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Review

Arsenic stimulates release of cytochrome *c* from isolated mitochondria via induction of mitochondrial permeability transition

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Abstract

Arsenic trioxide, As(III), is a known environmental toxicant, co-carcinogen, and potent chemotherapeutic agent. In model experiments with isolated rat liver mitochondria, As(III) stimulated a dose-dependent, cyclosporin A-sensitive release of cytochrome *c* via induction of mitochondrial permeability transition and subsequent swelling of mitochondria. Mitochondrial GSH does not seem to be a target for As(III) which, however, appears to cause oxidative modification of thiol groups of pore forming proteins, notably adenine nucleotide translocase. In mouse embryonic fibroblasts, 10 μM As(III) stimulated cytochrome *c* release and apoptosis via a Bax/Bak-dependent mechanism. At high concentrations (125 μM and higher), cells died by Bax/Bak-independent necrosis; at this concentration range As(III) targets mitochondria directly, particularly complex I of the mitochondrial respiratory chain. Since pyruvate, a substrate of complex I, is a predominant mitochondrial substrate in the cell, inhibition of complex I will cause mitochondrial instability and a decrease of $\Delta\psi$ that facilitates permeability transition and necrotic cell death.

Keywords: Arsenic; Apoptosis; Necrosis; Mitochondrial permeability transition; Cytochrome *c*

Contents

Introduction

Arsenic is an environmental contaminant, which exists predominantly as highly toxic derivatives in trivalent and pentavalent forms. Inorganic arsenic compounds are often more unstable, more toxic, and excreted more slowly than the organic forms (Waxman and Anderson, 2001). Arsenic is also

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used in cancer chemotherapy; recently the advantage of low doses of As(III) compared to traditional cytotoxic chemotherapeutic agents in the treatment of hematological malignancies has been shown (Soignet et al., 1998). Experiments performed in our laboratory revealed that at low concentrations sodium arsenite induces both apoptosis and necrosis when administered in vitro to normal T cells (Bustamante et al., 1997). Similar effects of arsenic compounds have been observed by others in different cellular systems.

Apoptosis and necrosis are two modes of cell death with distinct morphological and biochemical features. Apoptosis is an active process characterized by cell shrinkage, nuclear and cytoplasmic condensation, chromatin fragmentation, and phagocytosis of the apoptotic cell. In contrast, necrosis is a passive form of cell death associated with inflammation, resulting from cellular and organelle swelling, rupture of the plasma membrane, and spilling of cellular contents into the extracellular milieu. Lethal levels of different toxicants may trigger either apoptotic or necrotic cell death, depending on cell type and severity of insult. Further, effectuation of the apoptotic death program requires maintenance of a sufficient intracellular energy level (Stridh et al., 1999) and of a redox state compatible with caspase activation (Nobel et al., 1997). Thus, ATP depletion or severe oxidative stress may re-direct otherwise apoptotic cell death to necrosis.

Numerous data illustrate a key role of mitochondria in cell death (Orrenius, 2004). Induction of apoptosis often brings about impairment of different mitochondrial functions: release of cytochrome *c* and other proteins into the cytoplasm, failure of the electron transport system, dissipation of the mitochondrial transmembrane potential ($\Delta\psi$), and increased permeability of the inner mitochondrial membrane (mitochondrial permeability transition, MPT) due to opening of a non-specific pore.

Since phenylarsine oxide, a derivative of As(III), was shown to stimulate permeability transition in isolated rat liver mitochondria (Lenartowics et al., 1991), the induction of MPT was suggested as a key step in apoptosis induced by arsenic (Larochette et al., 1999). The goal of the present study was to investigate the mechanisms involved in As(III)-induced mitochondrial deterioration and how changes at the mitochondrial level promote cell death. Hence, we have studied the effect of As(III) on isolated rat liver mitochondria, and on wild-type and $Bax^{-/-}Bak^{-/-}$ mouse embryonic fibroblasts (MEF). The results demonstrate that high concentrations of arsenic directly target mitochondria and trigger necrotic cell death, whereas at low concentrations, As(III) induces apoptosis which is mediated by Bax and/or Bak.

Methods

Cells, antibodies, and chemical reagents. $Bax^{-/-}$ and $Bak^{-/-}$ "double knockout" (DKO) cells were a generous gift from Dr. Stanley Korsmeyer, Dana-Farber Cancer Institute, Boston, MA. Cytochrome *c* antibody and recombinant caspase-3 were obtained from BD-Pharmingen (San Diego, CA). BAPTA-AM was purchased from Molecular Probes (Eugene, OR), whereas arsenic trioxide and all other chemicals were obtained from Sigma (St. Louis, MO).

Quantification of DNA fragmentation. Apoptosis was monitored by propidium iodide (PI) staining, and fluorescence-activated cell sorting (FACS) analysis was carried out as described previously (McConkey et al., 1996). Following incubation with various agents in vitro, cells were pelleted by centrifugation and resuspended in phosphate-buffered saline containing 50 μg/ml propidium iodide, 0.1% Triton X-100, and 0.1% sodium citrate. Samples were stored at 4 °C for 16 h and vortexed prior to FACS analysis (FL-3 channel) (FACScan; Becton Dickinson, Mountain View, CA).

Quantification of necrosis. Cells (0.5×10^6) were harvested, washed with Ca^{2+}-free PBS, and resuspended in 0.5 ml of Ca^{2+}-free PBS; propidium iodide was added to a final concentration of 5 ng/ml. Samples were gently vortexed and read 1–2 min after addition of propidium iodide using the FL-3 channel on a flow cytometer.

Isolation of rat liver mitochondria. Male Harlan Sprague–Dawley rats (6–8 weeks old) were killed by CO_2 inhalation in accordance with the European directive systems of protection of vertebrate animals for scientific research. The liver was removed and minced on ice, resuspended in 50 ml of MSH buffer (210 mM mannitol, 70 mM sucrose, 5 mM Hepes, pH 7.5) supplemented with 1 mM EDTA, and homogenized with a glass homogenizer and Teflon pestle. Homogenates were centrifuged at $600 \times g$ for 8 min at 4 °C. The supernatant was decanted and re-centrifuged at $5.500 \times g$ for 15 min to form a mitochondrial pellet that was resuspended in MSH buffer without EDTA and centrifuged again at $5500 \times g$ for 15 min. The final pellet was resuspended in MSH buffer at a protein concentration of 80–100 mg/ml.

Estimation of functional activity of isolated mitochondria. Mitochondria were incubated in MSH buffer supplemented with 5 mM succinate, 1 μM rotenone, and 1 mM inorganic phosphate (P_i). Estimation of $\Delta\psi$ was performed using an electrode sensitive to the lipophilic cation tetraphenylphosphonium (TPP^+). Energized mitochondria rapidly accumulate TPP^+ from the incubation buffer and release this cation as $\Delta\psi$ decays. Ca^{2+} uptake and release by the mitochondria were monitored using a Ca^{2+}-sensitive electrode (Thermo-Orion Inc, Beverly, MA). Mitochondrial swelling was monitored continuously as changes in optical density at 540 nm (OD_{540}). Oxygen consumption was measured using a Clark-type oxygen electrode (Yellow Spring Instrument Co., OH, USA) at 25 °C. At the end of the incubation period, mitochondrial suspensions were centrifuged at $10,000 \times g$ for 5 min, and the resulting