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Hsp27 inhibits 6-hydroxydopamine-induced cytochrome *c* release and apoptosis in PC12 cells

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Abstract

Cellular stress may stimulate cell survival pathways or cell death depending on its severity. 6-Hydroxydopamine (6-OHDA) is a neurotoxin that targets dopaminergic neurons that is often used to induce neuronal cell death in models of Parkinson's disease. Here we present evidence that 6-OHDA induces apoptosis in rat PC12 cells that involves release of cytochrome *c* and Smac/Diablo from mitochondria, caspase-3 activation, cleavage of PARP, and nuclear condensation. 6-OHDA also induced the heat shock response, leading to increased levels of Hsp25 and Hsp70. Increased Hsp25 expression was associated with cell survival. Prior heat shock or overexpression of Hsp27 (human homologue of Hsp25) delayed cytochrome *c* release, caspase activation, and reduced the level of apoptosis caused by 6-OHDA. We conclude that 6-OHDA induces a variety of responses in cultured PC12 cells ranging from cell survival to apoptosis, and that induction of stress proteins such as Hsp25 may protect cells from undergoing 6-OHDA-induced apoptosis.

Keywords: Apoptosis; Caspase; Heat shock response; Hsp27; Parkinson's disease; Cytochrome *c*; Smac/Diablo; Rat

6-Hydroxydopamine (6-OHDA), a hydroxylated analogue of dopamine, is commonly used in model systems to mimic Parkinson's disease, since it induces death of dopaminergic neurons both in vivo and in vitro [1,2]. The cellular events that occur as a result of 6-OHDA exposure are not entirely understood. Production of reactive oxygen species [3–5] and/or inhibition of complex I are both reported to mediate 6-OHDA-induced cell death [6]. There is general agreement, however, that the mode of cell death is by apoptosis in a variety of cell culture model systems, including primary mesencephalic dopaminergic neurons [7,8], MN9D [9], and PC12 cells [2,10].

Apoptosis is a highly regulated form of cell death that occurs under physiological and pathological conditions. The activation of caspase proteases is central to this process [11,12]. Caspase activation can be triggered by the release of cytochrome *c* from the mitochondria, which occurs in response to several apoptotic stimuli including neurotoxins [13,14]. In the cytosol, cytochrome *c* interacts with Apaf-1, which in the presence of dATP leads to clustering and autoactivation of caspase-9 [13,14]. Active caspase-9 causes cleavage and activation of downstream caspases, e.g., caspase-3. Caspases mediate the degradation of a number of proteins critical for cell homeostasis such as the cytoskeletal protein, fodrin, and the DNA repair enzyme, poly(ADP-ribose) polymerase (PARP) [15].

While exposure of cells to severe stress can induce cell death, transient or milder stress conditions stimulate cells to activate protective strategies that involve the induction of prosurvival proteins [16,17]. For example, increased expression of heat shock protein 27 (Hsp27) and Hsp70 occurs after exposure of cells to non-lethal elevations in temperature or oxidants [18]. Hsp27

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belongs to a sub-family of stress proteins, the small Hsps, which are detectable in virtually all organisms. Increased expression of Hsp27 renders cells more resistant to lethal levels of a variety of toxic insults [19–21]. Hsp27 inhibits apoptosis [20], by interfering with caspase activation at several different levels [22–24]. It can interact directly with mitochondria, thereby preventing release of cytochrome *c* [23] and Smac/Diablo [25]. In the cytosol, it can also sequester both cytochrome *c* and pro-caspase-3, thus preventing the correct formation/function of the apoptosome [22,24].

The present study was undertaken to examine the effect of 6-OHDA on the induction of cell death and stress responses, and in particular, to examine the role of Hsp27 in 6-OHDA-induced cell death in PC12 cells.

Materials and methods

Materials. All chemicals were purchased from Sigma unless indicated otherwise. Mouse monoclonal antibody against Hsp70 and rabbit polyclonal antibodies against Hsp25 (rat homologue of human Hsp27) were obtained from StressGen Biotechnologies. Mouse monoclonal antibodies against PARP were obtained from Biomol and cytochrome *c* was from BD Pharmingen. Rabbit polyclonal antibody against caspase-3 was obtained from Cell Signalling Technology and Smac/Diablo antibody was a kind gift from Prof. Seamus Martin, Dept. of Genetics, Trinity College, Dublin, Ireland. Goat secondary antibodies conjugated to horseradish peroxidase were from Pierce. Goat secondary anti-rabbit IgG conjugated to Alexa-546 was from Molecular Probes. Ac-Asp-Glu-Val-Asp-α-(4-methyl-coumaryl-7-amide) (DEVD-MCA) was from the Peptide Institute, Osaka, Japan. Protein molecular weight markers were obtained from New England Biolabs. T4 polynucleotide kinase was from Promega. [γ-^{32}P]ATP was from ICN. Effectene transfection reagent was from Qiagen. PCI-neo Mammalian expression vector was obtained from Promega. Rat pheochromocytoma PC12 cells were obtained from the ECACC.

Cell culture and treatments. PC12 cells were maintained in RPMI 1640 medium supplemented with 10% horse serum, 5% fetal calf serum, 50 U/ml penicillin, 50 μg/ml streptomycin, and 2 mM D-glutamine, at 37 °C in a humidified 5% CO_2 atmosphere. They were passaged once weekly. Experiments were performed using dishes coated with poly-L-lysine (10 μg/ml for 3 h) to assist cell adhesion. For determining Hsp induction cells were seeded at a density of 5×10^6 in a 25 cm^2 culture flask and cultured for 24 h, prior to treatment (heat shock or addition of 6-OHDA). To subject the cells to heat shock, the culture flasks were sealed by wrapping parafilm around the lids and immersed in a water bath at 41.5 °C for 1 h. Cells were then allowed to recover at 37 °C in a humidified 5% CO_2 atmosphere for various times. For treatment with 6-OHDA, stock solutions of 6-OHDA were made freshly in sodium metabisulphite (1 mg/ml) prior to each experiment. Cells were exposed to various concentrations of 6-OHDA as indicated in the figure legends.

