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Neuroprotective effects of MAPK/ERK1/2 and calpain inhibitors on lactacystin-induced cell damage in primary cortical neurons

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ABSTRACT

The dysfunction of the proteasome system is implicated in the pathomechanism of several chronic neurodegenerative diseases. Lactacystin (LC), an irreversible proteasome inhibitor, induces cell death in primary cortical neurons, however, the molecular mechanisms of its neurotoxic action has been only partially unraveled. In this study we aimed to elucidate an involvement of the key enzymatic pathways responsible for LC-induced neuronal cell death. Incubation of primary cortical neurons with LC (0.25–50 μg/ml) evoked neuronal cell death in concentration- and time-dependent manner. Lactacystin (2.5 μg/ml; 6.6 μM) enhanced caspase-3 activity, but caspase-3 inhibitor, Ac-DEVD-CHO did not attenuate the LC-evoked cell damage. Western blot analysis showed a time-dependent, prolonged activation of MAPK/ERK1/2 pathway after LC exposure. Moreover, inhibitors of MAPK/ERK1/2 signaling, U0126 and PD98052 attenuated the LC-evoked cell death. We also found that LC-treatment resulted in the induction of calpains and calpain inhibitors (MDL28170 and calpeptin) protected neurons against the LC-induced cell damage. Neuroprotective action of MAPK/ERK1/2 and calpain inhibitors were connected with attenuation of LC-induced DNA fragmentation measured by Hoechst 33342 staining and TUNEL assay. However, only MAPK/ERK1/2 but not calpain inhibitors, attenuated the LC-induced AIF (apoptosis inducing factor) release. Further studies showed no synergy between neuroprotective effects of MAPK/ERK1/2 and calpain inhibitors given in combination when compared to their effects alone. The obtained data provided evidence for neuroprotective potency of MAPK/ERK1/2 and calpain, but not caspase-3 inhibition against the neurotoxic effects of LC in primary cortical neurons and give rationale for using these inhibitors in the treatment of neurodegenerative diseases connected with proteasome dysfunction.

1. Introduction

The ubiquitin-proteasome system (UPS) is crucial for non-lysosomal degradation of damaged or misfolded proteins in eukaryotic cells (Ciechanover, 2006). UPS dysfunction can lead to many abnormalities in proper cellular function (e.g. inhibition of proliferation, induction of differentiation, protein aggregation, and induction of apoptosis) which can vary in dependence on cell type (proliferating vs. postmitotic) (Genin et al., 2010; Tai and Schuman, 2008). Many experimental and clinical evidences point to contribution of proteasome dysfunction in pathomechanism of many neurodegenerative conditions such as Parkinson's disease (PD), Alzheimer's disease (AD), Hunthington's disease (HD), and amyotrophic lateral sclerosis (ALS) (Keck et al., 2003; Layfield et al., 2003; Lindsten and Dantuma, 2003; McNaught et al., 2003). Since there is still a lack of clinical effective neuroprotective drugs (Faden and Stoica, 2007; Lipton, 2007), investigation of the mechanism of proteasome-mediated neuronal cell death could be helpful in finding new targets for neuroprotection.

Lactacystin is a *Streptomyces* metabolite and an irreversible specific blocker of the activity of mammalian 26S/20S proteasome, and is used in experimental studies to elucidate not only the mechanism of UPS dysfunction in neuronal cell death but also it serves as a model substance for searching new protective agents (Fenteany and Schreiber, 1998; Lee et al., 2001; Li et al., 2007a,b; Reaney et al., 2006; Suh et al., 2005; Xie et al., 2010). It was

Abbreviations: AIF, apoptosis inducing factor; DIV, day in vitro; ERK1/2, extracellular signal-regulated kinase 1/2; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GDNF, glia-derived neurotrophic factor; JNK, c-Jun N-terminal kinase; LC, lactacystin; LDH, lactate dehydrogenase; MAPK, mitogen-activated protein kinase; mTOR, mammalian target of rapamycin; MTT, 3-[4,5-dimethylthylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; NAC, N-acetyl-L-cysteine; ROS, reactive oxygen species; UPS, ubiquitin-proteasome system.

