Neuromuscular recovery using calcium protease inhibition after median nerve repair in primates

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ABSTRACT Inhibition of calcium-activated neutral protease, in muscle and nerve, by the tripeptide leupeptin after median nerve transection and epineural repair in monkeys (Cebus apella) was studied. Results indicate that inhibition of the protease after nerve repair facilitates morphologic recovery in denervated thenar muscles and in distal thenar nerve branches. In addition, functional recovery was facilitated in leupeptin-treated animals after nerve repair as measured by sensory and motor conduction velocities. Toxicologic testing showed that leupeptin, administered at 18 mg/kg, intramuscularly, twice daily, for 6 months did not adversely affect hematology, clotting, or plasma complement component C3 profiles. These data indicate that leupeptin is an effective and safe adjunct to peripheral nerve repair.

Calcium-activated neutral protease (CANP) has been identified biochemically in skeletal muscle homogenates (1) and in soluble extracts of peripheral nerve (2). There are two forms of CANP. One form requires millimolar calcium for activation and the other form is activated by micromolar calcium (3–6). CANP may play a role in normal myofibrillar and neurofibrillar proteolysis (2, 7, 8). Additionally, other studies have reported that the activity of this enzyme increases in such pathologies as Duchenne and Becker dystrophies (9), muscle atrophy (10–12), and Wallerian degeneration (13–17).

In a study from our laboratories (18), CANP was immunocytochemically localized in normal primate muscle and normal primate peripheral nerve. In vivo administration of the thiol-protease inhibitor leupeptin abolished the immunoreactivity in muscle and peripheral nerve (18). These data suggested that binding of leupeptin to CANP prevented the interaction with anti-CANP antibody, presumably at the active site, and further demonstrated the accessibility of intracellular CANP to injected leupeptin. These data and our previous studies (13–16) further strengthened our hypothesis that inhibition of muscle and neural CANP with leupeptin subsequent to peripheral nerve repair might facilitate neuromuscular recovery.

This study was undertaken to test this hypothesis in a primate median nerve model (Cebus apella), which closely approximated anatomic and regenerative aspects of the human. Excellent functional results after peripheral nerve repair in humans still cannot be consistently obtained or reliably predicted (19, 20). Therefore, adjunctive methods to nerve repair that facilitate neuromuscular recovery may provide useful therapies in humans.

MATERIALS AND METHODS

Neuromicrosurgery and Treatment. Ten young adult Cebus monkeys (C. apella) (3–4 kg) were preanesthetized with ketamine and atropane, intubated tracheally, and maintained on halothane anesthesia with cardiac monitoring. By using sterile technique and an operative microscope, all animals underwent a right median nerve transection, using nerve-holding forceps (Accurate Instruments, Westbury, NY) and a sharp 35-mm nerve blade, 1.5 cm proximal to the distal wrist crease. Both proximal and distal nerve stumps in five treated animals were immediately bathed and the right thenar muscles were injected with leupeptin at 18 mg/kg, dissolved in sterile 0.9% NaCl (0.25 ml, total volume). In the remaining five control animals, the transected proximal and distal nerve stumps were bathed in 0.25 ml of sterile 0.9% NaCl only. Subsequently, all animals underwent an immediate epineural repair of their median nerves using 10-0 nylon, a 70-μm needle, and an average maximum of four circumferential suture points. To ensure that the animals did not rupture their nerve repairs, fiberglass long arm casts were applied for 4 weeks to hold the wrists in volar flexion and the elbow at 45°–50° of flexion.

Beginning on post-operative day 1 and continuing for 6 months, treated animals received intramuscular injections in the hind limbs of leupeptin at 18 mg/kg in sterile 0.9% NaCl, twice daily, with 7 hr between the injections. Control animals were not injected since it was determined in a large group of rats (16) and a separate smaller group of Cebus monkeys (13) that control injections of saline only had no effects on neuromuscular recovery after nerve repair.

Conduction Velocities. At 6 and 8 weeks after nerve repair, all animals were anesthetized with ketamine and atropane and sensory and motor nerve conduction velocities were tested across the repair site. Similar conduction velocity tests were also performed on the (unoperated) left median nerves in all animals.

