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Noise, Calpain, Calpain Inhibitors, and Neuroprotection: A Preliminary Report of Tinnitus Control

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Abstract: Frequently, noise-induced hearing loss is associated with the symptom of tinnitus. Preliminary results in the animal model after noise exposure suggest that the calpain inhibitor leupeptin may protect against noise-induced hearing loss. A final common pathway for cell destruction and cell death (i.e., apoptosis) is the calpain hypothesis. Calpain is a normal, intracellular, cytosolic protease activated by excess intracellular calcium. Calpain inhibitors (AK275, AK295) have been shown to provide neuroprotection in the central nervous system. A collaboration of basic science and clinical research efforts focusing on calpain antagonists and inhibitors was established in New York in 1997; the initiators are attempting to develop neuroprotective drug therapy regimens for hearing and balance system complaints, particularly hearing loss, tinnitus, and vertigo. Both calpain inhibitors and antagonists are being developed and are being investigated with perfusion techniques of the inner ear, in vitro and in vivo, for their effects on peripheral and central portions of the cochleovestibular system.

Keywords: calpain, intratympanic drug therapy, leupeptin, neurodegeneration, neuroprotection, protease

The goal of this study is to introduce otologists, neurootologists, and other professionals attempting tinnitus control to a group of proteases called *calpains* and to neuroprotective agents called *calpain antagonists and inhibitors* and to cite their application to attempts to control tinnitus with intratympanic drug therapy (ITDT). The innovative application of calpain antagonists and inhibitors for the treatment of cochleovestibular complaints of hearing loss, tinnitus, and vertigo, highlighted by attempts for tinnitus control, was initiated in New York in 1997 and continues to be supported by the Martha Entenmann Tinnitus Research Center, Inc., as part of an ongoing effort directed at the development of a neuropharmacology for tinnitus control. A collaboration was established in 1997 among Drs. Alfred Stracher and Abraham Shulman of the State University of New York Health Sciences Center at Brooklyn, NY, and Dr. Richard J. Salvi of the State University at Buffalo, NY, the goal being to develop neuroprotective drug therapy regimens for hearing and balance system complaints, particularly hearing loss, tinnitus, and vertigo. The researchers embarked on conducting in vitro and in vivo studies of the neuroprotective effects of calpain antagonists and inhibitors in the inner ear [1–3].

Frequently, noise exposure is accompanied by loss of hearing and the associated complaint of tinnitus. Neuroprotective drug therapy regimens have been applied to such central nervous system (CNS) pathological processes as ischemia, trauma, hemorrhage, and neurodegeneration. Such etiologies are hypothesized to affect the inner ear [3]. The innovative application of such therapeutic regimens for treating the symptom of tinnitus of the severe disabling type and for treating inner ear complaints of hearing loss, vertigo, and ear blockage has been the basis for development of neurochemistry protocols for tinnitus control, including ITDT via the round window. One such category of drugs be-

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ing investigated for noise protection are the calpain antagonists and inhibitors.

This study provides (1) a brief introduction of the group of proteases called *calpains* and their antagonists and inhibitors; (2) a preliminary report of results of neuroprotection with leupeptin, a calpain inhibitor, in an animal model for noise exposure and hearing; and (3) the clinical application of calpain as an innovative treatment method. These antagonists and inhibitors can provide neuroprotection for complaints of the cochleovestibular system. highlighted by attempts for tinnitus control with ITDT for a predominantly cochlear-type tinnitus.

CALPAINS AND CALPAIN ANTAGONISTS

General Information

Neuroprotective drugs are hypothesized possibly to provide neuroprotection to the hearing and balance system for complaint of hearing loss, tinnitus, and vertigo [3]. Support for this hypothesis is provided by basic scientific research efforts with neurotrophic factors [4] and calpain, a cellular protease important in modulating cellular activity of biological phenomena, including cellular pathogenesis, learning and memory immune modulation, mitosis, and apoptosis [5,6].

