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L-β-ODAP alters mitochondrial Ca^{2+} handling as an early event in excitotoxicity

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ABSTRACT

The neurotoxin β-N-oxalyl-L-α,β-diaminopropionic acid (L-β-ODAP) is an L-glutamate analogue at α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA)/kainate receptors in neurons and therefore acts as an excitotoxic substance. Chronic exposure to L-β-ODAP present in *Lathyrus sativus* L. (*L. sativus*) seeds is proposed as the cause of the neurodegenerative disease neurolathyrism, but the mechanism of its action has not been conclusively identified. A key factor in excitotoxic neuronal cell death is a disturbance of the intracellular Ca^{2+} homeostasis, including changes in the capacity of intracellular Ca^{2+} stores like the endoplasmic reticulum (ER) or mitochondria. In this study, aequorin and other Ca^{2+} indicators were used in N2a neuroblastoma cells to investigate alterations of cellular Ca^{2+} handling after 24 h exposure to L-β-ODAP. Our data demonstrate increased mitochondrial Ca^{2+} loading and hyperpolarization of the mitochondrial membrane potential (Ψ_m), which was specific for L-β-ODAP and not observed with L-glutamate. We conclude that L-β-ODAP disturbs the ER–mitochondrial Ca^{2+} signaling axis and thereby renders the cells more vulnerable to its excitotoxic effects that ultimately will lead to cell death.

1. Introduction

Chronic intake of the neuro-excitatory amino acid β-N-oxalyl-L-α,β-diaminopropionic acid (L-β-ODAP), present in *Lathyrus sativus L.* (*L. sativus*) seeds, is proposed to be responsible for the pathogenesis of the neurodegenerative disease neurolathyrism [1]. Much of the neuropathology caused by this neurotoxin appears to involve mitochondrial [2] and enzymatic dysfunctions [3–6]. However, most of its effects can be attributed to structural similarities with L-glutamate and its ability to induce excitotoxicity by interacting with glutamate receptors [7], in particular AMPA receptors for which L-β-ODAP is an agonist [8–10]. L-glutamate exerts excitotoxic effects in several neurodegenerative diseases through a disturbed intracellular Ca^{2+} response. More specifically, a massive Ca^{2+} entry into the cells is caused by the activation of two types of ionotropic glutamate receptors: N-methyl-D-aspartate (NMDA) receptors and Ca^{2+}-permeable α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptors [11]. A clear relationship has been described between excessive Ca^{2+} entry and L-glutamate-triggered neuronal injury which is thought to be mediated by inappropriate activation of Ca^{2+}-dependent proteases, lipases, phosphatases, endonucleases and the formation of oxidative free radicals all of which may lead to cell death [12].

Under normal conditions, neurons control intracellular Ca^{2+} levels through a complex interplay between Ca^{2+} entry, Ca^{2+} extrusion, Ca^{2+} buffering and internal Ca^{2+} storage. Internal Ca^{2+} stores, such as the endoplasmic reticulum (ER) and mitochondria, dynamically participate in the generation of cytoplasmic Ca^{2+} signals. The ER is involved in rapid signaling events associated with cell stimulation, supported by Ca^{2+} release channels (inositol 1,4,5-trisphosphate [IP_3] receptor and ryanodine receptor) and sarco-/endoplasmic

Abbreviations: AEQ, aequorin; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid; ATP, adenosine triphosphate; BCIP/NBT, 5-bromo-4-chloro-3-indolyl/nitro blue tetrazolium; BK, bradykinin; ER, endoplasmic reticulum; FCCP, p-trifluoromethoxy carbonyl cyanide phenyl hydrazone; HBSS, Hanks' balanced salt solution; IP_3, inositol 1,4,5-trisphosphate; KRB-Hepes, Krebs-Ringer buffer; L-β-ODAP, β-N-oxalyl-L-α,β-diaminopropionic acid; *L. sativus*, *Lathyrus sativus*; Ψ_m, mitochondrial membrane potential; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt; NMDA, N-methyl-D-aspartate; NP-40, Nonidet P-40; $PBSD^+$, phosphate buffered salt with divalent cations; Rh-123, Rhodamine 123; RIPA, radio-immunoprecipitation assay buffer; SERCA, sarco-/endoplasmic reticulum Ca^{2+}-ATPase; SOCE, store-operated Ca^{2+} entry; Tg, thapsigargin.

