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Activation of p53 and the pro-apoptotic p53 target gene PUMA during depolarization-induced apoptosis of chromaffin cells

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Abstract

The pathogenesis of non-glutamatergic, depolarization-induced cell death is still enigmatic. Recently, we have shown that veratridine induces apoptosis in chromaffin cells, and we have demonstrated protective effects of antioxidants in this system, suggesting a role for Na^+ channels and oxidative stress in depolarization-induced cell death. We examined the possible contribution of p53, a transcription factor that has a major role in determining cell fate, and the mitochondrial apoptosis pathway in veratridine-induced cell death of cultured bovine chromaffin cells. Nuclear condensation and fragmentation were detected several hours after a 60-min exposure to 30 μM veratridine. Apoptosis was associated with a transitory increase in p53 protein levels. Veratridine induced transcription of the pro-apoptotic p53 target gene PUMA, but not of bax or pig3. Using transient transfection experiments, we found that wild-type p53, but not the mutant form p53-273H, was sufficient to induce cell death in the chromaffin cells, which was caspase-9 dependent. The down-regulation of either p53, by overexpressing p53-273H, or caspase-9 activity using a dominant-negative caspase-9 mutant protected chromaffin cells against veratridine-induced toxicity. Our data demonstrate the importance of p53 and the downstream activation of the mitochondrial apoptosis pathway in depolarization-induced apoptosis.

Introduction

Apoptosis is an essential physiological process for the selective elimination of cells. The apoptotic pathway can be divided into three stages: a phase of induction (pre-mitochondrial), a phase of decision (mitochondrial), and a phase of degradation (post-mitochondrial). Mitochondria that during years were postulated to be the energy supply of eukaryotic cells have emerged as the headquarter of apoptosis signaling pathway. The decision phase, which contains the point of no return, corresponds to the permeabilization of the mitochondrial membranes and the release of pro-apoptotic proteins, such as the cytochrome *c*, AIF, pro-caspases, and Smac/DIABLO, into the cytosol. Once in the cytoplasm Apaf-1, caspase-9 and cytochrome *c* assemble and form the apoptosome, a large caspase-activating complex that plays a central role in the initiation and execution phases of apoptosis. Indeed, active forms of caspase-9 or Apaf-1 are required to form a functional apoptosome complex, and cells expressing mutant caspase-9 are insensitive against apoptotic stimuli, including chemotherapeutics (Osaki et al., 1997). In cellular necrosis, mitochondria also may undergo permeability transition (Ravagnan et al., 2002; Baines et al., 2005; Nakagawa et al., 2005). A large, specific pore—the mitochondrial permeability transition pore (MPTP)—opens, and solutes of up to 1.5 kDa pass freely in and out of the mitochondrial matrix (Bernardi, 1999; Crompton, 1999; Jordan et al., 2003; Saelens et al., 2004).

Veratridine is an alkaloid obtained from *sabadilla* seeds and from the rhizome of *hellebore* that inhibits the complete inactivation of sodium channels, maintaining the channel open with a small but steady sodium current by generating a change in the three-dimensional conformation of the sodium channels (Sutro, 1986). Veratridine as a depolarizing agent can also be used in the

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study of neuronal death, and provides a model system to study glutamate-independent, depolarization-induced cell death pathways which can be relevant for white matter injury (LoPachin et al., 2001; Stys et al., 1992; Stys and Lopachin, 1998). We have already shown that in bovine chromaffin cell cultures, a well-established model to study secretory machinery (Bader et al., 2002), veratridine induces a delayed cellular death, which has the features of apoptosis such as chromatin condensation and DNA fragmentation, mitochondrial depolarization, cytochrome *c* release, and caspase activation (Jordan et al., 2000, 2002). Conversely, the molecular mechanisms underlying the overall signaling response of chromaffin cells to veratridine toxicity are not well characterized. Wild-type p53 protein has been shown to be capable of inducing apoptosis (Yonish-Rouach et al., 1991; Ramqvist et al., 1993; Jordan et al., 1997). Recently, the roles of several p53 targets genes in mediating the p53 apoptotic response have been queried through loss-of-function analysis using knockout mouse models. These studies have demonstrated that the p53 targets including bax and PUMA (p53 up-regulated modulator of apoptosis) play cell-type-specific roles in p53-mediated apoptosis (Schuler and Green, 2001). PUMA encodes a BH3-only protein and it could be a principal mediator of cell death in response to diverse apoptotic signals (Jeffers et al., 2003).

