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Calpain Inhibitors as Neuroprotective Agents in Neurodegenerative Disorders

Alfred Stracher, Ph.D.

Department of Biochemistry, Health Science Center at Brooklyn, State University of New York, Brooklyn, NY

Abstract: It seems plausible to hypothesize that in all forms of neurodegeneration or other forms of tissue degeneration, a common pathway exists which when deciphered could lead to our understanding of a variety of diseases which result in tissue necrosis as well as offer potential for therapeutic intervention.

A relatively recent interest has been our preliminary studies on the role of neurodegeneration in hearing loss and tinnitus, particularly that associated with noise. These studies grew out of a collaboration emanating from early discussions with Professor Abraham Shulman of the State University of New York, Health Science Center at Brooklyn, Department of Otolaryngology, and Dr Richard J. Salvi, of the Center for Communication Disorders and Sciences, Hearing Research Laboratories, State University of New York at Buffalo.

Further studies in this very promising area of research are continuing for noise induced hearing loss protection and tinnitus control. A brief review of calpain is presented.

Keywords: leupeptin; calpain; neurodegeneration; proteasis; tinnitus

It seems plausible to hypothesize that in all forms of neurodegeneration or other forms of tissue degeneration, a common pathway exists which when deciphered, could lead to our understanding a variety of diseases which result in tissue necrosis as well as offer potential for therapeutic intervention.

In recent years progress toward elucidating this common pathway has been accelerated through the studies of a number of laboratories, including our own, on the role of the protease calpain in this process. Thus, in a variety of such disorders as stroke, spinal cord injury, traumatic nerve injury, Parkinson's disease, amyotrophic lateral sclerosis (ALS), Alzheimer's disease, muscular dystrophy, cataract formation, and others, unregulated calpain proteolysis, initiated via dysregulation of calcium ion homeostasis, participates in the pathogenesis of these diseases and is a potentially unifying mechanistic event.

Calpains are a homologous family of Ca^{2+} activated proteases of which the two most common forms are ubiquitous and constitutively expressed. The two forms, referred to as calpain I and calpain II also are called μ-calpain and m-calpain, the former being activated at micromolar concentrations (1–20 μM) of Ca^{2+} and the latter at millimolar concentrations intracellularly (250–750 μM). Both forms are expressed as heterodimers, consist of an 80 kD catalytic subunit and a 30 kD regulatory subunit, and exist in most tissues in the body, being particularly rich in muscle, where calpain was discovered. The proportions of the two isoforms varies among different tissues. In the central nervous system, at least 95% of calpain exists as the m-form, which apparently is similar in most tissues except for the erythrocyte, which contains exclusively μ-calpain, and the platelet, which contains 90% μ-calpain. Although calpain is activated in most tissues by calcium concentrations that exceed intracellular levels under normal homeostasis, phospholipids and glycolipids have been found to reduce the calcium requirement in vitro, suggesting a possible activation mechanism by association with the membrane.

Although calpain has been studied extensively since its discovery [1], its precise role in normal cellular function still is unknown. Calpain does not cause ex-

Reprint requests: Alfred Stracher, Ph.D., Department of Biochemistry, Health Science Center at Brooklyn, Box 8, 450 Clarkson Avenue, Brooklyn, NY 11203.

tensive proteolysis when measured against a variety of substrates in vitro. Rather, its action appears to be more limited, resulting in partial degradation rather than destruction of a protein. This type of action suggests that calpain activation by Ca^{2+} may be more in the capacity of a modulator of biological processes as a result of calcium flux. Limited proteolysis by calpain may act also as a signal for initiating a more extensive degradative process by invading cells, as has been suggested by our studies in muscular dystrophy, or in the normal course of events in tissue protein turnover [2,3].

Although the precise biological role of calpain in physiological and pathological conditions is not known, the current evidence indicates that calpain activity is directed preferentially directed to proteolytic modification of cytoskeletal-membrane proteins and other proteins located at the inner surface of the plasma membrane. In addition, in vitro studies have shown that calpain can produce specific limited proteolysis of myofibrillar proteins, cytoskeletal proteins, hormone receptors, protein kinases, and several other proteins, such as neurofilament protein, vimentin, and hormone receptors. In spite of a detailed structural knowledge of calpains, the precise structural details of the protein substrates which are recognized by calpains and the nature of the peptide bond cleaved by these enzymes are not known [4].

