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Cathepsin X promotes 6-hydroxydopamine-induced apoptosis of PC12 and SH-SY5Y cells

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ABSTRACT

The cysteine carboxypeptidase cathepsin X is an important player in degenerative processes under normal ageing and pathological conditions. In the present study, we investigated the potential role of cathepsin X in 6-hydroxydopamine (6-OHDA)-induced toxicity in the pheochromocytoma cell line PC12 and neuroblastoma cell line SH-SY5Y. Cells exposed to 6-OHDA demonstrated alterations in the protein level of cathepsin X and activity of cathepsin X. Downregulation of cathepsin X expression by siRNA attenuated the neuronal death caused by 6-OHDA. Treatment with specific cathepsin X inhibitor AMS36 protected cells against 6-OHDA mediated cytotoxicity, resulting in reduced cell death and apoptosis. Furthermore, AMS36 reversed 6-OHDA-induced loss of tyrosine hydroxylase and attenuated 6-OHDA-induced activation of caspase-3, triggering apoptosis, intracellular generation of reactive oxygen species and mitochondrial dysfunction, including the release of cytochrome *c* and an imbalanced Bax/Bcl-2 ratio. Moreover, AMS36 interfered with NF-κB activation by blocking degradation of IκBα, preventing NF-κB translocation to the nucleus. Our data provide the first evidence that inhibition of cathepsin X protects both, PC12 and SH-SY5Y cells against 6-OHDA toxicity and indicate that cathepsin X may be responsible for dopamine neuron death, involved in the pathogenic cascade event for the neurodegenerative disorders, such as Parkinson's disease.

1. Introduction

Cathepsins are cysteine proteases long believed to be responsible for terminal protein degradation in lysosomes. However, this view has changed dramatically since they have been found to regulate a number of other important physiological and pathological processes (Kos et al., 2009; Obermajer et al., 2006). Recent studies have suggested that cathepsin X is an important player in degenerative processes during normal ageing and in neurodegenerative diseases. It is expressed abundantly in various immune cells such as monocytes, macrophages and dendritic cells (Kos et al., 2005) and in mouse brain, with a preference for glial cells and aged neurons (Wendt et al., 2007). In animal models of Alzheimer disease (AD) its association with amyloid plaques has also been observed (Hafner et al., 2013; Wendt et al., 2007). The modes of degeneration mediated by cathepsin X, however, are far from being completely understood. This neurodegeneration comprises the sequential cleavage of C-terminal amino acids of γ-enolase, abolishing its neurotrophic activity (Obermajer et al., 2009). In this way γ-enolase mediates neuronal cell survival and neuritogenesis, and the neuroprotective role in amyloid-related degeneration can be abolished (Hafner et al., 2012; 2013). In addition to role of cathepsin X in neurodegeneration, several studies have demonstrated the involvement of cathepsin X in inflammatory processes (Jevnikar et al., 2011; Obermajer et al., 2008), implying a role for cathepsin X in inflammation-induced neurodegeneration (Stichel and Luebbert, 2007).

Parkinson's disease (PD) is a chronic neurodegenerative disorder resulting from progressive loss of dopaminergic neurons in the substantia nigra, striatal dopamine depletion and motor impairments (Chung et al., 2003; Lotharius and Brundin, 2002). Although the causes of PD still remain unclear, evidence suggests that

Abbreviations: AD, Alzheimer's disease; ELISA, enzyme-linked immunosorbent assay; FBS, foetal bovine serum; HS, horse serum; LDH, lactate dehydrogenase; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt; NF-kappaB, nuclear factor-kappaB; 6-OHDA, 6-hydroxydopamine; PBS, phosphate-buffered saline; PD, Parkinson's disease; PI, propidium iodide; ROS, reactive oxygen species; TH, tyrosine hydroxylase; ΔΨm, mitochondrial membrane potential.

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inflammation is the fundamental process contributing to neuron death in PD (McGeer and McGeer, 2004; Qian et al., 2010), accompanied by oxidative stress (Beal, 2003), mitochondrial dysfunction (Winklhofer and Haass, 2010) and activation of the apoptotic cascade (Blum et al., 2001; Singh and Dikshit, 2007).

