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Arsenic increases lipopolysaccharide-dependent expression of interleukin-8 gene by stimulating a redox-sensitive pathway that strengthens p38-kinase activation☆

Emilie Bourdonnay[a], Claudie Morzadec[a], Olivier Fardel[a,b], Laurent Vernhet[a,*]

[a] Institut de Recherche sur la Santé, l'Environnement et le Travail (IRSET), EA-4427 Signalisation et Réponses aux Agents Infectieux et Chimiques, Université de Rennes 1, 2 avenue du Professeur Léon Bernard, 35043 Rennes, France
[b] Département HITC, Centre Hospitalier Universitaire (CHU) Rennes, 2 rue Henri Le Guilloux, 35033 Rennes, France

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ABSTRACT

Inorganic arsenic is an immunotoxic metalloid that causes or exacerbates deleterious inflammatory states. Notably, arsenic can increase inflammation-related gene expression induced by lipopolysaccharide (LPS) in monocytes/macrophages. Molecular mechanisms mediating such effects remain however poorly understood. In the present study, we determined molecular basis of arsenic trioxide (ATO) effects on LPS-dependent expression of interleukin-8 (IL-8) gene in human monocytic cells. Pre-treatment with non cytotoxic concentrations of ATO for 48 h increase IL-8 gene expression induced by LPS in monocytic U937 cells and in some, but not all, primary cultures of blood monocytes. Actinomycin D blocks induction of IL-8 mRNA levels in LPS-stimulated U937 cells pre-treated or not with ATO, which suggests that the metalloid strengthens LPS-dependent transcriptional regulation of IL-8 expression. ATO increases LPS-dependent expression of IL-8 by enhancing p38-kinase activity induced by LPS in U937 cells. This increment of LPS-dependent p38-kinase activity is caused by the ATO-related production of reactive oxygen species (ROS) and the subsequent activation of the tyrosine kinase Src. The antioxidant N-acetylcysteine almost totally inhibits ROS production and Src kinase activation in ATO-pre-treated cells. In addition, it markedly prevents the increase of both p38-kinase phosphorylation and IL-8 gene expression in LPS-stimulated cells pre-treated with ATO. Finally, as observed with the metalloid, pre-treatment of U937 cells with hydrogen peroxide markedly increases LPS-dependent expression of IL-8 gene. In conclusion, our study demonstrates that ATO increases LPS-dependent expression of the inflammatory IL-8 gene by strengthening the p38 kinase activity induced by LPS through stimulation of a ROS/Src kinase signalling pathway.

1. Introduction

Inorganic arsenic (iAs) is a major environmental toxicant to which millions of humans are exposed over the world, mainly through contaminated drinking water. Chronic exposure to this metalloid favours development of keratosis, atherosclerosis, hepatic fibrosis, chronic bronchitis and neuronal diseases (Das and Sengupta, 2008). In addition, it increases incidence of non-melanoma skin, lung and bladder cancers (Rahman et al., 2009). Mechanistic studies suggest that iAs can induce or exacerbate several of these diseases by promoting inflammatory states (States et al., 2009). Indeed, chronic exposure to low concentrations of iAs stimulate expression of inflammatory molecules such as interleukin-6 (IL-6) and tumor necrosis factor α (TNFα) in liver and/or atherosclerotic lesions in mice (Das et al., 2005; Srivastava et al., 2009). iAs also increases expression of IL-8, TNFα and granulocyte-macrophage stimulating growth factor (GM-CSF) in human primary cultures of keratinocytes and endothelial cells, or in human immortalized non-tumorigenic urothelial cells (Germolec et al., 1996; Yen et al., 1996; Klei and Barchowsky, 2008; Escudero-Lourdes et al., 2010). These cytokines and growth factors can contribute to hepatic fibrosis, atherosclerosis or tumor growth and progression (Tilg et al., 2006; Schuett et al., 2009; Gambichler et al., 2006; Black and Dinney, 2007).

Abbreviations: iAs, inorganic arsenic; ATO, arsenic trioxide; ROS, reactive oxygen species; IL, interleukin; TNF, tumor necrosis factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; LPS, lipopolysaccharide; PBMC, peripheral blood mononuclear cells; MAP-kinases, mitogen-activated protein kinases; RT-qPCR, reverse transcription-real-time quantitative PCR.

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* Corresponding author. Tel.: +33 2 3 23 48 07; fax: +33 2 23 23 47 94.
E-mail address: laurent.vernhet@univ-rennes1.fr (L. Vernhet).

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Besides keratinocytes or endothelial cells, development of inflammation is above all controlled by monocytes and macrophages. These cells secrete several inflammatory molecules in response to various stimuli, notably once activated by lipopolysaccharide (LPS). Different reports have shown that iAs can also alter *in vivo* and *in vitro* pro-inflammatory response of LPS in human monocyte/macrophages. First, chronic exposure of individuals to iAs through drinking water is significantly associated with an increased response of peripheral blood mononuclear cells (PBMC) to LPS (Soto-Pena et al., 2006). In this study, *ex vivo* secretion of GM-CSF by LPS-stimulated PBMC was positively correlated with increment of total iAs in urine. Arteel et al. (2008) have also demonstrated that chronic consumption of iAs-contaminated drinking water significantly increases the number and size of necroinflammatory liver foci caused by high doses of LPS in mice. In addition, ATO increases *in vitro* secretion of inflammatory cytokines induced by LPS in human macrophages. It notably increases expression of the pro-angiogenic chemokine IL-8 (Waught and Wilson, 2008). Although iAs can promote LPS-dependent expression of pro-inflammatory cytokines, molecular basis of its effects remains however to be established.