Since unregulated activity of calpain would seriously damage the cytoskeleton structure and result in serious damage to the cells, it is not surprising that calpain activity is very tightly regulated. Even calpain I (μM form) requires 1 to 20 μM ionic calcium for half maximal activity which exceeds the normal physiological intracellular concentration of the metal ion, and therefore, presumably calpain is inactive inside the cells. It has been suggested that under certain conditions, the 80kD subunit of calpain I can undergo autoproteolysis, resulting in the removal of a 5kD fragment. The resulting 75 kD fragment requires considerably less Ca^{2+} ion for its proteolytic activity. The exposure of different cells to various agonists such as angiotensin II, alpha adrenergic agents, thrombin, and plasmin that can increase intracellular Ca^{++} levels then may activate the proteolytic activity of the 75 kD fragment. Another important regulation of calpain activity is accomplished by its natural endogenous inhibitor, calpastatin. The amino acid sequence of calpastatin from various species is highly conserved, contains repetitive peptide domains and each domain is capable to bind and inactivate one molecule of calpain. However, a detailed knowledge of calpain-calpastatin interaction and the importance of this interaction on the biological activity of calpain in physiological and pathological conditions at the cellular level remains to be elucidated.

CALPAIN INHIBITORS AS THERAPEUTIC AGENTS

Commencing with our early studies on the use of protease inhibitors of calpain as potential therapeutic agents in muscle wasting diseases and myasthenia gravis, subsequent studies in a variety of laboratories have demonstrated the validity of these early studies when applied to a growing number of neurodegenerative diseases. Be it by direct evidence or by strong circumstantial evidence, calpain proteolysis clearly contributes to many neurological disorders [2,3]. Use of calpain inhibitors clearly implicates the Ca^{2+}/calpain system in focal and global ischemia, peripheral nerve injury, and spinal cord injury. Although the evidence is less direct, calpain proteolysis may contribute to the neurodegeneration associated with Alzheimer's disease, Parkinson's disease, multiple sclerosis, Huntington's disease as well as amyotrophic laterial sclerosis. More work clearly is required to establish a direct link between these diseases and the Ca^{2+}/calpain hypothesis[1].

In order to demonstrate the feasibility of the approach we have taken in using the calpain inhibitor leupeptin as a therapeutic agent, I will describe two areas of research in which we have been engaged over the last 20 years, one of which is quite recent.

Neuromuscular Degeneration

Our laboratory has been concerned with the mechanism of muscle protein degradation for the past 17 years. We were among the first to recognize the therapeutic potential of protease inhibitors, especially the low molecular weight microbial inhibitors first isolated and characterized by Umezawa and coworkers. We have been especially interested in the tripeptide inhibitor leupeptin (acetyl-leucyl-leucyl-argininal) which has been shown to be a potent inhibitor of the Ca^{2+}-activated protease, calpain. This protease, first described in muscle by Goll and coworkers, is thought to carry out one of the earliest proteolytic events in the "cascade" of proteolysis, eventually leading to muscle protein turnover. It is thought to act at the level of the myofibrillar organization since myosin, actin and α-actinin are poor substrates for this enzyme. Its ability to "nick" the myofibril may disrupt its organization sufficiently to make the contractile proteins now accessible to other degradative enzymes present in muscle tissue. Otherwise, little is known of calpain's "site" of action in muscle or in other cellular systems. Since it is a ubiquitous enzyme, its role might be in protein turnover or regulation of cellular events by limited proteolytic processing. It is not known whether it acts at the level of the membrane or cytosol although some studies suggest translocation

to the membrane on activation. Despite this lack of mechanistic knowledge, it is known that in neuromuscular degenerative conditions, such as muscular dystrophy or denervation atrophy, the activity of this enzyme is increased several fold suggesting that it plays some essential role in the increased protein degradation which accompanies these disorders.

It was this type of information, which led us to consider the possibility that protease inhibitors might have some benefit as therapeutic agents by lowering the protease activity and consequently decreasing the atrophy. At the time, little was known of the role of calpain, and most studies pointed to the lysosomal proteolytic enzymes Cathepsins D, B, H, L as being most concerned with degradative events. Our initial studies in tissue culture, therefore, utilized the inhibitors pepstatin, specific for Cathepsin D and leupeptin, which inhibits cathepsins B, H, and L. In a series of publications starting in 1976 we were able to show a delay in atrophy and degeneration of muscle cells in culture using a combination of pepstatin and leupeptin. We later showed that leupeptin alone was sufficient to achieve this result. We went on to show that similar effects could be seen *in vivo* when leupeptin was injected directly into the affected muscles of dystrophic chickens or by intraperitoneal injection into genetically dystrophic mice [3]. A more striking effect could be seen in denervation atrophy [3]. If the sciatic nerve of a rat is severed, approximately 40% of the muscle mass will atrophy over a 12 day period. If a similar experiment is carried out, however, and leupeptin is injected into the affected muscle, only a 3–8% loss in muscle mass takes place. These studies were verified by measuring myofiber diameters.