Muscle and Nerve Biopsy. At 3 months after nerve repair, animals were anesthetized as before and, by using a 3.5× loupe, the nerve repair site was re-explored to assure that it was intact and free of neuroma. All animals underwent the following procedures: (i) right and left biopsies of the thenar opponens pollicis muscle with a fixed-length 10-mm Rayport muscle biopsy clamp (Baxter V. Mueller, McGray Park, IL) and (ii) right and left nerve biopsies of the motor branch of the median nerve supplying the opponens pollicis. The right nerve biopsy was taken consistently at 3.0 cm distal to the nerve repair site. The left nerve biopsy was taken at the corresponding anatomic location.

At 6 months after nerve repair, all animals again underwent the following similar muscle and nerve biopsies: (i) right and left biopsies of the thenar abductor pollicis muscle using the Rayport clamp and (ii) right and left biopsies of the motor branch of the median nerve supplying the abductor pollicis. The right nerve biopsy was taken at a fixed point 3.0 cm distal to the nerve repair site. The left nerve biopsy was taken at the corresponding anatomic location.

Morphologic and Immunocytochemical Methods. For histological analysis, muscle samples were frozen in isopentane cooled by liquid nitrogen. Transverse sections were cut at 10

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μm in a cryostat and stained with hematoxylin and eosin. Muscle and nerve samples were also fixed in 3% (vol/vol) glutaraldehyde/0.1 M cacodylate buffer (pH 7.2) for transmission electron microscopy. These samples were postfixed in 1% osmium tetroxide, washed in buffer, and dehydrated in an ascending ethanol series. Thereafter, the samples were cleared in propylene oxide, infiltrated in propylene oxide/Polybed 812 resin [1:1 (vol/vol); Polysciences], and embedded in pure resin for 48 hr at 60°C in both longitudinal and transverse orientation. One-micrometer-thick survey sections were cut on a Sorvall MT-2B ultramicrotome and stained with 0.5% toluidine blue. Ultrathin 600-Å sections were cut, stained with uranyl acetate and lead citrate, and examined in a Hitachi 12 electron microscope.

The antibody was prepared against CANP purified from human blood platelets as described by Truglia and Stracher (21). Cross reactivity with muscle CANP was demonstrated by Western analysis of rabbit muscle extracts and immunohistopathology.

For light microscopic CANP immunocytochemistry, 10-μm muscle and nerve samples were cut frozen, rinsed in ice-cold, isotonic phosphate-buffered saline (PBS), and incubated in mouse anti-CANP antibody at a 1:10 dilution in PBS overnight at 4°C. After washing in PBS, sections were incubated in goat anti-mouse IgG conjugated to fluorescein isothiocyanate at a 1:100 dilution. After rinsing in PBS, sections, covered with a dilute glycerin solution and a coverslip, were examined and photographed in a Leitz Dialux fluorescence microscope. As a control, excess purified CANP was adsorbed to the antibody and alternate slides were incubated in this adsorbed solution.

For electron microscopic CANP immunocytochemistry, 50-μm muscle and nerve sections were cut frozen and incubated as described above in CANP antisera, then incubated in goat anti-mouse IgG (1:100 dilution), rinsed in PBS, and immersed in mouse peroxidase-antiperoxidase (1:50 dilution). The chromogen used was 2.5% (wt/vol) diaminobenzidine containing 3% (vol/vol) H₂O₂. After a PBS wash, samples were fixed in 1% osmium tetroxide and prepared as described above for transmission electron microscopy. As a control for ultrastructural immunocytochemistry, alternate 50-μm sections were incubated as before in antibody adsorbed to an excess of purified CANP and prepared for transmission electron microscopy. Sections were stained by uranyl acetate and lead citrate or were unstained.

Toxicology. Two times at monthly intervals prior to surgery and six times at monthly intervals thereafter, animals were anesthetized with ketamine and atropine and 10 ml of blood was drawn from the femoral vein. Blood samples were analyzed for numbers of leukocytes, erythrocytes, and platelets; hematocrit; prothrombin time; and partial thromboplastin time. Plasma was analyzed for C3.

