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The study of the mechanism of arsenite toxicity in respiration-deficient cells reveals that NADPH oxidase-derived superoxide promotes the same downstream events mediated by mitochondrial superoxide in respiration-proficient cells

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ABSTRACT

We herein report the results from a comparative study of arsenite toxicity in respiration-proficient (RP) and -deficient (RD) U937 cells. An initial characterization of these cells led to the demonstration that the respiration-deficient phenotype is not associated with apparent changes in mitochondrial mass and membrane potential. In addition, similar levels of superoxide ($O_2^{\cdot-}$) were generated by RP and RD cells in response to stimuli specifically triggering respiratory chain-independent mitochondrial mechanisms or extramitochondrial, NADPH-oxidase dependent, mechanisms. At the concentration of 2.5 μM, arsenite elicited selective formation of $O_2^{\cdot-}$ in the respiratory chain of RP cells, with hardly any contribution of the above mechanisms. Under these conditions, $O_2^{\cdot-}$ triggered downstream events leading to endoplasmic reticulum (ER) stress, autophagy and apoptosis. RD cells challenged with similar levels of arsenite failed to generate $O_2^{\cdot-}$ because of the lack of a functional respiratory chain and were therefore resistant to the toxic effects mediated by the metalloid. Their resistance, however, was lost after exposure to four fold greater concentrations of arsenite, coincidentally with the release of $O_2^{\cdot-}$ mediated by NADPH oxidase. Interestingly, extramitochondrial $O_2^{\cdot-}$ triggered the same downstream events and an identical mode of death previously observed in RP cells.

Taken together, the results obtained in this study indicate that arsenite toxicity is strictly dependent on $O_2^{\cdot-}$ availability that, regardless of whether generated in the mitochondrial or extramitochondrial compartments, triggers similar downstream events leading to ER stress, autophagy and apoptosis.

1. Introduction

The mitochondrial electron transport chain is a site in which electrons escaping from the respiratory complexes may be accepted by molecular oxygen, thereby forming the superoxide radical ($O_2^{\cdot-}$). Various damaging agents and conditions, often associated with enhanced mitochondrial Ca^{2+} accumulation (Rizzuto et al., 2012; Orrenius et al., 2015), directly or indirectly trigger this sequence of events, which leads to H_2O_2 formation and eventually, after interaction with transition metals, to the formation of a more reactive radical species, the hydroxyl radical (Turrens, 2003; Venditti et al., 2013; Zorov et al., 2014). While these events are a common cause of mitochondrial damage (Venditti et al., 2013; Zorov et al., 2014; Orrenius et al., 2015), with an ensuing impairment in energy production, these organelles may respond to dysfunction with enhanced formation of H_2O_2, which might then exit the mitochondria and produce damage in a variety of extramitochondrial targets (Zorov et al., 2014).

A second important source of $O_2^{\cdot-}$ is NADPH oxidase that, while more densely expressed in neutrophils and other phagocytes (Babior, 1999; Bedard and Krause, 2007), can also be found in endothelial (Jones et al., 1996; Bedard and Krause, 2007) and other non-phagocytic cells (Cave et al., 2006; Bedard and Krause, 2007). Hence, NADPH oxidase-dependent $O_2^{\cdot-}$ formation might also be engaged in specific toxicity paradigms (Bedard and Krause, 2007). Contrary to the former condition, however, $O_2^{\cdot-}$ produced by NADPH oxidase is at least in part extracellular, with the possibility that the resulting H_2O_2 readily penetrates plasma, and eventually, mitochondrial membranes to produce hydroxyl

Abbreviations: Ant, antimycin A; Apo, apocynin; CHOP, GADD153/C/EBP homologous protein; Cf, caffeine; CsA, cyclosporin A; DHR, Dihydrorhodamine 123; DPI, diphenyleneiodonium; ER, endoplasmic reticulum; GRP78, glucose-regulated protein 78; H_2O_2, hydrogen peroxide; LC3, microtubule-associated protein light chain 3; 3-MA, 3-methyladenine; MDC, monodansylcadaverine; MPT, mitochondrial permeability transition; $O_2^{\cdot-}$, superoxide; PBS, phosphate-buffered saline; PMA, phorbol-12-myristate-13-acetate; RD cells, respiration-proficient cells; RP cells, respiration-proficient cells; Ry, ryanodine; Rot, rotenone.