A pharmacological basis for the efficacy of calpain antagonists includes a property described as *neuroprotection* [3], a term that refers to processes that protect neuronal function from injury or that improve such function after injury. Common etiological agents that cause injury to the CNS have been hypothesized to have similar effects on the inner ear. The chief etiologies to be considered include ischemia, trauma, or hemorrhage and neurodegenerative disease. For the inner ear, such injury is reflected in interference in function secondary to cellular and neuronal death. Clinical complaints include hearing loss, tinnitus, vertigo, and other abnormal auditory and vestibular sensations. Among the clinical neurootological syndromes are Menière's disease, endolymphatic hydrops, fluctuating hearing loss syndrome, sudden hearing loss syndrome, and sudden tinnitus syndrome.

Pharmacological agents considered to be neuroprotective have been identified: calcium channel blockers, free radical scavengers, corticosteroids, antagonists of glutamate *N*-methyl-D-aspartate (NMDA) and non-NMDA receptors, various thrombolytic agents, and calpain antagonists. Neuroprotective agents are proposed for pharmacological therapeutic management for the symptom of tinnitus, particularly of the severe disabling type. Such drugs for the CNS have been developed on the basis of what is known of the neuroexcitotoxic theory of tissue damage. Possibly, interventions aimed at reducing cellular calcium overload can help to protect against excitotoxic death [7].

A class of drugs—protease inhibitors (and specifically the protease inhibitor LXIC)—may provide neuroprotection for noise exposure and associated complaints about the hearing and balance system (e.g., hearing loss, tinnitus, and vertigo) [1, 2].

Proteases and Proteolysis

Proteases are enzymes that attack peptide bonds either in proteins or in polypeptides. They include proteinases, which attack large protein molecules (e.g., albumin, myosin, peptidase [proteases that act on smaller peptide molecules]). Proteases are divided into exopeptidases and endopeptidases. The exopeptidases attack the outer portion of the protein, and the endopeptidases attack the inner portion of the protein molecule [8, 9].

Proteolysis is an extensive process that results in degradation of proteins to amino acids [10–13]. Substrates (e.g., proteins and peptides) contain information that determines their proteolytic susceptibility [6]. Cellular proteolysis is a complex process that occurs in all compartments of cells. Compartments of mammalian cells (e.g., lysosomes, endosomes, secretory granules, membrane formations, transport vesicles, and mitochondria) interact and have multiple ways of controlling proteolytic processes.

The proteases are classified by the nature of the active site in which proteolysis occurs. Four distinct classes of proteases have been identified on this basis: (1) serine, (2) cysteine, (3) aspartic, and (4) metallic [6]. The metalloprotein proteases contain metal ions (e.g., usually zinc) at the active center [14, 15].

The functions of cellular proteases are diverse [6]. Essentially, proteases function to create biologically active molecules or to destroy biologically active proteins and peptides. The second (destructive) group are catabolic proteases that have a role in removing defective or abnormal or normal polypeptides from cells. These two general types of protease functions do not interfere with the multiple processes in which the cellular proteases participate. Such processes include reorganization of cytoskeleton, myoblast fusion and differentiation, memory, protein synthesis, hormone activation and inactivation, fertilization, growth and aging, and creation of immunologically recognizable molecules and degradation of endocytized material and necrosis. Furthermore, cellular proteases play an important role in such diseases as muscular dystrophy, diabetes, cachexia, cancer, and multiple sclerosis [2, 16–20]. The proteases produce irreversible modifications that influ-

ence physiological processes involved in essential biological systems.

The term *protease* is synonymous with *peptide hydrolase.* These terms include all enzymes that cleave peptide bonds. Proteases can be subdivided into exopeptidases and endopeptidases. Exopeptidase action is directed at the amino or carboxyl termini of the peptide. Endopeptidase enzymes cleave peptide bonds internally in peptides and usually cannot accommodate the charge the amino or carboxyl termini amino acids at the active site. The term *endopeptidases* has been recommended to be used synonymously with *protease.*

A further classification of proteases is based on the effects of protease inhibitors on enzyme activity [6]. Serine proteases are inhibited by diisopropyl fluorophosphate. Cysteine proteases are inhibited by low concentrations of P-hydroxy mercuribenzoate and alcolating reagents. Aspartic proteases are inhibited by pepstatins. Metalloproteases are inhibited by chelating agents (e.g., ethylenediaminetetraacetic acid). Metal chelators may inhibit metal-activated proteases in addition to metalloproteases. Metal-activated proteases bind metals loosely and usually contain cysteine or serine as the catalytic residues. Often, metals have to be added during purification procedures for metal-activated enzymes.