Biochemistry. The effect of anti-CANP antibody on CANP activity was investigated as follows. A 50-μl sample of CANP was mixed with 200 μg (5 × 10⁵ cpn; 25 μl) of 1⁴C-labeled casein and 400 μl of buffer. The reaction was stopped and the proteins were precipitated by adding 500 μl of 50% (wt/vol) trichloroacetic acid. Ice-cold casein (50 μl at 6 mg/ml) was added to aid protein precipitation. The mixture was centrifuged at 12,000 × g for 2 min to pellet the precipitate. Supernatant (450 μl) containing the non-trichloroacetic acid-precipitable 1⁴C-labeled peptides was pipetted into 6 ml of Aquasol-2 (New England Nuclear) scintillation fluid and radioactivity was measured to less than 1% error. Standard buffer, to which CaCl₂, EGTA, 2-mercaptoethanol, or antibody was added, contained 40 mM KCl, 25 mM 2-[Tris(hydroxymethyl)methylamino]-1-ethanesulfonic acid, and 1 mM NaH₂PO₄ (pH 7.4). Standard conditions for determining protease activity are 4 mM CaCl₂ and 10 mM 2-mercaptoethanol in standard buffer incubated with sample and 200 μg of 1⁴C-labeled casein for 30 min at 22°C. Sample antibody was diluted in standard buffer 1:3, 1:10, and 1:25.

Statistical Analysis. Means (± SD) were calculated for muscle and nerve samples: first in each animal and then in the control and treated animal groups at the 3- and 6-month biopsy points. This was done separately in the right and the left hand. For nerve conduction velocity measurements, a mean value (± SD) was calculated for the control group and for the treated group, in both hands at each time point tested. For toxicologic testing, means (± SD) were obtained in the control and treated animals after six monthly values were obtained. To test the significance of sample means between control and treated animals a one-way analysis of variance was used. Where handedness was a variable, right hands were compared only to right and left hands were compared to left. Thus, P values expressed in the text and in graphs reflect the significance of sample means.

RESULTS

Muscle Morphology. Opponens pollicis myofibers biopsied at 3 months in the left (unoperated) hand of control animals showed typical histological structure and a diameter of 40.51 ± 2.7 μm (mean ± SD). Opponens myofibers biopsied at 3
months in the left (unoperated) hand of leupeptin-treated animals were somewhat larger, showed normal structure, and had a diameter of $46.2 \pm 3.6 \mu m$ (mean $\pm$ SD). The difference between the mean values was statistically significant ($P = 0.025$).

The opponens pollicis myofibers biopsied at 3 months in the right (nerve-repaired) hand of control animals showed histological and ultrastructural features consistent with denervation, including persistent myofiber angulation, large round myofibers, central nuclei, and increased fibrosis (Fig. 1A). These myofibers had a diameter of $22.27 \pm 2.68 \mu m$ (mean $\pm$ SD). In contrast, the nerve-repaired right opponens myofibers in treated animals were larger and averaged $37.60 \pm 3.87 \mu m$ in diameter (mean $\pm$ SD). These myofibers generally retained their typical polygonal shape with minimal denervation changes (Fig. 1B). The mean difference in diameters was significant ($P = 0$).

At 6 months, abductor pollicis myofibers demonstrated similar results. The left control myofiber diameter was $40.57 \pm 3.72 \mu m$ (mean $\pm$ SD), and left treated myofibers were larger with a diameter of $45.72 \pm 2.29 \mu m$ (mean $\pm$ SD). This difference was statistically significant ($P = 0.03$). The denervated abductor pollicis myofibers in the right (nerve-repaired) hand showed a striking difference in diameter between controls ($28.25 \pm 0.71 \mu m$) and treated ($43.65 \pm 5.30 \mu m$) animals (mean $\pm$ SD; $P = 0$).

**Muscle Immunocytochemistry.** Light and electron microscopic immunocytochemistry for CANP in left opponens and left abductor myofibers of control animals revealed the enzyme was associated with endomysial collagen fibrils, the myofiber basal lamina, sarcolemma, and Z-bands, as we have reported (18) (Fig. 2A). CANP was not detected in the left opponens and left abductor myofibers of leupeptin-treated animals (Fig. 2B).

Immunocytochemistry of the denervated opponens and abductor in the right (nerve-repaired) hand of control animals showed that CANP was widely dispersed within myofibers (Fig. 3). CANP was not detected in denervated opponens and abductor myofibers in the right (nerve-repaired) hand of leupeptin-treated animals (Fig. 4). In all muscle sections incubated in adsorbed antibody, CANP was not detected.

**Nerve Morphology.** As a morphologic indicator of axon regrowth, axons were counted $3.0 \, cm$ distal to the nerve repair on the right median nerve branch to the opponens (3 months) and the right branch to the abductor (6 months). Axons were also counted in the left nerves at the corresponding anatomic location of all animals to establish normal values. Ten randomly selected light microscopic and 10 randomly selected electron microscopic section fields per nerve were quantitated. Constant magnification of fields was used for light and electron microscopy.

