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The dual role of mitochondrial superoxide in arsenite toxicity: Signaling at the boundary between apoptotic commitment and cytoprotection

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ABSTRACT

Arsenite toxicity is in numerous cellular systems dependent on the formation of reactive oxygen and or nitrogen species. This is also true in U937 cells in which the metalloid selectively promotes the formation of mitochondrial superoxide (mito$O_2^{-\cdot}$) rapidly converted to diffusible H_2O_2. We tested the hypothesis that, under the same conditions, mito$O_2^{-\cdot}$ also mediates the triggering of a parallel survival signaling. We found that a low concentration of the metalloid causes an early activation of nuclear factor erythroid 2 p45-related factor 2 (Nrf2), and a downstream signaling leading to enhanced GSH biosynthesis, *via* a mechanism sensitive to various treatments/strategies selectively preventing mito$O_2^{-\cdot}$ formation. Under the same conditions, the toxic effects mediated by arsenite, leading to delayed mitochondrial permeability transition (MPT)-dependent apoptosis, were also prevented. Additional studies revealed remarkable similarities in the kinetics of mito$O_2^{-\cdot}$ formation, MPT induction, Nrf2 activation and GSH biosynthesis, prior to the onset of apoptosis in a small portion of the cells. Importantly, mito$O_2^{-\cdot}$ formation, as well as the ensuing toxic events, were significantly potentiated and anticipated under conditions associated with inhibition of *de novo* GSH biosynthesis triggered by the metalloid through Nrf2 activation.

We conclude that, in the arsenite toxicity paradigm under investigation, mito$O_2^{-\cdot}$ represents the only trigger of two opposite pathways leading to activation of the Nrf2 signaling and/or to a MPT-dependent apoptotic death. The first pathway, through enhanced GSH biosynthesis, mitigates the extent of further mito$O_2^{-\cdot}$ formation, thereby limiting and delaying an otherwise rapid and massive apoptotic death.

1. Introduction

Arsenite is a widely diffused environmental toxicant. Epidemiological studies have demonstrated that human exposure to the metalloid, mainly resulting from the ingestion of contaminated drinking water, significantly increases the risk to develop various types of cancer and other chronic pathologies (Flora, 2011; Jomova et al., 2011). Although various mechanisms have been thus far proposed to explain the carcinogenic and toxic effects of the metalloid, in particular those resulting from its direct binding to thiol residues of target molecules (Shen et al., 2013; Watanabe and Hirano, 2013; Mandal, 2017), the involvement of reactive oxygen species (ROS) in these events is nowadays widely accepted (Flora, 2011; Jomova et al., 2011; Ellinsworth, 2015). Indeed, numerous studies have used various approaches to provide an indication of ROS formation and to infer the involvement of these species in the resulting lesions through the demonstration of a protective effect mediated by antioxidant supplementation (Flora, 2011; Jomova et al., 2011; Rao et al., 2017).

Less studies have instead addressed the question of the origin and identity of the ROS produced in response to arsenite, and the limited amount of information available indicates that the mechanism (s) leading to superoxide ($O_2^{-\cdot}$) formation is largely dependent on the cell type and treatment condition. For example, the involvement of NADPH oxidase has been reported in some cell types (Smith et al., 2001; Straub

Abbreviations: AA, L-ascorbic acid; BSO, DL-buthionine-[S,R]-sulfoximine; CsA, cyclosporin A; DHR, dihydrorhodamine 123; DTNB, dithiobis-(2-nitrobenzoic acid); EB, extracellular buffer; γ-GCS, γ-glutamylcysteine synthase; GCLC, γ-GCS catalytic heavy subunit; GCLM, γ-GCS regulatory light subunit; GSH, glutathione; Keap1, Kelch-like ECH-associated protein 1; Mito$O_2^{\cdot-}$, mitochondrial superoxide; MPT, mitochondrial permeability transition; NO, nitric oxide; Nrf2, nuclear factor erythroid 2 p45-related factor 2; $O_2^{\cdot-}$, superoxide; PBS, phosphate buffered solution; RD cells, respiration-deficient cells