In general, the cysteine proteases are true intracellular proteases usually found in the cytosol or in lysosomes. The highly reducing environment in cells is important for their function. Lysosomes enzymes from macrophages are released at sites of inflammation and may damage normal tissue and structural proteins in the area.

The metal dependence of the metalloproteases is not clear. Most are inhibited by metal chelators and are termed *metal-dependent proteases.* Zinc, manganese, and cobalt (but not calcium) prevent inactivation by metallochelators [21].

Calpains

The calpains (calcium-dependent papain-like proteinases) are a group of cysteine endopeptidases that require calcium ions for activity [2, 6, 16, 17, 20–22]. Calpains may be related more closely to one of the other families of cysteine proteases.

The calpains are a homologous family of calcium-activated proteases, the two most common forms of which are termed *calpain I* and *calpain II.* They also are called *micromolar calpain* and *millimolar calpain.* Microcalpain is activated at micromolar concentrations (i.e., 1–20 μM of calcium) and calpain II at millimolar concentrations intracellularly (250–750 mM). The small subunit is the same in both enzymes, whereas the larger subunits are the products of different genes [23, 24]. A tissue may contain either or both forms of the enzymes. Calpain exists in most tissues in the body, particularly in rigid muscle, where calpain was discovered. The two isoforms vary among different tissues.

In the CNS, at least 95% of calpain exists as the m-form. It is similar in most tissues except in the erythrocyte, which contains exclusively microcalpain, and the platelet, which contains 90% microcalpain.

The calpains do not appear to have general proteolytic activity but rather provide specific limited cleavage of substrates, giving rise to specific physiological responses. For example, calpains have been implicated in myloblast fusion. In brain membrane, associated calpain II will degrade fodrin when stimulated by an influx of calcium into a neuron [25, 26]. This results in a disruption between the cytoskeleton and the membrane, causing reorganization of the cell structure. Such changes are thought to be involved ultimately in establishing long-term memory.

Regulation of cellular protease activity is affected in many ways. Among these are compartmentalization, synthesis, and degradation of proteases; inhibitors and activators; metabolite concentration; and substrate susceptibility. Endogenous intracellular inhibitors are considered to be important for the control of cellular proteases. Fluctuation in protease activities in cells can be due to changes in inhibitor rather than protease concentrations and also to induction of proteolytic activities. Two types of polypeptide inhibitors recently discovered in cells are *cystatins* and *stefins* [27], which inhibit lysosomal cathepsins, and *calpastatin*, an inhibitor of calpains.

Calpain is a normal, intracellular, cytosolic protease activated by excess intracellular calcium. Ischemic injury damages calcium homeostasis. Increased glutamate results in excessive calcium entry into the cell by NMDA and α-methyl-propionic acid receptors, voltage-gaited ion channels, or activated intracellular calcium pods.

The precise biological role of calpain is not known. Current evidence indicates that calpain activity is directed preferentially to proteolytic modification of cytoskeletal membrane proteins and other proteins located at the inner surface of the plasma membrane. 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.

Calpain initiates intracellular proteolysis and destroys intracellular and membrane proteins. A final common pathway for cell destruction and cell death (i.e., apoptosis) is the calpain hypothesis [22, 29].

Regulation of calpain activity is controlled tightly by the intracellular concentration of calcium. Calpain activation in most tissues occurs 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. The activity of calpain appears to be limited, resulting in partial degradation rather than destruction of a protein [2]. This type of action has been suggested by Stracher [2] to reflect calpain activation by calcium, and to be more in the capacity of a modulator of biological processes as a result of calcium flux. Limited proteolysis by calpain may signal initiation of more extensive degrading processes by invading cells. This effect is suggested by work on muscular dystrophy and in the normal course of events in turnover of tissue protein. Calpain is presumed to be inactive inside the cell. Exposure of the cell to different agonists that increase intracellular calcium levels activates calpain's proteolytic activity.