The mean number of axons was consistently higher and statistically significant in the right leupeptin-treated nerves compared to the right control nerves at both biopsy intervals. The only exception to this was the number of unmyelinated axons at 6 months in the abductor branch where there was no statistical significance between treated and control animals. The effects of treatment on axon numbers in the left (unoperated) hand of all animals were not significant. Figs. 5 and 6 summarize these data.

As an additional morphologic indicator of distal axon regrowth, mean thickness of the myelin sheath was measured.
in the opponens nerve (3 months) and abductor nerve (6 months) in both hands of all animals. The thickness of the myelin sheath (mean ± SD) in the left nerve branches was not affected by leupeptin treatment as follows: left opponens control thickness was 0.72 ± 0.10 μm; left opponens treated thickness was 0.82 ± 0.20 μm (P = 0.490); left abductor control thickness was 0.87 ± 0.006 μm; and left abductor treated thickness was 0.80 ± 0.006 μm (P = 0.440).

In contrast, in the right opponents and abductor nerve branches, myelin sheath thickness was affected by leupeptin treatment. At 3 months, in the right opponents nerve, the thickness was 0.15 ± 0.14 μm in controls but was significantly increased to 0.51 ± 0.17 μm in treated animals (mean ± SD; P = 0.007). By 6 months, the thickness in the right abductor branch in controls was 0.41 ± 0.006 μm whereas the

leupeptin-treated animals had an increased thickness in the right abductor branch of 0.64 ± 0.004 μm (mean ± SD; P = 0.016).

Nerve Immunocytochemistry. Light and electron microscopic immunocytochemistry on control opponents and abductor nerves in the left (unoperated) hand revealed CANP to be associated with endoneurial collagen fibrils, basal lamina of Schwann cells of myelinated and unmyelinated axons, axolemma, and neurofilaments of both axon types. CANP was not detected in left opponents and abductor nerves of leupeptin-treated animals and in sections of nerve incubated in adsorbed antibody, which is consistent with our previous study (18).

In denervated opponents and abductor nerves of control animals, CANP was detected in myelinated and unmyelinated axons in association with basal lamina, axolemma, and neurofilaments and was most pronounced 3 months after nerve repair. In the denervated opponents and abductor nerves of leupeptin-treated animals, CANP was not detected after nerve repair.

Nerve Conduction Velocities. At 6 and 8 weeks after nerve repair, leupeptin-treated animals showed faster motor and sensory conduction velocities across the repair site when compared to control animals. These results are summarized in Figs. 7 and 8. Conduction velocity testing in the left (unoperated) hands of all animals established normal values.

Biochemistry. Antibody to CANP diluted at 1:3, 1:10, 1:25 completely inhibited all protease activity.

Toxicology. Complete blood counts with clotting times after intramuscular administration of 18 mg/kg, twice daily, for 6 months were not affected in treated animals when compared to controls. In controls, mean values (± SD) were as follows: leukocytes, 5.09 ± 0.520 x 10³ cells per mm³; erythrocytes, 5.85 ± 0.008 x 10⁶ cells per mm³; hematocrit, 42.4 ± 0.44%; platelets, 3.24 ± 0.200 x 10⁵ cells per mm³;

![Fig. 5. Myelinated axons 3.0 cm distal to the nerve repair site by light microscopy. Axons in leupeptin-treated animals are significantly increased over controls. At 3 months, P = 0.019; at 6 months, P = 0.040.](image1)

![Fig. 6. (A) Myelinated axons 3.0 cm distal to the nerve repair site by electron microscopy. Axons in leupeptin-treated animals are significantly increased over controls. At 3 months, P = 0.019; at 6 months, P = 0.040. (B) Unmyelinated axons 3.0 cm distal to the nerve repair site by electron microscopy. There is a significant increase in leupeptin-treated animals in the opponents nerve at the 3-month interval. At 3 months, P = 0.002; at 6 months, P = 0.05. Solid arrows, mean normal number of axons in the left opponents nerve; open arrows, mean normal number of axons in the left abductor nerve. Stippled bars, control; hatched bars, leupeptin-treated animals.](image2)

