures 3E and F). Regenerative fibers with central nuclei were usually the only indication of exercise injuries. No differences were observed in the degree of regeneration between leupeptin-treated (Figure 3F) and untreated (Figure 3E) exercised mice.

Discussion

The repeated administration of leupeptin, a potent inhibitor of cathepsin B, caused a paradoxical increase of cathepsin B activity in mouse skeletal muscles. Sutherland and Greenbaum[6] found an increase in cathepsin B activity in mouse skeletal muscle after a single high dose (200 mg/kg) of leupeptin injection but not after a low dose (20 mg/kg). The increase appeared 24 hours after the injection. The present results show that a low dose (15.5 mg/kg) of leupeptin also induces a stimulation of cathepsin B activity if administered repeatedly for 3–9 days. A single injection of leupeptin at the same dose did not affect the activity of cathepsin B in mouse skeletal muscles (unpublished data). The repeated administration of leupeptin for 9 days also increased the activities of the other acid proteases studied, cathepsin C and D, but not the activity of alkaline protease, which is probably derived from mast cells.[19] The activity of β-glucuronidase also increased, but those of the other nonproteolytic lysosomal hydrolases assayed did not. Histopathologic alterations were not observed in the skeletal muscles. The results suggest that

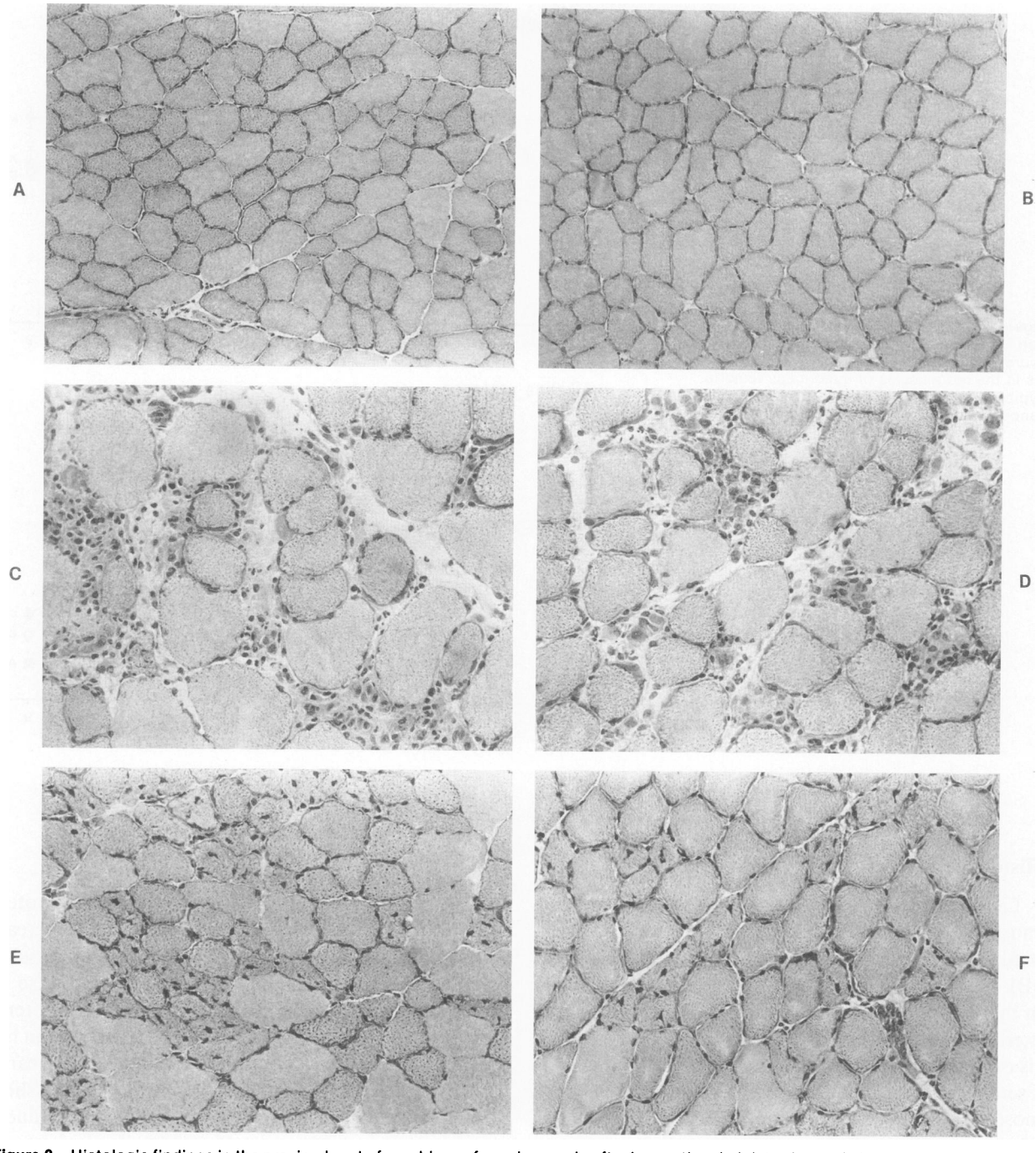

Figure 3 – Histologic findings in the proximal part of quadriceps femoris muscle after leupeptin administration and physical exertion. **A** – Transverse section from the muscle of control mice with normal histologic structure. (H&E, × 104) **B** – Representative figure from the muscle of leupeptin-treated mice. The structure was normal after the treatment of 9 days (H&E, × 104). **C** – Focal necrosis and infiltration of inflammatory phagocytes 3 days after exertion. No leupeptin treatment. (H&E, × 163) **D** – Changes similar to those in **C**. Leupeptin-treated exercised mice 3 days after exertion. (H&E, × 163) **E** – Central nucleated regenerative muscle fibers 9 days after exertion. No leupeptin treatment. (H&E, × 163) **F** – Changes similar to those in **E**. Leupeptin treatment. (H&E, × 163)

muscle fibers probably respond to the repeated administration of the protease inhibitor leupeptin by stimulating the acid proteolytic capacity, as observed by the increase in the rate of acid autolysis.

It has been presumed that the therapeutic effect of leupeptin in muscular dystrophies is due to the inhibition of the increased and possibly injurious activity of cathepsin B.[8,10] This interpretation is based on the effects of leupeptin *in vitro*.[4,7] The doses used in the therapeutic studies were similar to those used in this study but