Another regulation outside of calcium for calpain activity is its natural endogenous inhibitor, calpastatin [30]. Calpastatin is a protein containing repetitive peptide domains. Each domain is capable of binding and activating one molecule of calpain. The specific interaction of calpain-calpastatin and its importance for biological activity of calpain in physiological and pathological conditions at a cellular level is under investigation. Calpastatin is a specific protein inhibitor of the calpains. It is equally effective in inhibiting calpain I and calpain II and does not inhibit any other type of protease [30]. Calpastatin binds to the large subunit of calpain in the presence of high concentrations of calcium and is not broken up by the protease. The mechanism of action of the inhibitor is not understood. An activator of calpain has been isolated from brain [31].

Several calpain antagonists have been shown to be neuroprotective both in vitro and in vivo during ischemia in brain, spinal cord, and peripheral nerve injury. Stracher [2] demonstrated that oral administration of leupeptin, a calpain inhibitor, improved muscle recovery and neuron recovery after median nerve section and repair. Long-term administration of leupeptin did not cause any adverse effects [16, 17].

CALPAIN FINAL PATHWAY AND APOPTOSIS

The calpain final common pathway for apoptosis is theorized to underlie basic processes of ischemia, trauma, hemorrhage, neurodegeneration, and protein modification—all of which affect membrane permeability by increasing such permeability, producing glutamate, and increasing calcium entry into the cell [22, 29, 32]. Processes involved in apoptosis, initiated by the underlying etiologies of ischemia, trauma, hemorrhage, and neurodegeneration in the inner ear, are hypothesized to became clinically manifest by complaints highlighted by the symptom of tinnitus [3].

Calcium is required for the function of all cells in the body. Precise calcium homeostasis at intracellular levels is critical for many neuronal processes. The glutamate neurotoxicity theory involves calcium hemostasis. It is modeled as a three-stage process analogous to long-term potentiation [7]. In the induction stage, extracellular glutamate initiates cell death by activating neuronal membrane receptors and triggering a set of defined intracellular derangements, particularly intracellular calcium overload. In the amplification stage, modulatory events amplify the derangements, increase their intensity, and recruit additional neurons into the injury process. In the expression stage, death is expressed when these derangements set in motion the final cascade directly responsible for neuronal disintegration, described as *calcium cascade neurotoxicity.*

Calpain activation results from an increased cytosolic concentration with consequent widespread degradation of multiple substrates (e.g., receptors, kinases, cytoskeleton) resulting in multiple cellular dysfunction, instability, and cell death [2]. An interaction may be considered to take place between protein synthesis and protein degradation. This dynamic interaction is continuous. An imbalance leads to dysfunction involving all tissues. Amino acids are the building blocks for protein synthesis on which protein degradation is superimposed. An imbalance between protein synthesis and protein degradation leads to dysfunction. When synthesis is greater than degradation, hypertrophy results. When degradation is greater than protein synthesis, atrophy results.

Products of protein degradation can be classified as extracellular or intracellular [2]. Extracellular protein degradation products include the digestive proteases. Intracellular protein degradation products can be subdivided into lysosomal or cytoplasmic. For example, lysosomal intracellular products of degradation include proteases, cathepsins, and the like. Nonlysosomal protein degradation is manifested by proteosomes, calpains, metalloproteases, and caspases.

Caspases are a family of proteases containing the amino acid cysteine in their active site. Target proteins are cleaved at specific aspartic acids and so are called *caspases* [33]. The caspases have been identified as critical mediators of apoptosis in mammals. Multiple protein substrates of caspases have been found. The

functional significance of the substrates is poorly understood, though they are involved in cleavage of multiple proteins. None are known to have direct physiological significance in the morphological changes and nuclear degradation that are hallmarks of apoptosis. The actin-modulating protein gelsolin is the most prominent direct substrate of caspase 3 in murine embryos [22, 34]. Data indicate that the gelsolin fragment mediates in part the morphological changes of apoptosis. Blockage or enhancement of gelsolin cleavage may retard or increase apoptosis (or do both) in multiple cell types. Gelsolin is found widely in adult mammalian tissues. It is downregulated in many human neoplastic lesions. Observations suggest that gelsolin downregulation in tumors may be a mechanism by which tumors escape apoptosis.

