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Mitochondrial impairment induced by 3-nitropropionic acid is enhanced by endogenous metalloprotease activity inhibition in cultured rat striatal neurons

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HIGHLIGHTS

- We investigate endogenous metalloproteases role in neuronal mitochondrial activity.
- Metalloprotease inhibition potentiates mitochondrial impairment induced by 3-NP.
- NMDAR undergoes relocalization after 3-NP and metalloprotease inhibition.

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ABSTRACT

Metalloproteases from the metzincin family mediate molecule processing at the cell membrane termed ectodomain shedding (ES). This mechanism enables the generation of intracellular and extracellular fragments from cell membrane molecules that exert additional functions involved in cell processes including cell death, beyond those of full length molecules. Micotoxin 3-nitropropionic acid (3-NP) induces striatal neuronal degeneration *in vivo* and *in vitro* through mitochondrial complex II inhibition. In this study, we hypothesized that metalloproteases regulate mitochondrial activity in cultured rat striatal neurons undergoing degeneration. To test this idea, striatal neuronal cultures characterized by NeuN and GAD-67 expression were treated with 3-NP together with the metalloprotease inhibitor GM6001 and their mitochondrial activity was evaluated by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay. Our results showed that metalloprotease inhibition potentiated mitochondrial activity impairment induced by 3-NP whereas the inhibitor alone had no effect. These results indicate that metalloproteases regulate and promote mitochondrial functionality in striatal neurons undergoing degeneration induced by 3-NP. Since NMDA receptor is involved in the excitotoxic neuronal death triggered by 3-NP and is known to undergo ES, we analyzed NMDAR subunit NR1 phenotypic distribution by immunofluorescence. 3-NP and GM6001 induced abnormal perinuclear NR1 accumulation that was not observed with 3-NP or GM6001 alone. This observation suggests that metalloproteases are involved in NR1 cellular reorganization induced by 3-NP, and that their inhibition results in abnormal NR1 distribution. Together results indicate that endogenous metalloproteases are activated during striatal neurodegeneration induced by 3-NP eliciting an adaptative or compensatory response that protects mitochondrial functionality.

1. Introduction

There are two different metalloprotease families: the matrix metalloproteases (MMP) and the a disintegrin and metalloproteases (ADAMs), from the superfamily of Metzicins. Almost 30 MMPs have been reported initially described by their ability to cleave extracellular matrix molecules; however, their catalog of substrates has been diversified and includes cell membrane molecules [2,23]. On the other hand, more than 30 ADAMs have been reported and were first described as potential integrin-binding proteins involved in sperm–egg fusion although now are known to cleave cell membrane molecules [23]. MMPs and ADAMs

Abbreviations: 3-NP, 3-nitropropionic acid; ADAM, a disintegrin and metalloprotease; DIV, days *in vitro*; ES, ectodomain shedding; GAD-67, gamma-amino butyric acid decarboxilase-67; GFAP, glial fibillary acidic protein; GM6001, Ilomastat; MMP, matrix metalloprotease; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide; NeuN, neuronal nuclear antigen; NR1, NMDAR subunit 1; PDL, poly D-lysine; RIP, regulated intracellular proteolysis.

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are dependent upon Zn^{++}, share similar catalytic domains and prodomains, and are known to be activated by free radicals or intracellular Ca^{++} rise ([23,28] and references therein). In recent years, metalloproteases have gained attention because they mediate ectodomain shedding (ES). This cell mechanism is defined as the extracellular domain (ectodomain) proteolytic release from cell membrane molecules. Metalloproteases, also known as sheddases, cleave membrane molecules in the stem region, <30 amino acids upstream the transmembrane domain [23]. For some time, it was believed that ES down-regulated cell membrane molecule function, since it was considered to fully depend on their ectodomain, thus ES was believed to be the initial step of a degradation pathway. However, several studies have demonstrated that ES enables resultant extracellular and intracellular fragments to carry out additional functions beyond those of native full length molecules [2,23]. Moreover, ES is a switch that enables a second proteolytic cleavage termed Regulated Intracellular Proteolysis (RIP). This cleavage is mediated by the intracellular cleaving proteases and yield soluble functional intracellular fragments [10,23]. Notably, fragments generated after ES and RIP have been involved in cell survival among other cell processes [3,10,23,24], whereas metalloproteases are involved in neurodegenerative diseases at multiple levels [27].

In humans and rodents, 3-nitropropionic acid (3-NP) micotoxin acute exposure induces neurodegeneration in the putamen and caudate nucleus. For this reason, it has been employed *in vivo* to model the disease phenotype associated with neuronal degeneration of these brain regions such as Huntington disease [5,30]. Also, 3-NP has been used *in vitro* to induce striatal neuronal degeneration. The mechanism mainly associated to 3-NP effect is succinate dehydrogenase irreversible inhibition in the mitochondrial complex II. This mitochondrial oxidative metabolism blockade induces, among other cell dysfunctions, free radicals, energy deficit, loss of membrane potential and neurotransmitter release that switches on excitotoxicity; the well established neuronal death mechanism mediated by NMDAR over activation with glutamate. Together, these events culminate with neuronal degeneration [5,30].