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reticulum Ca^{2+}-ATPase (SERCA) pumps residing in the endomembrane. The released Ca^{2+} is taken up by mitochondria that are in close apposition to the ER release sites [13–15]. Ca^{2+} uptake in mitochondria occurs via a uniporter and accumulated Ca^{2+} may leave the organelle via an antiporter in exchange for Na^+ or H^+. The ER–mitochondrial tandem is importantly involved in the pathways leading to cell death. Disruption of ER Ca^{2+} homeostasis triggers cellular stress responses that may lead to neurodegeneration by affecting post-translational protein processing [16], whereas mitochondria avidly take up Ca^{2+} during Ca^{2+} overload conditions [17], causing a reduction in the rate of ATP synthesis or the release of cytochrome c, resulting in necrosis or apoptosis, respectively.

The aim of the present work was to investigate the effect of chronic exposure of cells to sub-toxic L-β-ODAP concentrations on neuronal Ca^{2+} homeostasis. To resolve the early events and effects of this neurotoxin on Ca^{2+} handling, we made use of N2a neuroblastoma cells. These cells have a normal intracellular Ca^{2+} homeostasis and allow detailed Ca^{2+} studies in subcellular compartments. They do not express functional AMPA- or NMDA-receptors and are relatively resistant to L-β-ODAP- or L-glutamate-induced cell death. This approach made it possible to specifically investigate the intrinsic effects of the neurotoxin on cellular Ca^{2+} handling and to clearly separate these effects from the major disturbance of cellular Ca^{2+} homeostasis that occurs as a consequence of the cell death process. Since L-β-ODAP is often reported to be a glutamate-analogue in excitotoxic events [7–10], all experiments were performed with L-β-ODAP in parallel with L-glutamate, a prototypic excitotoxic amino acid [18]. Our results show that chronic exposure to L-β-ODAP, but not to L-glutamate, modestly potentiates ER Ca^{2+} loading, while strongly increasing cytoplasmic Ca^{2+} changes, the negative mitochondrial potential and the uptake of Ca^{2+} into the mitochondria. These changes indicate an early 'sensitizing' effect of L-β-ODAP that will attract more Ca^{2+} to the mitochondria that may ultimately lead to cell death. We conclude that L-β-ODAP alters cellular Ca^{2+} handling in a manner that prepares the way toward cell death that will be precipitated by its excitotoxic actions. These observations shed new light on the multiple faces of L-β-ODAP neurotoxicity.

2. Experimental procedures

2.1. Cell culture

Parental N2a mouse neuroblastoma cells (N2a, ATTC number CCL131) and N2a cells stably transfected with mitochondrial aequorin (N2a-combiAEQ) were grown in Dulbecco's Modified Eagle Medium (D-MEM; Invitrogen) with 4500 mg/mL D-glucose, Glutamax™ I and 110 mg/L sodium pyruvate, supplemented with 5% (v/v) fetal calf serum, 100 U/mL penicillin and 100 μg/mL streptomycin at 37 °C in a humidified incubator at 5% CO_2/95% air (v/v). Medium for N2a-combiAEQ cells was supplemented with 200 μg/mL hygromycin B. For some experiments cells were treated for 6 h in D-MEM without glucose containing 5% (v/v) fetal calf serum, 100 U/mL penicillin, 100 μg/mL streptomycin and 2 mM L-glutamine.

2.2. Cell viability/cell death assays

N2a cells were grown in 24-well plates (Becton Dickinson, Erembodegem, Belgium) and treated with L-β-ODAP (kindly provided by S.L.N. Rao) or L-glutamate (2 mM, 6 or 24 h). In some experiments, the cells were additionally exposed to p-trifluoromethoxy carbonyl cyanide phenyl hydrazone (FCCP, 100 nM, 6 h) after 24 h pre-treatment with L-β-ODAP, L-glutamate or NMDA (2 mM, 24 h). Cell viability was assayed with the CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay (Promega, Leiden, Netherlands). This assay measures 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt (MTS) which is reduced to a colored formazan product by metabolic active cells. The assay buffer was added to the cells as 1:10 (μL buffer/μL medium), and after a 2 h incubation (37 °C, dark) absorbance was assessed at 490 nm using a plate reader (Victor-3, type 1420, Perkin Elmer, Brussels, Belgium). Absorbance was evaluated as a percentage of control, corrected for a background signal (medium with assay buffer, without cells). Apoptotic cell death was determined with the Caspase-Glo® 3/7 assay (Promega, Leiden, Netherlands), estimating the activity of caspase-3 and -7. Caspase-Glo 3/7 reagent was diluted in the treatment medium after the exposure to L-β-ODAP or L-glutamate (1:3 or 1:6 (v/v)), incubated for 1 hour at room temperature and luminescence was measured with a plate reader (Victor-3, type 1420, Perkin Elmer, Brussels, Belgium).