ANIMAL MODEL NOISE-INDUCED HEARING LOSS AND EAR PROTECTION

Several calpain antagonists have been shown to be neuroprotective in vitro and in vivo during ischemia and brain, spinal cord, and peripheral nerve injury [17, 35, 36]. Stracher [2] demonstrated a neuroprotective action after oral administration of leupeptin, a calpain inhibitor, in the recovery of muscles and neurons after median nerve transection and repair [16, 17]. No long-term adverse side effects were reported with leupeptin. Its limitation is difficulty in crossing the blood-brain barrier. Initially, leupeptin was investigated for its neuroprotective activity against noise-induced hearing loss [1].

In an experimental setting, leupeptin was introduced into directly the inner ear using an osmotic pump [37]. First, investigation determined whether leupeptin, the carrier solution, or surgery had any negative effects on normal auditory function. The implantation of the pump and perfusion of leupeptin in the chinchilla had no effect on auditory evoked response threshold. This finding suggests that leupeptin has no short-term adverse effect on auditory function.

Second, researchers investigated whether leupeptin could protect cochlear sensory cells from acoustic overstimulation. The leupeptin was administered 4 days prior to the start of noise exposure to optimize its effect. After exposure, hearing loss was assessed at regular intervals. The test animals were sacrificed approximately 4 weeks later and right and left cochleas removed, stained with succinate dehydrogenase histochemistry, fixed, and dissected as a flat-surface preparation [38, 39]. A threshold shift was demonstrated as a function of frequency at 0 days and 7 days after exposure. The preliminary results suggest that leupeptin may protect the ear from acoustic overstimulation.

The anatomical data obtained from the animal in which the thresholds have been demonstrated revealed hair-cell loss in the untreated control ear near the 4-kHz region of the cochlea. However, little evidence of damage was found in the leupeptin-treated ear.

Figure 1A is a surface preparation view of the organ of Corti taken from the untreated ear in the region of maximum damage. Most of the outer hair cells are missing from all three rows of outer hair cells; however, the inner hair cells still are present. Figure 1B shows the same region of the organ of Corti in the leupeptin-treated ear. The inner hair cells and nearly all the outer hair cells were present in the leupeptin-treated ear.

Preliminary results similar to these have been obtained from other animals exposed to the same noise or a high level of noise. Some deterioration in auditory thresholds has been noted when the osmotic pumps were running for more than 14 days. The cause is now under investigation (to determine whether it is due to the depletion of the pump, to degradation of leupeptin maintained in the pump, or to other factors).

Preliminary studies suggest that leupeptin may protect hair cells in the inner ear from acoustic trauma.

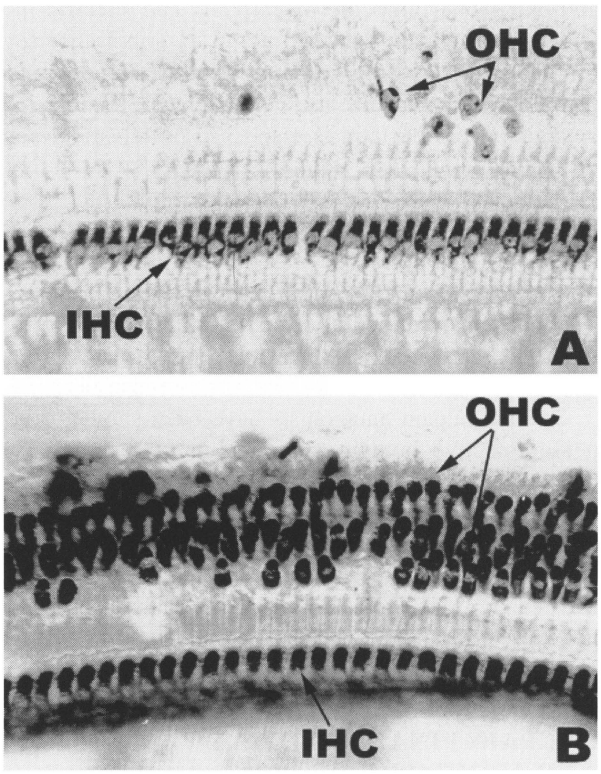

Figure 1. Surface preparation of the organ of Corti in the 4-kHz region of the cochlea. (A) Normal control ear. (B) Leupeptin-treated ear. *OHC* = outer hair cells; *IHC* = inner hair cells.