To elucidate the molecular pathways of neuronal death in PD and to improve neuroprotective strategies, a number of *in vitro* and *in vivo* models have been developed. Many of them utilize the experimental neurotoxin 6-hydroxydopamine (6-OHDA), which is thought to induce toxicity that mimics the neuropathological and biochemical conditions, in PD (Eminel et al., 2004; Hanrott et al., 2006; Rodriguez-Blanco et al., 2008; Zhang et al., 2011). 6-OHDA can be transported into neurons by plasma membrane dopamine transporters and inhibits the mitochondrial electron transport chain complexes I and IV, leading to neuronal damage by producing reactive oxygen species (ROS) (Paradies et al., 2011; Simola et al., 2007). The latter may cause activation of down-stream signalling pathways, resulting in mitochondrial dysfunction, and activation of caspases and consequent triggering of apoptosis (Blum et al., 2001). In addition to the caspase cascade, much attention has been paid to the role of the endosomal/lysosomal proteolytic system in 6-OHDA-induced cell death (Takai et al., 1998). Some inhibitors of lysosomal proteases, such as pepstatin A, an inhibitor of cathepsin D (Lee et al., 2006) and Z-Fy(t-Bu)-DMK, an inhibitor of cathepsin L (Xiang et al., 2011), have been reported as potential therapeutics with neuroprotective effects on 6-OHDA-induced cell death of dopaminergic neurons. Therefore, lysosomal proteases may be important targets for protecting neurons against neurotoxin induced degeneration.

Here, we have established the potential role of lysosomal cysteine cathepsin X in neuronal cell lines PC12 and SH-SY5Y following exposure to 6-OHDA. We showed that the specific inhibitor for cathepsin X, AMS36, significantly reverses 6-OHDA-induced degeneration in PC12 and SH-SY5Y cells. Detailed study on PC12 cells has further demonstrated that the inhibitor can interfere with the multiple signalling pathways involved in the mitochondrial dysfunction and apoptosis induced by 6-OHDA.

2. Materials and methods

2.1. Cell culture and drug treatment

Rat pheochromocytoma cells (PC12) were purchased from American Type Culture Collection (ATCC; CRL-1721, Manassas, VA, USA). They were maintained in RPMI medium supplemented with 5% foetal bovine serum (FBS), 10% heat-inactivated horse serum (HS), 2 mM L-glutamine, 50 units/ml penicillin and 50 μg/ml streptomycin (Sigma) in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C. For differentiation, PC12 cells were treated with nerve growth factor (NGF; 50 ng/ml) for 5 days and allowed to differentiate into neuron-like cells. Human neuroblastoma cells (SH-SY5Y) were obtained from ATCC (CRL-2266, Manassas, VA, USA). The cells were grown in Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% FBS, 2 mM L-glutamine, 50 units/ml penicillin and 50 μg/ml streptomycin. The cells were maintained at 37 °C in a humidified atmosphere of 95% air and 5% CO_2, and grown to 80% confluence. For experiments indicated below, undifferentiated SH-SY5Y cells were used. Immediately before treatment, 6-OHDA (Sigma, St. Louis, MO, USA) was dissolved in phosphate buffer saline (PBS), pH 7.4, containing 0.01% ascorbic acid at a concentration of 10 mM. The specific irreversible inhibitor of cathepsin X, AMS36, was synthesized according to the modified procedure of Sadaghiani et al. (2007) and the first four steps were accomplished according to the described procedure of Jackson et al. (1998) with minor modifications as presented in Fig. S1 (Supplementary data). AMS36 was prepared as a stock solution of 10 mM in DMSO. Prior to cell treatment, complete medium was replaced with reduced-serum medium (2% FBS, 2% HS for PC12 and 2% FBS for SH-SY5Y cells). After pretreatment with AMS36 (1–10 μM) or control (0.1% DMSO) for 2 h, cells were exposed to 6-OHDA (10–100 μM) for the time period indicated. An inhibitor of cathepsin B CA074-Me was used at concentration 10 μM 2 h prior 6-OHDA treatment.