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et al., 2008; Flora, 2011; Li et al., 2014; Ellinsworth, 2015), in which the relevance of this pathway of $O_2^{-\cdot}$ production is likely dependent on the level of expression of NADPH oxidase itself. Activation of this pathway may then result in mitochondrial dysfunction, thereby recruiting an additional mechanism of $O_2^{-\cdot}$ formation (Li et al., 2014). In this perspective, the selective activation of mitochondrial $O_2^{-\cdot}$ ($mitoO_2^{-\cdot}$) formation is more likely to take place in cell types poorly expressing NADPH oxidase. Evidence of $mitoO_2^{-\cdot}$ formation has been provided in a limited number of studies (Flora, 2011; Jomova et al., 2011; Guidarelli et al., 2016a; Guidarelli et al., 2016b). Additional factors affecting the cell type-dependence of the ROS response to arsenite are related to the expression of nitric oxide (NO) synthase, as the diffusion-limited reaction of $O_2^{-\cdot}$ with NO leads to the formation of peroxynitrite, a highly reactive and toxic species (Liu et al., 2005; Jomova et al., 2011; Ellinsworth, 2015). Cells not expressing NO synthase will be eventually damaged by H_2O_2, the dismutation product of $O_2^{-\cdot}$, with different sub-cellular concentrations of the oxidant expected to result from $mitoO_2^{-\cdot}$ formation, associated with the effect of manganese superoxide dismutase, or NADPH-oxidase-derived $O_2^{-\cdot}$, associated with the action of cupper/zinc superoxide dismutase.

These simple considerations, while representing only a small part of a more complicate scenario, nevertheless emphasize the importance of learning more on the site in which ROS are being generated in response to arsenite. These mechanisms necessarily impact on the identity, and sub-cellular concentrations, of the downstream species produced and are also critical to correctly address the study of the indirect effects of arsenite, for example at the level of gene expression, induction of damage on different sub-cellular targets, apoptotic and even survival signaling responses.

By keeping these considerations in mind, we initially characterised the effects of arsenite in promonocytic U937 cells to understand more on the antileukemic and toxic effects of the metalloid (Guidarelli et al., 2015; Guidarelli et al., 2016a). Our interest was also stimulated by the observation that in these cells low concentrations of arsenite (*e.g.*, 2.5 μM) selectively promote $mitoO_2^{-\cdot}$ formation, in the absence of detectable effects mediated by NADPH oxidase (Guidarelli et al., 2016b). This notion was established using various approaches and significantly greater concentrations of the metalloid were necessary in order to promote detectable NADPH oxidase activation. As an example, 10 μM arsenite caused in respiration-deficient U937 cells (which fail to produce $mitoO_2^{-\cdot}$ in response to 2.5 μM arsenite) a NADPH oxidase inhibitor-sensitive DHR fluorescence response similar to that mediated by 2.5 μM arsenite in respiration-proficient cells.

Thus, the metalloid caused the selective formation of $mitoO_2^{-\cdot}$ and we were indeed able to selectively suppress this response with rotenone, an inhibitor of complex I (Degli Esposti, 1998), or using the same cells manipulated to induce respiratory deficiency (Guidarelli et al., 2016a; Guidarelli et al., 2016b). It is important to remind that U937 cells are highly glycolytic, and therefore maintain high ATP levels, and remain viable, under conditions of impaired function/activity of the mitochondrial respiratory chain (Brand and Hermfisse, 1997).

An additional advantage of the specific cell type employed is related to the expression of high affinity transporters of L-ascorbic acid (AA) in both the plasma and mitochondrial membranes (Azzolini et al., 2013; Fiorani et al., 2015a; Fiorani et al., 2015b), a condition favoring the selective accumulation of AA in mitochondria after exposure to low extracellular concentrations of the vitamin (Azzolini et al., 2013; Fiorani et al., 2015a). Under these conditions, intramitochondrial AA very rapidly and effectively scavenged $mitoO_2^{-\cdot}$ (Guidarelli et al., 2016a). Using these treatments, we were able to demonstrate a selective suppression of ROS formation mediated by arsenite, and hence establish the pivotal role of $mitoO_2^{-\cdot}$ and its down-stream products in the induction of the deleterious effects elicited by the metalloid (Guidarelli et al., 2017).