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radical-dependent damage in the intramitochondrial compartment. Obviously, there is the alternative, or additional, possibility that intracellular $O_2^{\cdot-}$ and/or H_2O_2-derived hydroxyl radicals, initiate events and produce effects on a variety of extra-mitochondrial targets.

The very last important consideration is that $O_2^{\cdot-}$, regardless of whether generated in the intra- or extra-mitochondrial compartments, can interact with nitric oxide to generate peroxynitrite, a highly reactive nitrogen species (Beckman and Koppenol, 1996).

Based on the above information and on the fact that different drugs promote either mitochondrial or NADPH-dependent $O_2^{\cdot-}$ formation, or both events, an important question to be asked is on the different impact of the $O_2^{\cdot-}$ produced in the mitochondrial *vs* extra-mitochondrial compartments in the context of a specific toxicity paradigm.

Arsenite is a widely diffused environmental contaminant (Rodriguez-Lado et al., 2013) and oxidative stressor reported to produce $O_2^{\cdot-}$ mostly through NADPH oxidase (Smith et al., 2001; Straub et al., 2008; Flora, 2011), with some reports also postulating a role for mitochondria (Liu et al., 2005; Guidarelli et al., 2016), or even an involvement of these organelles downstream to NADPH oxidase activation (Li et al., 2014). As these different results are most likely dependent on the specific cell type employed, we recently performed an initial characterization of the U937 cell clone utilised in our laboratory. We found that low concentrations of arsenite induce U937 cell apoptosis through a mitochondrial permeability transition (MPT)-mediated mechanism (Guidarelli et al., 2015), entirely dependent on mitochondrial $O_2^{\cdot-}$ formation (Guidarelli et al., 2016). Suppression of mitochondrial $O_2^{\cdot-}$ formation with rotenone (Rot), or the respiration-deficient phenotype, was invariably associated with prevention of the toxic effects of arsenite. In addition, the effects of $O_2^{\cdot-}$ were not mediated by downstream interactions with nitric oxide to generate peroxynitrite.

The present study was performed with the aim of further characterising the mechanism of mitochondrial $O_2^{\cdot-}$ formation elicited by arsenite which, using different strategies, turned out to be entirely based on interference with the electron transport in the respiratory chain. A second important aim of this study was to investigate the mechanism of arsenite toxicity in respiration-deficient (RD) cells, derived from the same U937 cell line (RP cells) employed in the above studies, *i.e.*, under conditions in which the mitochondrial mechanism of $O_2^{\cdot-}$ formation cannot take place. We postulated that these cells, resistant to arsenite, would have been eventually killed by greater concentrations of the metalloid, thereby enabling us to ask the question of whether $O_2^{\cdot-}$ formation is a critical, or a dispensable event in arsenite toxicity. The results obtained provided evidence for the formation of NADPH oxidase-derived $O_2^{\cdot-}$, that turned out to be critical also in the ensuing lethal response. We were also surprised to observe that mitochondrial and NADPH oxidase-derived $O_2^{\cdot-}$ are equally effective in triggering apoptosis, in both circumstances preceded by the endoplasmic reticulum (ER) stress and autophagic responses.

2. Materials and methods

2.1. Chemicals

Sodium arsenite, Rot, diphenyleneiodonium (DPI), apocynin (Apo), phorbol-12-myristate-13-acetate (PMA), caffeine (Cf), antimycin A (Ant A), ryanodine (Ry), monodansylcadaverine (MDC), Hoechst 33,342, as well most reagent-grade chemicals were purchased from Sigma-Aldrich (Milan, Italy). Dihydrorhodamine 123 (DHR), MitoSOX Red, MitoTracker Green and MitoTracker Red CMXRos were purchased from Molecular Probes (Leiden, The Netherlands).

