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Research paper

Impact of high cholesterol in a Parkinson's disease model: Prevention of lysosomal leakage versus stimulation of α-synuclein aggregation

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ABSTRACT

Parkinson's disease is characterized by accumulation of intraneuronal cytoplasmic inclusions, Lewy bodies, which mainly consist of aggregated α-synuclein. Controversies exist as to whether high blood cholesterol is a risk factor for the development of the disease and whether statin treatment could have a protective effect. Using a model system of BE(2)-M17 neuroblastoma cells treated with the neurotoxin 1-methyl-4-phenylpyridinium (MPP^+), we found that MPP^+-induced cell death was accompanied by cholesterol accumulation in a lysosomal-like pattern in pre-apoptotic cells. To study the effects of lysosomal cholesterol accumulation, we increased lysosomal cholesterol through pre-treatment with U18666A and found delayed leakage of lysosomal contents into the cytosol, which reduced cell death. This suggests that increased lysosomal cholesterol is a stress response mechanism to protect lysosomal membrane integrity in response to early apoptotic stress. However, high cholesterol also stimulated the accumulation of α-synuclein. Treatment with the cholesterol-lowering drug lovastatin reduced MPP^+-induced cell death by inhibiting the production of reactive oxygen species, but did not prevent lysosomal cholesterol increase nor affect α-synuclein accumulation. Our study indicates a dual role of high cholesterol in Parkinson's disease, in which it acts both as a protector against lysosomal membrane permeabilization and as a stimulator of α-synuclein accumulation.

1. Introduction

Parkinsonís disease (PD) is the second most common neurodegenerative disease worldwide, causing motor symptoms such as tremor, rigidity, postural instability and bradykinesia. It is characterized by a progressive loss of midbrain dopaminergic neurons in the substantia nigra and other brainstem nuclei (Tofaris, 2012). The hallmarks of PD are the presence of Lewy bodies and cytoplasmic inclusions composed of mainly α-synuclein fibrils. α-Synuclein is a small, 140-amino acid protein enriched in presynaptic terminals. Its function has not yet been fully characterized, but it plays a role in the modulation of dopaminergic signaling by regulating storage, exocytosis and neurotransmitter release (Cookson, 2009). α-Synuclein contains a highly amyloidogenic domain with a high propensity to aggregate and form oligomeric structures and fibers (Giasson et al., 2001) and an N-terminal amphipathic α-helical domain, which is formed when the protein interacts with lipid-containing microdomains (Fortin et al., 2004). The mechanism of α-synuclein aggregation in idiopathic PD is still elusive, but high cholesterol has been shown to promote its aggregation in cultured cells (Bar-On et al., 2008). Moreover, oxidative stress caused by autoxidation of e.g., dopamine, as well as the malfunction of the degradation machinery, have been suggested to play role (Bourdenx et al., 2014; Goodwin et al., 2013). Although α-synuclein clearance is likely to occur through different mechanisms, recent reports have underscored the important contribution of lysosomal pathways and, in particular, chaperone-mediated autophagy (CMA). Posttranslational modifications of α-synuclein, especially dopamine-induced modifications could impair and even block the CMA degradation pathway (Martinez-Vicente et al., 2008).

Abbreviations: carboxy-H_2DCFDA, 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate; CMA, chaperone mediated autophagy; DAPI, 2-(4-amidinophenyl)-1H -indole-6-carboxamidine; $\Delta\Psi_m$, mitochondrial membrane potential; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LDL, low-density lipoprotein; LMP, lysosomal membrane permeabilization; LAMP-2, lysosomal associated membrane protein-2; MβCD, methyl-ß-cyclodextrin; MPP, 1-methyl-4-phenylpyridinium; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; PD, Parkinson's disease; PUFA, polyunsaturated fatty acid; ROS, reactive oxygen species; SRB, sulforhodamine B; TMA-DPH, 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene *p*-toluenesulfonate; U18666A, 3-β-[2-(diethylamine)ethoxy]androst-5-en-17-one.

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Oxidative stress is a well-documented mechanism for the destruction of cellular macromolecules and triggering of apoptotic cell death. Reactive oxygen species (ROS)-induced lysosomal membrane permeabilization (LMP) might contribute to direct neuronal cell death by the release of hydrolytic proteases into the cytosol (Dehay et al., 2010). Moreover, the release of lysosomal proteases, cathepsins, to the cytosol has been shown to trigger apoptotic cell death in several systems (Johansson et al., 2010; Kagedal et al., 2001). Interestingly, we have recently studied the effect of intracellular cholesterol levels in the neurodegenerative lysosomal storage disorder, Niemann Pick's disease C1, and found that the accumulation of lysosomal cholesterol increases lysosomal stability and prevents LMP (Appelqvist et al., 2012). Similarly, using the cholesterol transport inhibitor U18666A, which mimics the Niemann Pick's disease C1 phenotype, LMP was diminished.

The neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) induces a PD-like syndrome that is almost indistinguishable from PD in humans (Przedborski et al., 2001). In the brain, MPTP is metabolized, mainly by astrocytes, to the active substance MPP^+, which is subsequently taken up and concentrated within dopaminergic neurons by dopamine transporters (Singer and Ramsay, 1990). MPP^+ interferes with complex I of the mitochondrial electron transport chain, generating ROS (Przedborski et al., 2001) and subsequent oxidative damage. Of note, several reports have identified mitochondrial complex I deficiency in the nigrostriatum of parkinsonian patients, indicating a close relationship between idiopathic and MPP^+-induced diseases (Navarro and Boveris, 2009). PD is incurable, and standard treatments include L-DOPA, monoamine oxidase B (MAO-B) inhibitors and dopamine agonists, which temporarily reduce motor dysfunction. Recent studies have suggested that cholesterol-lowering drugs, such as statins, might be beneficial in PD (Bar-On et al., 2008; Friedman et al., 2013), although contradictory findings have been presented (Huang et al., 2015).