Analysis of cellular morphology. For morphological analysis, cells were scraped from flasks, and 100 μl of the cell suspension (approximately 50×10^4 cells) was cytocentrifuged onto glass slides. After air-drying the preparations were stained using RAPI-DIFF II stain pack (Triangle Biomedical Sciences). Cells were scored by counting at least 300 cells from each sample, from three different experiments.

DNA fragmentation. Following experimental treatments, cells were removed from the culture flask by scraping. Cells were centrifuged at 775*g* for 5 min at 4 °C. The pellet was washed once with phosphate-buffered saline (PBS), and lysed in 300 μl of a buffer containing 100 mM Tris–HCl, pH 8.5, 5 mM EDTA, 0.2 M NaCl, 0.2% (w/v) SDS, and 0.0001% (w/v) proteinase K. The cell suspension was incubated for 3 h at 50 °C, followed by addition of 30 μl of 3 M $C_2H_3NaO_2$ and 660 μl of 96% ethanol. The cell suspension was kept overnight at −20 °C. The lysate was then centrifuged at 21,000*g* for 10 min. The supernatant was discarded, the pellet was resuspended in 70% ethanol and centrifuged at 21,000*g* for 10 min. The pellet was air-dried at room temperature for 30 min. The pellet was resuspended in 50 μl buffer containing 10 mM Tris–HCl, pH 7.5, 1 mM EDTA, and 100 μg/ml RNase. The sample was incubated for 1 h at 37 °C. The sample was then centrifuged at 21,000*g* for 10 min, supernatant was removed, resuspended in 5× loading buffer (25% Ficoll and 0.25% bromophenol blue), and resolved on a 1.5% agarose gel for 2 h at 60 V. The bands were then visualised under UV light.

Detection of caspase activity. The activity of group II caspases, DEVDases, was determined fluorometrically as developed by Nicholson et al. [26] with some modifications [27]. Briefly, lysate from 2.5×10^6 cells and substrate (DEVD-MCA) were combined in reaction buffer {100 M *N*-2-hydroxyethyl-piperazine-*N*′-2-ethanesulphonic acid (HEPES), pH 7.5, 10% sucrose, 0.1% 3[(3cholamidopropyl)-dimethylammonio]-1-propanesulphonate, 5 mM dithiothreitol, 10^{-4}% Nonidet P40, and 50 μM DEVD-MCA} and added in triplicate to a microtitre plate. Substrate cleavage leading to the release of free MCA was monitored at 37 °C using a Wallac Victor multilabel counter (excitation 355 nm, emission 460 nm). Fluorescent units were converted to micromoles of MCA released using a standard curve generated with free MCA and subsequently related to protein concentration.

Preparation of whole cell extracts. Following experimental treatments the cells were removed from the culture flask by scraping. The cells were centrifuged at 775*g* for 5 min at 4 °C. The pellet was washed once with PBS and lysed in 100 μl of a buffer containing 20 mM HEPES, pH 7.5, 350 mM NaCl, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.1 mM EGTA, 1% Nonidet P-40, 0.5 mM dithiothreitol, 0.1% phenylmethylsulphonyl fluoride, and 1% aprotinin. The cell suspension was incubated on ice for 15 min and then centrifuged at 21,000*g* for 30 s. The supernatants were stored at −70 °C until further analysis by Western blotting. Protein content was determined using a Bio-Rad protein assay kit with bovine serum albumin (BSA) as standard.

Western blotting. Samples were resuspended in Laemmli's sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) sample buffer and boiled for 5 min. Proteins (20–25 μg per lane) were then resolved on 10–12% SDS–PAGE gels and electrophoretically transferred onto nitrocellulose for 1.5 h at 100 V. Membranes were blocked for 1 h in PBS containing 0.05% Tween 20 and 5% (w/v) non-fat dried milk. The membranes were then incubated for 1 h at room temperature with antibodies to Hsp25 (1:2000), Hsp27, cytochrome *c*, Hsp70 (1:1,000), Smac/Diablo (1:200) or actin (1:500). Alternatively, the membranes were incubated overnight at 4 °C with antibodies to caspase-3 or PARP (1:1000). This was followed by 1 h incubation at room temperature with appropriate horseradish peroxidase-conjugated goat IgGs (1:10,000 or 1:2000 for detection of caspase-3). Protein bands were then visualised using Supersignal West pico Western blot detection kit (Pierce).

Determination of cytochrome c and Smac/Diablo release. Cells were trypsinised and centrifuged at 150*g* for 5 min at 4 °C. The pellet was washed once with PBS. The cells were then lysed using 100 μl cell lysis and mitochondria intact (CLAMI) buffer containing (250 mM sucrose and 70 mM KCl in PBS, 0.1 mM phenylmethylsulphonyl fluoride, 1 mM dithiothreitol, 5 μg/ml pepstatin, 10 μg/ml leupeptin, 2 μg/ml aprotinin, and 25 μg/ml calpain inhibitor 1). Digitonin (10 μl of 20 mg/ml solution) was added to the samples on ice for 5 min and then the cell suspension was centrifuged at 3000*g* for 10 min at 4 °C. The supernatant was removed and stored as the cytosolic fraction, at −20 °C. The pellet was resuspended in 100 μl CLAMI buffer and stored as the mitochondrial fraction, at −20 °C. Samples were analysed by Western blotting.