Histological results to date suggest that leupeptin can reduce significantly the amount of hair cell loss from acoustic overstimulation. Functional measures demonstrate that leupeptin may reduce the amount of hearing loss during the early stage of recovery from acoustic trauma. Its long-term effectiveness requires further investigation. Future investigation will attempt to identify the mechanism underlying neuroprotection and acoustic trauma (i.e., protection against both short-duration, high-level exposure and long-duration, moderate-level exposure). This preliminary report will be followed by both in vivo and in vitro experimentation.

INTRATYMPANIC DRUG THERAPY

Neuroprotective agents have been investigated for various injuries to the CNS (i.e., ischemic stroke, aneurysmal rupture, and traumatic brain and spinal cord injury) [29]. ITDT experience in patients with a predominantly cochlear-type tinnitus were initiated in 1997 and have continued since, with application of the technique as reported by Sakata et al. [40] since 1982. Patient selection is based on completion of the Medical Audiologic Tinnitus Patient Protocol and identification of a predominantly cochlear-type tinnitus.

Patients who have a predominantly cochlear-type tinnitus and report no significant degree of tinnitus control with instrumentation or perfusion (or both) of the inner ear after ITDT with steroids are being recommended for a follow-up attempt at tinnitus control using the microcatheter as developed by Arenberg et al. with a minipump. The pump ensures control of the drug to be delivered (i.e., amount, rate of delivery, and duration of use). Significant improvement was reported with associated complaints of hearing loss, ear blockage, and tinnitus. Originally, Menière's disease patients were treated with this technique for control of vertigo. The application of this technique using dexamethasone specifically for tinnitus has been reported [40]. The efficacy of dexamethasone for tinnitus control is hypothesized possibly to reflect its neuroprotective activity. Calpain inhibitors and antagonists are being developed and should be investigated with perfusion techniques into the inner ear, to observe the effects on the peripheral or central (or both) portions of the cochleovestibular system.

The development of the noise-induced hearing loss model and ear protection using calpain antagonists is considered an innovative application for attempting to develop a neuropharmacology for a predominantly cochlear-type tinnitus. The approach melds both basic scientific and clinical research. Preliminary data in the noise-induced animal model protected by the calpain antagonist leupeptin are encouraging. Processes involved in apoptosis initiated by the underlying etiologies of ischemia, trauma, hemorrhage, and neurodegeneration are hypothesized to result in a series of inner-ear complaints highlighted by tinnitus.

Neuroprotective drug therapy directed at the calpain protease final common pathway for cell destruction is now being investigated. The clinical application of calpain inhibitors and antagonists highlighted by the calpain antagonist LX1C is part of an investigation for control of a predominantly cochlear-type tinnitus (unpublished proposal) This approach reflects new neurochemistry protocols in attempting tinnitus control [3].

CONCLUSIONS

Neuroprotective drug therapies for CNS etiologies of ischemia, hemorrhage, and trauma are hypothesized to have innovative applications for control of the symptom of tinnitus, particularly of the severe disabling type. Such neuroprotective drug therapy should be considered for attempts at tinnitus control, for prevention and prophylaxis, and for short- and long-term treatment.

Preliminary data in the noise-induced hearing loss animal model with the calpain antagonist leupeptin suggest neuroprotection and that underlying mechanisms of tinnitus production may be influenced in both the peripheral and the central portions of the cochleovestibular system. ITDT for inner ear disease treatment and particularly for tinnitus control is recommended. Tinnitus control for a predominantly cochlear-type tinnitus may be achieved by intratympanic application of drugs for perfusion of the inner ear. This modality of therapy method is hypothesized to produce secondary plastic changes within the central auditory system.

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