2.2. Transfection

Cathepsin X was silenced with small interfering RNA (siRNA) oligonucleotides targeting CatX mRNA (CatX siRNA: sc-44661, Santa Cruz Biotechnology). PC12 cells were transiently transfected with CatX siRNA using Lipofectamine™ 2000 (Invitrogen), according to the manufacturer's protocol. Briefly, PC12 cells were seeded in 24-well culture plates (5×10^4/well) in 500 μl of growth medium without antibiotics. For transfection, Lipofectamine 2000 was gently mixed before use, diluted in Opti-MEM I medium without serum (Invitrogen) and incubated for 5 min at room temperature. After the incubation, diluted Lipofectamine 2000 was combined with the diluted siRNA oligomer (resulting concentration of RNA was 40 nM) in Opti-MEM I medium, gently mixed and incubated for further 20 min at room temperature. Next, 100 μl of transfection complex was added to each well containing cells and medium and incubated at 37 °C in a humidified atmosphere of 95% air and 5% CO_2 for 6 h. Afterwards, the medium with transfection complex was replaced with the complete growth medium for 24 h. Control siRNA (sc-37007, Santa Cruz Biotechnology) was used as a negative control. The effect of siRNA silencing after 24 h was tested by western blotting and by activity assay using specific substrate (Fig. S2A and S2B; Supplementary data).

2.3. MTS assay

Cell viability was determined using a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt (MTS) that can be reduced to purple-coloured formazan by intact cells. PC12 and SH-SY5Y cells were seeded in 96-well culture plates (2×10^4/well). The next day, cells were treated as described above and, after 24 h, cell viability was assessed using the CellTiter 96® Aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI), in accordance with the manufacturer's instructions. The absorbance was measured with an automatic microplate reader (Safire) at a wavelength of 492 nm. Results are presented as a percentage of the control.

2.4. LDH Assay

Cell toxicity was measured by determining the activity of lactate dehydrogenase (LDH) released into the medium when cell membranes are damaged. PC12 and SH-SY5Y cells were seeded on 24-well culture plates (5×10^4/well). The released LDH was determined in the cell medium after treatment, while total LDH was determined after cell lysis. Total and released LDH activity were determined following specifications of the CytoTox-ONE™ Homogeneous Membrane Integrity Assay (Promega). Fluorescence was measured with an automatic microplate reader (Safire) at an excitation wavelength of 560 nm and an emission wavelength of 590 nm. Released LDH was normalized to total LDH and results are shown as a percentage of the control.

2.5. Staining with PI

Cytotoxicity was also determined with the propidium iodide (PI) fluorescence method. PC12 cells were seeded on 24-well culture plates (5×10^4/well) and next day, treated as described above. After 24 h treatment, cells were labelled with PI (30 μM) for an additional 30 min at 37 °C. Cells were then analysed for cytotoxicity by flow cytometry on FACS Calibur (BD Bioscience). The percentage of PI positive (PI^{pos}) cells was evaluated using FlowJo software (Ashland) and recorded as a percentage of the control.

2.6. Apoptosis detection by flow cytometry

Apoptosis was detected and quantified using an Annexin V-FITC Apoptosis Detection Kit (Sigma, St. Louis) in accordance with the manufacturer's instructions. PC12 cells were cultured on 24-well plates. 24 h after treatment, they were washed twice with cold PBS and resuspended in 500 μl of binding buffer. FITC-labelled Annexin V (5 μl) and PI (10 μl) were added to cells, which were incubated in the dark at room temperature for 15 min. Cell apoptosis was measured using a FACS Calibur flow cytometer (BD Bioscience). Annexin V-positive, PI-negative cells were scored as early apoptotic cells, and cells double-stained with both Annexin V and PI were considered late apoptotic cells. Control cells were negative for both stains.

2.7. Cathepsin X activity

Cathepsin X activity in non-differentiated and differentiated PC12 cells and SH-SY5Y cells was measured with the cathepsin X-specific, intramolecularly quenched fluorogenic substrate Abz-Phe-Glu-Lys(Dnp)-OH synthesized by Jiangsu Vcare Pharmatech Co. (China). Cell lysates were prepared in lysis buffer (0.05 M sodium acetate, pH 5.5, 1 mM EDTA, 0.1 M NaCl, 0.25% Triton X-100) and protein concentration determined by the BioRad Protein Assay Kit. An aliquot of 100 μg of lysate proteins was incubated at 37 °C, followed by measurement of cathepsin X activity using 10 μM Abz-Phe-Glu-Lys(Dnp)-OH The fluorometric reaction was quantified at 37 °C at an excitation wavelength of 320 nm and emission wavelength of 420 nm on a microplate reader (Tecan Safire). Results are presented as change in fluorescence as a function of time ($\Delta F/\Delta t$).