The present study was undertaken to elucidate the impact of altered intracellular cholesterol in PD using dopamine-producing BE(2)-M17 neuroblastoma cells treated with MPP^+ to induce PD-like cytotoxicity. We show that MPP^+ exposure results in lysosomal cholesterol accumulation, which could be mimicked by pre-exposing cells to U18666A. Our main findings are that cholesterol has a dual role in Parkinson's disease, conferring protection against LMP-induced cell death and stimulating α-synuclein accumulation. Lovastatin reduces cell toxicity, probably by preventing ROS production, but has no effect on α-synuclein accumulation.

2. Materials and methods

2.1. Cell culture and treatment

The dopaminergic neuroblastoma cell line BE(2)-M17 used in this study was purchased from ATCC, (American Type Culture Collection, LGC Standards GmbH, Wesel, Germany). Cells were cultured in Eagle's minimal essential medium/Ham's F12 (1:1) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin (all from Gibco, Paisley, UK) at 37 °C in a humidified atmosphere with 5% CO_2. For the experiment, the cells were trypsinized and seeded at a density of 50,000 cells/cm^2, and after 72 h the PD-inducing agent MPP^+ was added (1–5 mM for 6–48 h). When indicated, the cells were pretreated with Bafilomycin A1 (10 nM for 30 min), Z-FA-FMK (10 μM for 24 h, Enzyme Systems Products, Livermore, CA, USA), pepstatin A (100 μM for 16 h), E64d (10 μM for 16 h), Z-VAD-FMK (10 μM for 1 h), lovastatin (1–10 μM for 48 h), methyl-β-cyclodextrin (MβCD; 0–1 mM for 1 h and 24-h chase) (Rosenbaum et al., 2010), U18666A (0–5 μg/ml for 24 h) and α-tocopherol (10 μM for 48 h). If not stated otherwise chemicals were obtained from Sigma-Aldrich, St. Louis, MO, USA.

2.2. Measurements of viability and apoptosis

Viability was analyzed by the sulforhodamine B (SRB) assay according to the manufacturer's instructions. Briefly, cells were fixed by adding 50% trichloroacetic acid (TCA) to the growth medium for 1 h at 4 °C. After rinsing in water, a 0.4% SRB solution was added for 20 min. The dishes were then dried, and the incorporated dye was dissolved in 10 mM Tris base. Absorbance was measured at 595 nm using a Wallac 1420 Victor Plate Reader (PerkinElmer, Waltham, MA, USA). Caspase-3 activity was analyzed using the fluorescent substrate Ac-DEVD-AMC (Becton-Dickinson, Mountain View, CA, USA) according to the manufacturer's instructions. Fluorescence was correlated with protein content, as determined by the BioRad DC Protein Assay (Bio-Rad Laboratories, Hercules, CA, USA).

2.3. Determination of ROS and mitochondrial membrane potential

ROS were analyzed using the cell-permeable probe 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (carboxy-H2DCFDA, Molecular Probes, Eugene, OR, USA). Cells were labeled with 25 μM carboxy-H2DCFDA for 30 min at 37 °C and then analyzed with a Wallac 1420 Victor Plate Reader (λ_{ex}485 nm, λ_{em}535 nm). The results are given as the mean green fluorescence. Loss of mitochondrial membrane potential was assessed with the cationic carbocyanine dye JC-1 (Molecular Probes). Cells were labeled with 5 μg/ml JC-1 for 10 min at 37 °C, washed and resuspended in PBS and then analyzed in a flow cytometer equipped with a 488 nm argon laser (CyFlow® Cube 8, Partec, Münster, Germany). Data from 10,000 cells were collected, and the ratio between the median green and red fluorescence was calculated.

2.4. LDL uptake

LDL uptake was analyzed using fluorescently labelled LDL (LDL Bodipy FL; Molecular Probes). Cells were labelled with 10 μg/ml LDL Bodipy FL in serum-free culture medium for 6 h, washed, resuspended in PBS and then analyzed in a flow cytometer (Gallios, Beckman Coulter, Brea, CA, USA) using the 488 nm laser and a 550 nm short pass filter together with a 525 nm bandpass filter. Data from 10,000 cells were collected and results are presented as median green fluorescence.

2.5. Cellular fractionation

Cytosolic and membrane fractions were collected using a Proteo Extract™ Subcellular Proteome Extraction Kit (Calbiochem, Darmstadt, Germany) according to the manufacturer's instructions. Aliquots were mixed with Laemmli Sample Buffer (Bio-Rad Laboratories) at a 2:1 ratio and subjected to Western blot. To establish equal loading, rabbit anti-lactate dehydrogenase (LDH; 1:50,000; Abcam, Cambridge, UK) was used.

2.6. Cholesterol measurement

Total and esterified cholesterol level was measured in cell lysates using an Amplex Red Cholesterol Assay Kit (Invitrogen, Paisley, UK), as described by the manufacturer. The cholesterol level was correlated with the protein content, as determined by the Bio-Rad DC Protein Assay.

2.7. Immunocytochemistry

Cells cultured on glass coverslips were fixed in 4% paraformaldehyde for 20 min at 4 °C. After washing in PBS, the cells were