the frequency of administration was lower.[8,9] Hence, it is possible that the activity of cathepsin B increases, rather than decreases, in muscular dystrophies. Aoyagi et al[20] found that the activity of cathepsin C, a thiol-dependent protease, increased in the muscles of dystrophic mice during a leupeptin treatment of 8 days. By contrast, the activities of several aminopeptidases decreased. Hence, the effects of repeated leupeptin administration are more extensive and different from those anticipated from their effects *in vitro*. The increase in proteolytic capacity is probably due to increased synthesis or to decreased breakdown of proteolytic enzymes, although disturbances in the balance of proteases and their endogenous inhibitors might affect the activities assayed. Both cytosolic and lysosomal inhibitors, for example, exist *in vivo* against cathepsin B.[21]

Physical exertion induced necrotic muscle fiber injuries, as observed earlier.[11,12] Damaged muscle fibers were infiltrated and replaced by inflammatory phagocytes. This process was well advanced by the third day after exertion. Thereafter, the muscle injuries were repaired by muscle fiber regeneration, which was highly advanced by the ninth day after exercise. The administration of leupeptin did not reduce degeneration or inflammation in the present study. The administration was not started until the day after exertion, because the aim of the study was to investigate whether leupeptin affects the repair of existing injuries. Leupeptin administration should have been started before the exertion if its effect on the appearance of exercise injuries was to be studied. It was interesting that leupeptin administration did not delay the phagocytosis of necrotic material or the regeneration of injured muscle fibers, although lysosomal function is augmented, both in heterophagocytosis of phagocytes and in autophagocytosis of muscle fibers adjacent to necrotic foci.[12] Leupeptin could transiently prevent the function of the lysosomal system in both macrophages and muscle fibers as well as in hepatocytes,[5] but the effective time with the dose used in this work could have been so short as to have had no effect on the whole repair process. The level of leupeptin rapidly declines in tissues after injection; eg, leupeptin disappears from rat liver within 6 hours of the injection (20 mg/kg).[5] Leupeptin-inactivating enzymes have been characterized in mouse liver.[22] The present observations on exercise injuries suggest that regenerative processes in dystrophic muscles are not disturbed by leupeptin treatment and that leupeptin can still be administered after the onset of muscle fiber injuries.

The increase in acid protease activities induced by leupeptin was also observable in exercised mice. This appeared to be due to the summation of the effects of exercise and leupeptin. The summation was evident on the ninth day after exertion. In addition, the administration of leupeptin induced an exercise response in the activities of cathepsin B, arylsulfatase, and ribonuclease. The cause of this potentiation by leupeptin is unclear. The summation of the effects of exercise and leupeptin on proteolytic activities suggests that the origins of the proteolytic changes may be different. It is likely that the therapeutic use of leupeptin in some deficiency diseases, such as in β-galactosidase α-neuraminidase deficiency,[13] and in neuromuscular dystrophies (which are possibly also deficiency diseases), is due to the regulation of protein breakdown, eg, by limited proteolysis, and thus the regulation of protein levels in cells.

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