iAs is a major pro-oxidant metalloid which produces reactive oxygen species (ROS) in many cell types, including monocytes/macrophages (Kumagai and Sumi, 2007; Lemarie et al., 2008). It increases ROS levels by inhibiting antioxidant enzymes or directly by activating enzymatic complex such as the reduced nicotinamide dinucleotide phosphate oxidase (Lemarie et al., 2008). Through ROS production, iAs can modulate key signalling pathways and cell functions. According to cell types, iAs can notably activate or inhibit the redox-sensitive mitogen-activated protein kinases (MAP-kinases) ERK and p38-kinase (Kumagai and Sumi, 2007). These kinases are well-known to mediate LPS-dependent expression of inflammatory molecules in monocytes/macrophages (Guha and Mackman, 2001). The present study was thus designed to determine whether iAs can increase LPS-dependent expression of IL-8 through activation of a redox-sensitive pathway involving MAP-kinases in human monocytic cells.

Our results mainly demonstrate that pre-treatment with ATO for 48 h potently increases LPS-dependent expression of IL-8 gene in the monocytic U937 cell line and in some, but not all, primary cultures of human peripheral blood monocytes. ATO effects in U937 cells likely result from an increase of LPS-dependent p38-kinase activity, which is controlled by ROS production and the subsequent activation of Src kinase.

2. Materials and methods

2.1. Chemical reagents

ATO, LPS from *Escherichia coli* (055:B5) and actinomycin D were purchased from Sigma–Aldrich. U0126 was from promega. Antibodies against phosphorylated forms of p38-kinase, ERK, JNK and Src family kinase (Y416) were purchased from Cell Signalling Technology.

2.2. Cell viability

Cytotoxic effects of ATO were analyzed by measuring the respective parts of apoptosis and necrosis using Annexin-5 conjugated to the fluorescent label Alexa^{TM} 568 and the green fluorescent DNA dye Sytox-Green, respectively, as previously described (Lemarie et al., 2006). Viable cells were defined as cells negative for both annexin-5 and Sytox-Green.

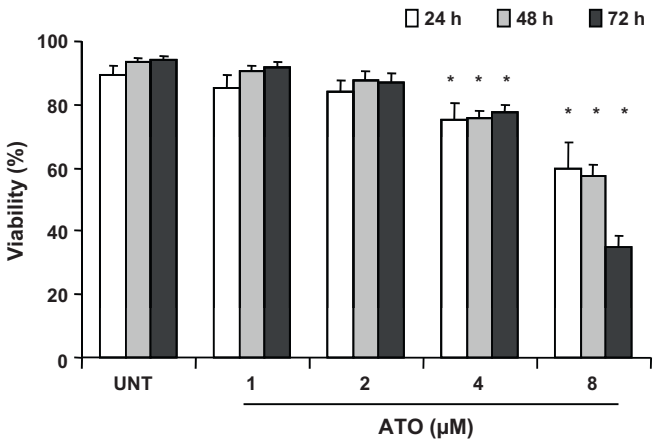

Fig. 1. Effect of ATO on U937 cell viability. U937 cells were either untreated (UNT) or treated with increasing concentrations of ATO for indicated times. Then, viability was quantified by measuring the respective parts of apoptosis and necrosis using Annexin-5 and Sytox Green, respectively. Viable cells were determined as cells negative for both dyes. Results are expressed as mean ± S.D. of four independent experiments. $^{*}p < 0.05$ versus UNT.

2.3. Cell culture

Human monocytic U937 cells were cultured in RPMI medium (Gibco) supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U/mL penicillin, and 100 mg/mL streptomycin. Peripheral blood mononuclear cells were first isolated from bloody buffy coats of healthy donors through Ficoll gradient centrifugation. Monocytes were then selected by a 1-h adhesion step, which routinely obtained > 90% of adherent CD14-positive cells as assessed by immunostaining. Monocytes were cultured in RPMI medium as described for U937 cells.

2.4. RNA isolation and reverse transcription-real-time quantitative PCR (RT-qPCR) assay

Total RNAs were extracted using the TRIzol method (Invitrogen) and then subjected to RT-qPCR analyses as previously described (Lemarie et al., 2008). RT-qPCR assays were performed using the SYBR Green methodology and an ABI Prism 7000 detector (Applied Biosystem). Specific gene primer sets were from Qiagen. Specificity of gene amplification was checked up at the end of qPCR through analysis of dissociation curves of the PCR products. Amplification curves were read with ABI Prism 7000 SDS software using the comparative cycle threshold method. Relative quantification of the steady-state target mRNA levels was calculated after normalization of the total amount of cDNA to a 18S RNA endogenous reference.

2.5. Determination of cytokine levels

Levels of IL-8 secreted in culture medium were quantified using IL-8 Duoset ELISA development system kit (R&D Systems). 96-Well plate, initially coated with 1 μg/mL mouse anti-human IL-8 antibody overnight at room temperature, was incubated for 2 h with U937 cell culture medium or recombinant human IL-8 standards. After washing, plates were processed according to the manufacturer's instructions. Sensitivity of the IL-8 Duoset ELISA is 20 pg/ml.

2.6. Western blot immunoassays

After treatment, U937 cells were harvested and lysed on ice with lysis buffer (50 mM Tris–HCl, 150 mM NaCl, 0.5% deoxycholate, 5 mM EDTA, 1% Triton X-100, 1 mM sodium orthovanadate, 1 mM