The specific characteristics of the U937 cell clone used in our laboratory can also be used to address additional questions, for example related to the role of $mitoO_2^{-\cdot}$ in the activation of specific cytoprotective signaling pathways, as the one connected to the nuclear factor (erythroid-2 related) factor 2 (Nrf2) (Abdul-Aziz et al., 2015; Tebay et al., 2015; Hourihan et al., 2016).

Oxidative and electrophilic stress are associated with an initial dissociation of Nrf2 from Kelch-like ECH-associated protein 1 (Keap1) and its subsequent translocation and accumulation in the nucleus (Tebay et al., 2015; Hourihan et al., 2016). The transcription factor promotes enhanced expression of an array of antioxidant enzymes (Abdul-Aziz et al., 2015; Tebay et al., 2015; Hourihan et al., 2016), which include γ-glutamylcysteine synthase (γ-GCS, (Abdul-Aziz et al., 2015; Tebay et al., 2015)), the rate-limiting enzyme of GSH biosynthesis (Griffith and Mulcahy, 1999; Franklin et al., 2009), comprising a catalytic heavy subunit, (GCLC) and a regulatory light subunit (GCLM). Activation of these pathways leading to enhanced GSH biosynthesis is of extreme importance for providing cytoprotection in a variety of toxicity paradigms (Flora, 2011; Hou et al., 2014; Forman, 2016).

It has been reported that arsenite induces the dissociation of Nrf2 from Keap 1 (Pi et al., 2003; Lau et al., 2013). Although some studies claimed a role for a direct binding of arsenite to either Keap 1 or Nrf2 (He and Ma, 2009; He and Ma, 2010), Nrf2 activation is often reported to depend also on ROS formation. This notion is normally established by studies showing that Nrf2 activation is sensitive to very high concentrations of *N*-acetylcysteine (Ray et al., 2015; Rossler and Thiel, 2017), or other antioxidants (Choudhury et al., 2016; Gong et al., 2016), with a need of a more selective approach linking the cytoprotective signaling to the specific sources from which ROS are released by the metalloid.

The study of the effect of $mitoO_2^{-\cdot}$ in the Nrf2 signaling, using selective strategies to prevent the formation of these species, would therefore allow a more clear definition of their role in Nrf2 activation. The approach of selectively targeting $mitoO_2^{-\cdot}$ to understand its role in a cytoprotective signaling appears even more important under conditions in which the same species is also involved in the triggering of events leading to apoptosis.

In this perspective, the respective timing of these responses appears of particular importance to determine the dynamics regulating critical steps of the final decision of the cells to survive or die. This information is necessary to further our knowledge, thus far limited to the demonstration that cells overexpressing Nrf2 (or downstream effectors) are particularly resistant to arsenite, and that down-regulation of these systems remarkably enhances the sensitivity of the cells to the metalloid (Wang et al., 2007; Jiang et al., 2009; Yang et al., 2012; Son et al., 2015; Chen et al., 2017).

The present study was designed to determine the role of $mitoO_2^{-\cdot}$ released in response to a low concentration (*i.e.*, promoting delayed apoptosis in a limited portion of cells) of arsenite in the induction/activation of Nrf2 (Pi et al., 2003; Li et al., 2013). Our results indicate that $mitoO_2^{-\cdot}$ is responsible for the induction of two opposite pathways associated with the early activation of the Nrf2 signaling and the delayed induction of mitochondrial permeability-transition (MPT)-dependent apoptosis. The first pathway significantly blunted and delayed the MPT-dependent apoptosis induced by the metalloid through a mechanism associated with enhanced GSH biosynthesis and inhibition of excessive $mitoO_2^{-\cdot}$ formation.

2. Materials and methods

2.1. Chemicals

Sodium arsenite, AA, rotenone, diphenyleneiodonium (DPI), apocynin (Apo), phorbol-12-myristate-13-acetate (PMA), DL-buthionine-[S,R]-sulfoximine (BSO), Hoechst 33342, GSH, dithiobis-(2-nitrobenzoic acid), (DTNB) as well most of the reagent-grade chemicals were purchased from Sigma-Aldrich (Milan, Italy). Cyclosporin A (CsA) was from Novartis (Bern, Switzerland). Dihydrorhodamine 123 (DHR)