In this framework, since fragments originated after ES have been involved in cell survival, we wanted to investigate whether endogenous metalloprotease activity participate in mitochondrial impairment induced by 3-NP. For this purpose we used the wide spectra inhibitor GM6001. In addition, due to its prominent role during neuronal death by excitotoxicity, we also analyzed NMDAR subunit NR1 cellular distribution after 3-NP treatment and metalloprotease inhibition, receptor subunit that is known to undergo ES.

2. Materials and methods

2.1. Animals

16–18 days Wistar rat embryos were obtained through cesarean incisions from pregnant rats obtained from the FQ animal facility from UNAM. Striatal tissue was dissected and maintained in cold Hank's Balanced Salt Solution (HBSS) without Ca^{++} and Mg^{++}. All animal handling and procedures were performed accordingly to ethical guidelines established by INNN.

2.2. Cell cultures

Striatal neuronal cultures were prepared as described elsewhere with minor modifications [13]. Briefly, dissected striatal tissue was mechanically dissociated using fire polished Pasteur pipettes and 100 μm nylon mesh in Dulbecco's modified Eagle's Medium (DMEM), 10% Fetal Bovine Serum (FBS), 100 U/ml penicillin and streptomycin. Suspended cells were centrifuged and resuspended in culture medium (Neurobasal, B27 supplement, 100 U/ml penicillin and streptomycin) and seeded (2.5×10^5 cells/cm^2) into culture plates or coverslips treated with poly-D-lysine (PDL). Cells were maintained at 37 °C and 5% CO^2, and 50% culture medium was replaced every 4 days. Cells were used for experiments after 7–9 days *in vitro* (DIV).

2.3. Reagents and antibodies

Salts, GM6001 and reagents were all from Sigma Chemical Co. (St. Louis, MO, USA). Cell culture media, supplements and Hoechst were from Life Technologies (Carlsbad, CA, USA), culture plates were from Costar (Tewksbury, MA, USA). Ab against NR1 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Abs against Neuronal Nuclear antigen (NeuN), gamma-amino butyric acid decarboxilase-67 (GAD-67) and glial fibillary acidic protein (GFAP) were from EMD Millipore (Billerica, MA, USA); Secondary Abs labeled with Dylight-488 or Dylight-594 were from Jackson Immunochemicals (West Grove, PA, USA).

2.4. MTT assay

Mitochondrial activity was evaluated as described previously. [32]. Striatal neurons were seeded into 96 well culture plates After 7–9 DIV cells were treated with metalloprotease inhibitor GM6001, after 15 min, 3-NP was added. Cells were incubated for 24 h at 37 °C and 5% CO_2, then, culture medium was replaced with fresh medium containing 500 μg/ml 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) and cells were incubated for additional 5 h. Finally, formazan crystals were dissolved with SDS-HCl solution and optical density was determined with a spectrophotometer at λ 595 nm. Data are expressed as mean triplicates after background subtraction ±standard error of the mean (SEM). Differences between treatments were analyzed by Student's-*t*-test and difference is claimed with a $p \leq 0.05$.

2.5. Immunofluorescence

Cells seeded into PDL-treated coverslips were washed with cold PBS and fixed with PBS-4%PFA-4% sucrose 15 min on ice. Then, cells were permeabilized with PBS-0.5%-tween-20 10 min. Cells were then incubated 1 h with 1 μg/ml of primary Ab in M1 buffer (140 mM NaCl, 20 mM HEPES, 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 5 mM KCl), washed and incubated 1 h with secondary Ab. Finally, nuclei were labeled with Hoechst, washed and mounted with homemade Mowiol-DABCO. Bright field, NeuN and GAD-67 images were acquired with a Leica microscope using a 20× or a 40× objective. Cell z stacks were acquired with a 60× N.A. 1.35 objective in an Olympus IX-81 (Olympus Corporation) inverted microscope coupled to a Hammamatsu Orca R2 CCD with Cell Sens Dimension software and Hg illumination. Stacks were processed with Cell Sens Dimension built in blind deconvolution algorithm. Two slices are shown corresponding to slide focal plane (0 μm) and 500 μm above it.

3. Results

Initially, striatal cell phenotype was characterized. In culture, most cells presented neuronal phenotype as observed by bright field illumination characterized by somas with large projections forming networks (Fig. 1A). Their neuronal phenotype was confirmed by NeuN staining, showing positive labeling in >95% of non degenerated cells (Fig. 1B and C), with very low numbers of GFAP positive cells (not shown), consistently with previous reports in which neuronal selective culture media was used [4,12]. NeuN is