In all these studies a direct correlation of calpain inhibition and muscle mass retention could be established. Calpain activities could be measured on isolated muscles using a C^{14}-casein assay developed in our laboratory and described in several of our publications. A number of other laboratories have corroborated our earlier findings concerning Ca^{++}-mediated degradation of muscle. Etlinger [6] and coworkers have shown that increased Ca^{++} can stimulate rates of overall proteolysis in several intact skeletal muscles when incubated in vitro. Calcium ionophore, when incubated with muscle cells, likewise increases overall rates of degradation. Libby and Goldberg [7] in isolated skeletal muscle and in fetal heart preparations, have shown that leupeptin decreased degradation while having no effect on protein synthesis and that in so doing increased the content of functional cell protein. Presumably, these results and our earlier findings suggest an important role for leupeptin sensitive proteases in muscle cellular protein degradation.

Based on these initial findings in a variety of model experiments, our laboratory has concerned itself with the use of leupeptin as adjunctive therapy in medial nerve transection and epineural repair[3]. It has been shown in several laboratories that the influx of Ca^{++} into an injured nerve is the trigger for the activation of calpain, which contributes to Wallerian degeneration, especially of neurofilaments. We have confirmed the calcium-induced degeneration of neurofilaments in vitro, in peripheral nerves of monkeys and humans, further suggesting a role for calpain in this process.

We have extended these studies over the last several years in the Capuchin monkey (Cebus appella) median nerve model[3]. Our first experiments began with a small group of four animals who underwent complete median nerve transection and repair at the wrist, with intramuscular leupeptin treatment of 12 mg/kg once daily. When compared to controls, leupeptin-treated animals showed inhibition of the atrophic process in denervated thenar muscles, increase myelinated and unmyelinated axon counts, increased axon diameters, and increased myelin sheath thickness in distal nerve segments. Increased numbers of Meissner's corpuscles also were observed in treated animals in (median-innervated) skin biopsies. Also, when compared to control animals after nerve repair, more rapid motor nerve conduction velocities in leupeptin-treated monkeys suggested that the enhanced morphological neuromuscular recovery correlated to functional recovery. Leupeptin (12 mg/kg) proved to be efficiently absorbed in plasma and to have no adverse effects on hematology or clogging profiles.

Because our previous studies in rats and in four Cebus monkeys indicated that leupeptin appeared to be effective in enhancing neuromuscular recovery after nerve repair at an intramuscular dose of 12 mg/kg once daily but that clotting profiles were negatively affected (in monkeys) by a single intramuscular dose of 24 mg/kg, the dose midpoint between 12–24 mg/kg, i.e. 18 mg/kg, was chosen to investigate if this higher dose administered more frequently, might exert a more pronounced and positive effect. However, before testing an 18-mg/kg IM dose after nerve repair, our study dealt with the effects of this new and more frequently administered dose of leupeptin on normal peripheral nerve in a monkey (Cebus apella) model.

Five monkeys received 18 mg/kg leupeptin intramuscularly twice daily for 8 months, with a period of 7 hours between each injection. Five monkeys served as controls and were not injected. The chronic effects of leupeptin were studied in normal animals beyond the time point we anticipated would be necessary for treatment after nerve repair.

1. Effects of Leupeptin on Normal Skeletal Muscle and Normal Peripheral Nerve in Primates (2)

Histological analysis of both control and leupeptin-trated flexor carpi radialis of myofibers demonstrated normal cellular structure with typically placed peripheral nuclei. Confirmation of normal myofiber structure was demonstrated by transmission electron microscopy in all samples. Control flexor carpi radialis myofibers had a mean diameter of 45.24 + 2.31 μm at 2 months and 51.60 + 2.1 μm at 4 months. Leupeptin-treated myofibers were noted to be significantly larger: 50.32 + 3.61 μm at 2 months and 64.82 + 2.1 μm at 4 months ($p = 0.002$ at 2 months; $p = 0.011$ at 4 months).