In the present study, we examined the role of p53 in veratridine-induced cell death and its implication in apoptosis of bovine chromaffin cell death.

Materials and methods

Chromaffin cell culture

Bovine chromaffin cells were isolated as previously described (Galindo et al., 2003; Neco et al., 2004). After washing the gland with a Ca^{2+}-free Locke's solution (Locke's medium) containing (in mmol L^{-1}): NaCl 154, KCl 5.6, $MgCl_2$ 1, HEPES 10, glucose 10, pH 7 to remove remaining erythrocytes, adrenal glands were incubated with Ca^{2+}-free Locke's medium containing 0.2% collagenase (Boehringer-Mannheim, Indianapolis, IN) and 0.5% bovine serum albumin (Calbiochem, La Jolla, CA) for 45 (3 × 15) min. Following medulla dissection and further incubation in collagenase solution for 30 additional minutes, chromaffin cells were separated from erythrocytes using a Percoll gradient. Cells were plated either onto poly-L-lysine (Sigma, St. Louis, MO; 0.5 mg/mL in borate buffer, PH 8.0)-coated 15-mm round glass coverslips ($2–3 \times 10^5$ cells/coverslips) for cell viability experiments or in 25 cm^2 flasks ($5–8 \times 10^6$ cell/culture flasks) for reporter assays, in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum, penicillin (100 IU/mL) and streptomycin (50 μg/mL) at 37°C under an atmosphere of 5% CO_2.

Veratridine exposures

Chromaffin cell cultures were rinsed twice with Krebs HEPES buffer (K–H) with the following ionic composition (in mM): NaCl 140, KCl 5.9, $MgCl_2$ 1.2, HEPES 15, glucose 10, $CaCl_2$ 2.5, pH 7.4, incubated for 1 h either in K–H or in K–H containing 30 μM veratridine at room temperature. Exposure was terminated by washing the cells three times with K–H solution. For vitamin E and cycloheximide treatments, drugs were added 12 h before veratridine exposure and maintained until the end of the experiment.

Cell viability experiments

To assess the cell viability, coverslips containing chromaffin cells were treated with 1 μg/mL Hoechst 33342 for 1 min. The chromatins of GFP-positive cells stained with Hoechst 33342 were examined with a standard epi-illumination fluorescence microscope (Axiophot, Zeiss, Germany). Cells with condensed or fragmented chromatin represented dead cells. A blinded observer counted the number of dead and alive cells in 10 microscopic fields (under 40× magnifications) for each coverslip and the mean was regarded as the representative value for the coverslip. The percentage of dead cells was determined in 3 or 4 coverslips for each experimental condition. The average percent apoptotic cells from at least three separate experiments for each condition is expressed in the text and figures as the mean ± SEM. Statistical significance was determined by Student's *t* test.

Immunoblotting

Chromaffin cell cultures were washed with cold PBS twice and then collected by mechanical scraping with 1 mL of PBS per tissue culture dish. The protein suspension was centrifuged at 12,000–14,000 rpm for 5 min. The supernatant was discarded, and the protein pellet was brought up in 150 μL of sample buffer. The protein from each condition was quantified spectrophotometrically (Micro BCA Protein Reagent Kit, Pierce, Rockford, IL), and an equal amount of protein (30 μg) was loaded onto each lane of the 10% SDS-PAGE, which was then run at 90 mV. After electrophoresis, proteins were transferred to Immobilon PVDF membranes. Non-specific protein binding was blocked with Blotto [4% w/v non-fat dried milk, 4% bovine serum albumin (Sigma) and 0.1% Tween 20 (Sigma)] in PBS for 1 h. The membranes were incubated with anti-p53 [1:50 dilution of anti-mouse monoclonal (Pab240) sc-99 Santa Cruz] or anti-PUMA (1:1000 dilution of polyclonal, Oncogene Research Products, San Diego CA) overnight at 4°C. After washing with Blotto, the membranes were incubated with a secondary antibody (1:5000 dilution of peroxidase-labeled anti-mouse, Promega, Madison, WI) in Blotto. The signal was detected using