2.2. Cell culture and treatment conditions

U937 human myeloid leukemia cells were cultured in suspension in RPMI 1640 medium (Sigma-Aldrich, Milan, Italy) supplemented with 10% fetal bovine serum (Euroclone, Celbio Biotecnologie, Milan, Italy), penicillin (100 units/ml) and streptomycin (100 μg/ml) (Euroclone), at 37 °C in T-75 tissue culture flasks (Corning Inc., Corning, NY, USA) gassed with an atmosphere of 95% air-5% CO_2. RD cells were obtained by culturing U937 cells in RPMI medium containing 110 μg/ml pyruvate, 5 μg/ml uridine and 400 ng/ml ethidium bromide for 4 days with medium changes every 2 days. Sodium arsenite was prepared as a 1 mM stock solution in saline A (8.182 g/l NaCl, 0.372 g/l KCl, 0.336 g/l $NaHCO_3$, and 0.9 g/l glucose) and stored at 4 °C. Cells (1×10^5 cells/ml) were exposed to arsenite in complete RPMI 1640 culture medium, as reported in the legends to the figures.

Experiments with peroxynitrite were performed using 15 ml plastic tubes containing 5×10^5 cells (2 ml) of pre-warmed saline A. Peroxynitrite, synthesized as previously described (Guidarelli et al., 2006), was rapidly added on the wall of the plastic tubes and mixed to equilibrate the peroxynitrite concentration in the culture medium. To avoid changes in pH due to the high alkalinity of the peroxynitrite stock solution, an appropriate amount of 1.5 N HCl was also added to the wall of the tubes prior to peroxynitrite.

2.3. DHR and MitoSOX red fluorescence assays

The cells were incubated for 30 min with 10 μM DHR or 5 μM MitoSOX Red prior to the end of the 16 h exposure to arsenite. After the treatments, the cells were washed three times and fluorescence images were captured with a BX-51 microscope (Olympus, Milan, Italy), equipped with a SPOT-RT camera unit (Diagnostic Instruments, Delta Sistemi, Rome, Italy) using an Olympus LCAch 40 x/0.55 objective lens. The excitation and emission wavelengths were 488 and 515 nm (DHR), and 510 and 580 nm (MitoSOX Red) with a 5-nm slit width for both emission and excitation. Images were collected with exposure times of 100–400 ms, digitally acquired and processed for fluorescence determination at the single cell level on a personal computer using the J-Image software. Mean fluorescence values were determined by averaging the fluorescence values of at least 50 cells/treatment condition/experiment.

2.4. Aconitase activity

After treatments, the cells were washed twice with saline A, resuspended in lysis buffer (50 mM Tris-HCl, 2 mM Na-citrate, 0.6 mM $MnCl_2$, pH 7.4) and finally sonicated three times on ice by using the Sonicator Ultrasonic Liquid Processor XL (Heat System-Ultrasonics, Inc., NY) operating at 20 W (30 s). The resulting homogenates were centrifuged for 5 min at 18,000 ×*g* at 4 °C. Aconitase activity was determined spectrophotometrically in the supernatants at 340 nm, as described in ref. (Gardner, 2002).

2.5. Cytotoxicity assay

After treatments with arsenite, the number of viable cells was estimated with the trypan blue exclusion assay. Briefly, an aliquot of the cell suspension was diluted 1:2 (v/v) with 0.4% trypan blue and the viable cells (*i.e.*, those excluding trypan blue) were counted with the hemocytometer.

2.6. Apoptosis detection

After treatments, the cells were incubated for 5 min with the cell-permeable DNA dye (Hoechst 33,342, 10 μmol/l) and then visualized by fluorescence microscopy to determine nuclear morphology (chromatin condensation and fragmentation). Cells with homogeneously stained nuclei were considered viable. Apoptotic DNA fragmentation in individual cells was also detected by using the comet assay (Singh et al., 1988). After treatments, the cells were resuspended at 2.0×10^4 cells/100 μl in 1.0% low-melting agarose in phosphate buffer saline (PBS, 8 g/l NaCl, 1.15 g/l Na_2HPO_4, 0.2 g/l KH_2PO_4, 0.2 g/l KCl)