![Fig. 7. Motor nerve conduction velocities across the nerve repair site in the right hand are significantly increased in leupeptin-treated animals. At 6 weeks, P = 0.001; at 8 weeks, P = 0.002. Solid arrow, mean normal velocity in the left hand; control; , leupeptin-treated animal.](image3)

![Fig. 8. Sensory conduction velocities across the nerve repair site in the right hand are significantly increased in leupeptin-treated animals. Solid arrow, mean normal velocity in the left hand; control; , leupeptin-treated animal. At 6 weeks, P = 0; at 8 weeks, P = 0.003.](image4)
prothrombin time, 10.3 ± 0.14 sec; partial thromboplastin time, 27.9 ± 0.70 sec; C3, 9.4 ± 1.5 mg/dl. In treated animals, mean values (± SD) were as follows: leukocytes, 5.54 ± 0.65 × 10^3 cells per mm^3; erythrocytes, 6.31 ± 0.06 × 10^6 cells per mm^3; hematocrit, 44.4 ± 0.35%; platelets, 3.29 ± 0.25 × 10^3 cells per mm^3; prothrombin time, 10.6 ± 0.14 sec; partial thromboplastin time, 27.3 ± 0.58 sec; C3, 10.7 ± 1.8 mg/dl. Differences in all means between treated and control animals were not significant.

**DISCUSSION**

Our results using a primate median nerve model suggest that the tripeptide leupeptin partially inhibits muscle denervation atrophy and enhances axon regrowth after immediate epineural nerve repair. This was demonstrated morphologically in treated animals by increased myofiber diameters with retention of normal myofiber morphology, increased numbers of myelinated and unmyelinated axons distal to the repair, and in increased myelin sheath thickness of distal axons.

The mechanism of leupeptin action in denervated muscle and nerve appears to be the direct inhibition of CANP in these tissues. The anti-CANP antibody used in this study completely abolished CANP protease activity, suggesting that anti-CANP and leupeptin bind to a similar site on CANP. This would explain the lack of immunoreactivity against anti-CANP in muscle and nerve of leupeptin-treated animals. Our previous study in normal tissue (18) and present immunocytochemical results indicate that the site of CANP in normal muscle are the Z-band, sarcolemma, and basal lamina. After denervation, nerve repair, and leupeptin treatment, it appears that the disassembly of the myofiber is prevented by inhibition of CANP at these sites. Thus, we suggest that the myofiber basal lamina and end-target muscle are retained. These structures are known to be positive neurotrophic reinervation targets (22-24). In normal primate nerve, the basal lamina, axolemma, and neurofilaments show immunoreactivity for CANP (18). After denervation, nerve repair, and treatment, leupeptin inhibited CANP associated with these structures. We suggest that the enhanced axon regrowth observed after repair and CANP inhibition by leupeptin may relate to retention of the axonal basal lamina in distal nerve segments. Leupeptin treatment may preserve this structure known to be a regenerative substrate for axonal adhesion and elongation (25, 26).

The ultimate determination of efficacy of any adjunctive treatment for peripheral nerve repair will of course relate to subsequent functional recovery. In this study, motor and sensory conduction were positively affected by leupeptin treatment. This was shown by faster nerve conduction velocity values in treated animals when compared to controls. These findings appear to correlate with the positive morphologic observations after treatment. Increased numbers of distal axons, especially myelinated axons possessing thicker myelin sheaths, are known to conduct more rapid depolarization impulses (27).

Finally, the dose, frequency, and method of administration of leupeptin used in this study were based on prior serum absorption studies in monkeys (13), indicating that the tripeptide is rapidly absorbed in serum 1 hr after intramuscular administration with a linear decline by 24 hr to only trace amounts. We have also noted (13) that a single intramuscular dose of 24 mg/kg negatively affected clotting profiles, presumably by the leupeptin inhibition of thrombokinase (28). Therefore, our use of 18 mg/kg, twice daily, was designed so that ensure levels of 2-4 μg/ml were consistently maintained. This treatment did not affect normal hematology, clotting, or C3 values.

With regard to C3 values, it appears that our twice-daily intramuscular leupeptin injections did not initiate formation of adverse antigen–antibody (immune) plasma complexes. These complexes may bind complement in a number of host tissue compartments, release C3, and ultimately cause hydrolytic enzyme destruction of tissues. Since no difference in C3 values was noted between leupeptin-treated and control animals, formation of immune plasma complexes does not appear to occur after leupeptin treatment.

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