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reported that the UPS inhibition by lactacystin, as well as by other proteasome inhibitors (MG132, epoxomicin, and carbobenzoxy-Leu-Leu-Leu-aldehyde) caused formation of protein aggregates (ubiqutin and α-synuclein) and induced apoptotic cell death in various types of primary neurons (cortical and ventral mesencephalic) and in neuronal cell lines (Csizmadia et al., 2008; Du et al., 2009; Dyllick-Brenzinger et al., 2010; Lee et al., 2001; MacInnes et al., 2008; Mytilineou et al., 2004; Rideout et al., 2005; Rideout and Stefanis, 2002). It was shown in cellular and animal models that apoptosis evoked by proteasome inhibitors in neuronal cells was accompanied by mitochondrial dysfunction, cytochrome c release, caspase-3 activation, elevated p53 expression and chromatin fragmentation (Du et al., 2009; Meriin et al., 1998; Perez-Alvarez et al., 2009; Qiu et al., 2000; Reaney et al., 2006; Suh et al., 2005; Sun et al., 2008). Some reports showed the engagement of activation of the cell death promoting kinase, JNK (c-Jun N-terminal kinase) in dopaminergic neurons in the lactacystin model of neuronal cell death (Li et al., 2008; Masaki et al., 2000; Meriin et al., 1998; Sang et al., 2002). Among agents which were effective in attenuation of the proteasome inhibition-evoked cell death were iron chelators, GDNF (glia-derived neurotrophic factor), dopamine D3 receptor agonists, rapamycin – a mTOR inhibitor, caspase-3 inhibitors, PAR (poly-ADP-rybolysation) inhibitors, JNK inhibitors, overexpression of Bcl-xl (Du et al., 2008; Keller and Markesbery, 2000; Lang-Rollin et al., 2005; Li et al., 2007a, 2010a,b; Pan et al., 2008; Qiu et al., 2000; Rideout et al., 2003; Sang et al., 2002; Wu et al., 2010; Zhu et al., 2008, 2010). On the contrary, some data showed a protective effect of proteasome inhibitors in glutamate, hydrogen peroxide, 6-OHDA or serum deprivation-induced cell death in various neuronal cells (Suh et al., 2005; van Leyen et al., 2005; Yamamoto et al., 2007). These effects were connected with the induction of heat shock proteins (HSPs) which might have increased cell tolerance to stressful conditions (Ahn and Jeon, 2006; Butts et al., 2005; Choy et al., 2011; Meriin et al., 1998; Suh et al., 2005; Yew et al., 2005). The above-mentioned studies point to a dual action of lactacystin in neuronal cell death promotion and neuroprotective effects depending on cell type and experimental conditions (Choy et al., 2011; Meriin et al., 1998; Butts et al., 2005; Suh et al., 2005; Yew et al., 2005). Regarding the molecular mechanism of LC influence on neuronal cell death and viability, little is known about the involvement of MAPK/ERK1/2 signaling in these processes. In contrast to the other members of MAPK family, JNK/SAPK and p38 kinases, the activation of which is widely accepted to be a cell-death promoting event, the ERK1/2 kinase could possess both, a cell prosurvival and cell death-promoting effect (Subramaniam and Unsicker, 2010). A growing number of studies conducted in recent years clearly underline the role of the ERK1/2 pathway activation as well as neuroprotective effects of MAPK/ERK 1/2 inhibitors in various in vitro and in vivo models of mainly non-apoptotic neuronal cell death (Subramaniam et al., 2004; Szydlowska et al., 2010; Yu et al., 2010). It is believed that the magnitude and duration of ERK1/2 activity determine its biological function.