2.3. Cytoplasmic Ca^{2+} measurements

Cytoplasmic Ca^{2+} measurements were performed with the bioluminescent protein aequorin and with the fluorescent Ca^{2+} indicator Fura-2. Aequorin is a Ca^{2+}-sensitive photoprotein derived from the jellyfish *Aequoria Victoria*. It is composed of an apoprotein and a hydrophobic prosthetic group, coelenterazine, and its polypeptide sequence includes three high affinity Ca^{2+}-binding sites. Ca^{2+} binding causes the rupture of the covalent link between the apoprotein and the prosthetic group, a reaction associated with the emission of one photon. The chimeric aequorin cDNA constructs were kindly provided by Dr. R. Rizzuto (University of Ferrara, Italy). N2a cells were transiently transfected with aequorin targeted to the cytoplasm (cyto-AEQ). To that purpose, cells cultured in 150 cm^2 flasks (TPP, Switzerland) were seeded on 18-mm gelatin-coated (2%, w/v) coverslips in 12-well culture plates (TPP, Switzerland) at a density of 45,000–70,000 cells per cm^2. One day after seeding, the cells were transfected with the aequorin construct in medium (0.4–0.7 μg Cyto-AEQ per well) using Lipofectamine 2000 reagent (3 μL/μg plasmide; Invitrogen). One day after transfection, the cells received a 24 h treatment with L-β-ODAP or L-glutamate (2 mM). After this treatment, the cells were incubated for 90 min in medium supplemented with 5 μM wild-type (W) coelenterazine (Invitrogen) at 37 °C and 5% CO_2/95% air (v/v) washed with HEPES-buffered Krebs-Ringer buffer (KRB-Hepes; 121 mM NaCl, 5.4 mM KCl, 0.8 mM $MgCl_2 \cdot 6H_2O$, 6 mM $NaHCO_3$, 5.5 mM D-glucose, 1.8 mM $CaCl_2 \cdot 2H_2O$ and 25 mM Hepes, pH 7.3) and kept at 37 °C for another 30 min. Ca^{2+} uptake was measured at 37 °C while stimulating the cells with bradykinin (BK, 300 nM–1 μM; Sigma) until complete recovery of the response to baseline level. Emitted light was measured using a low-noise photomultiplier with built-in amplifier-discriminator (Hamamatsu H7360-1) and collected using a photon-counting board (National Instruments PCI-6601).

The maximal luminescence level was determined by superfusion with digitonin (100 μM; Sigma) in a hypotonic solution composed of 10 mM $CaCl_2$ in H_2O, thus discharging the remaining AEQ pool (Fig. 1A). The emitted maximum signal can in principle be used for calibration purposes [19,20], but this yielded unrealistic $[Ca^{2+}]_i$ values (aequorin-based measurements shown in Fig. 1B were therefore expressed in arbitrary units) and for this reason, we repeated these experiments with Fura-2. For Fura-2 experiments, N2a cells were grown on gelatin-coated (2%, w/v) coverslips in 24-well plates, washed with HBSS-Hepes buffer (0.81 mM $MgSO_4 \cdot 7H_2O$, 0.95 mM $CaCl_2 \cdot 2H_2O$, 137 mM NaCl, 0.18 mM $Na_2HPO_4 \cdot 2H_2O$, 5.36 mM KCl, 0.44 mM KH_2PO_4, 5.55 mM D-Glucose, 25 mM Hepes, pH 7.4) and loaded with 5 μM Fura-2-AM (Invitrogen, Molecular Probes) in HBSS-Hepes containing probenecid (1 mM; Sigma) and pluronic acid (0.01%, w/v; Sigma). The cells were incubated at 37 °C for 30 min, washed with HBSS-