Histological analysis of both control and leupeptin-treated opponens pollicis myofibers from the 8-month time interval revealed normal structure. This was also confirmed by transmission EM. Control opponens pollicis myofibers had a mean diameter of 38.69 + 1.79 μm. As before, leupeptin-treated animals showed larger myofibers at 49.68 + 1.92 μm ($p = 0.003$ at 8 months).

Biochemical analysis of calpain activity in muscle showed that there was a 28–31% decrease in activity in leupeptin-treated flexor carpi radialis when compared to the same muscles in control animals.

2. Neuromuscular Recovery Using Calcium Protease Inhibition (by Leupeptin) After Median Nerve Repair in Primates (2)

To test the effects of leupeptin after nerve repair at the 18mg/kg dose, 10 *Cebus apella* underwent a right median nerve transection at the wrist immediate epineural repair. In five treatment animals the transected nerve was bathed in 18 mg/kg leupeptin in 0.9% saline for two minutes. The median innervated (right) thenar muscles were injected with the same dose of leupeptin. On the first post-operative day, the five treatment animals received the same dose of leupeptin as intramuscular injections in the hind limbs, twice daily for six months. Five control animals had their transected nerved bathed in 0.9% saline. Control animals were not injected with saline since it was previously determined in our prior series of rats and the separate smaller group of four Cebus monkeys that saline injections had no effect on neuromuscular recovery after repair. In all animals, right and left thenar muscle and nerve biopsies were performed at three and six months after repair. The complete data for this study may be founds in the reprint attached in the appendix.

The following summarizes our findings: Nerve repaired monkeys treated with 18 mg/kg leupeptin showed striking morphological results, reflected by increased myofiber diameters with typical polygonal structure, increased myelinated and unmyelinated axon counts distal to the repair, and increased myelin sheath thickness. More rapid motor and sensory conduction velocities in treated animals after repair suggested correlation between morphological and functional recovery. Leupeptin had no adverse effects on hematology or clotting (PT,PTT) profiles at the 18 mg/kg dose. Also significantly, this dose did not affect plasma C_3 values, indicating that repeated treatment did not induce formation of immune plasma complexes.

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

How may leupeptin enhance neuromuscular recovery by its mode of action (inhibition of neural and muscle calcium activated neutral protease)? The mechanism of leupeptin's action in denervated muscle and nerve appears to be the direct inhibition of calcium activated neutral protease in these tissues. In the Cebus appella model, biochemical assay of an antibody to the protease indicates that the antibody and leupeptin completely abolished all protease activity, suggesting competition at a similar site on the protease molecule. This finding may explain our observation that after immunohistochemistry, there was no immunoactivity in muscle and nerve of leupeptin treated animals. Our immunohistochemical results also indicate that the sites of the protease in normal and denervated muscle are the Z-band, sarcolemma and basal lamina. After denervation by nerve transection and subsequent repair, followed by leupeptin treatment, it appears that the disassembly of the myofiber is prevented by inhibition of the protease at these sites. Thus, the myofiber basal lamina and end-target muscle are retained. These structures are known to be positive neurotrophic reinnervation targets. In normal primate nerve, the basal lamina, axolemma and neurofilaments showed calcium protease immunoreactivity. After denervation by nerve transection and subsequent repair, followed by leupeptin treatment, leupeptin inhibited the protease immunoreactivity associated with these structures. It is possibly that the enhanced axon regrowth we have observed after repair and protease inhibition by leupeptin relates to retention of the axonal basal lamina in distal nerve segments. Leupeptin treatment may "preserve" this structure, which is known to be a regenerative substrate for axonal adherence and elongation.

Calpain Inhibitors in Noise-Induced Hearing Loss and Tinnitus

A relatively recent interest has been our preliminary studies on the role of neurodegeneration in hearing loss particularly that associated with noise and tinnitus. These studies grew out of a collaboration emanating from early discussions with Abraham Shulman M.D., of the State University of New York Health Science Center, Brooklyn, Department of Otolaryngology, and Dr. Richard J. Salvi, of the Center for Communication Disorders and Sciences, Hearing Research Laboratories, State University of New York at Buffalo.

This research hypothesized that a class of drugs described as protease inhibitors, particularly of the class called calpain inhibitors and specifically one called LXIC, may provide neuroprotection for noise exposure and associated complaints of the hearing and balance system e.g., hearing loss, tinnitus, and vertigo.

Early results supported this hypothesis and have shown that infusion of LXIC into the cochlea of chinchillas by the use of a mini pump prior to noise exposure at a level of 105dB for a period of up to 2 weeks provided substantial protection particularly when evaluated histologically [5].

Further studies in this very promising area of research is continuing.

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