In order to shed more light on the mechanism of the LC-induced neuronal cell death, in this study we tried to answer two questions. First, we verified whether the LC-induced neuronal cell damage depends on caspase-3 and/or calpains activation. This part of the study was based on a controversy about neuroprotective effects of caspase-3 inhibitors in the LC-evoked neuronal cell death (Choy et al., 2011; Rideout and Stefanis, 2002; Qiu et al., 2000). Secondly, since there was a lack of data about the involvement of the ERK1/2 activation in the proteasome inhibitors-mediated neuronal cell death, we aimed to investigate the role of this intracellular pathway in lactacystin toxicity in primary cortical neurons. Additionally, in the LC model of neuronal cell death, we tested some other potential neuroprotectants with various mechanism of action, e.g. an NMDA receptor antagonist, Ca^{2+} channel blocker, ROS scavengers chosen on the basis of their ability to inhibit detrimental pathways engaged in the mechanism of lactacystin neurotoxicity (oxidative stress, Ca^{2+} overload) (Lee et al., 2001; Li et al., 2007b; Perez-Alvarez et al., 2009).

2. Materials and methods

2.1. Chemicals

Neurobasal A medium, fetal bovine serum (FBS), supplement B27 were from Gibco (Invitrogen, Poisley, UK). The Cytotoxicity Detection Kit, BM Chemiluminescence Western Blotting Kit and *In Situ* Cell Death Detection Kit Fluorescein were from Roche Diagnostic (Mannheim, Germany). Primary antibodies: antiphospho-Tyr^{204} ERK1/2 (pERK 1/2, sc-7383), anti-ERK2 (ERK2, sc-474), anti-spectrin α II (sc-48382), anti-AIF (sc-5586), anti-GAPDH (GAPDH, sc-25778), protein markers and appropriate secondary antibodies were from Santa Cruz Biotechnology Inc. (CA, USA). All other reagents were from Sigma (Sigma–Aldrich Chemie GmbH, Germany).

2.2. Primary neuronal cell cultures

The experiments were conducted on primary cultures of mouse cortical neurons. The protocol for generating of the primary neuronal cultures was concordant with local and international guidelines on the ethical use of animals. Neuronal tissues were taken from Swiss mouse embryos at 15/16 day of gestation and were cultured essentially as described previously (Brewer, 1995; Jantas-Skotniczna et al., 2006). Briefly, pregnant females were anesthetized with CO_2 vapor, killed by cervical dislocation, and subjected to cesarean section in order to dissect fetal brains. The dissected tissues were minced separately into small pieces, then digested with trypsin (0.1% for 15 min at room temperature (RT)), triturated in the presence of 10% fetal bovine serum and DNAse I (150 Kunitz units/ml), and finally centrifuged for 5 min at 100 × *g*. The cells were suspended in Neurobasal medium supplemented with B27 and plated at a density of 1.5×10^5 cells per cm^2 onto poly-ornithine (0.01 mg/ml)-coated multi-well plates. This procedure typically yields cultures that contain >90% neurons and <10% supporting cells as verified by immunocytochemistry (data not shown). The cultures were then maintained at 37 °C in a humidified atmosphere containing 5% CO_2 for 7 days prior to experimentation.

2.3. Cell treatment

7 DIV cortical neurons were treated with lactacystin (0.025–50 μg/ml) for 24 and 48 h in order to prepare concentration dependent toxicity curve. For further studies with putative neuroprotective agents (memantine, nimodipine, N-acetyl-L-cysteine, 1,2,3,4-tetrahydroisoquinoline, and (±)-α-lipoic acid), a concentration of 2.5 μg/ml of lactacystin was used in a co-treatment scheme. An involvement of MAPK/ERK/1/2 pathway in lactacystin-evoked neurotoxicity was tested using MAPK/ERK1/2 inhibitors, U0126 (1–10 μM) and PD98052 (1–10 μM) which were added 30 min before treatment of cells with LC. The inhibitor of mTOR, rapamycin (0.1–1 μM) and the inhibitor of caspase-3 (Ac-DEVD-CHO, 10 μM) were also added to neurons 30 min before treatment of cells with LC. In the next part of the study, the calpain inhibitors, calpeptin (0.1–10 μM) and MDL28170 (0.1–10 μM) were added to cells concomitantly with LC. U0126 (10 mM, PD98052 (10 mM), rapamycin (1 mM), Ac-DEVD-CHO (10 mM), calpeptin (10 mM), MDL28170 (10 mM) stock solutions were prepared in dimethyl sulfoxide.