ARTICLE
Journal of Cellular Biochemistry 107:537–547 (2009)

Journal of Cellular Biochemistry

Redox-Sensitive Regulation of Gene Expression in Human Primary Macrophages Exposed to Inorganic Arsenic

Emilie Bourdonnay,[1,2] Claudie Morzadec,[1,2] Olivier Fardel,[1,2,3] and Laurent Vernhet[1,2*]

[1]*Inserm U620, Group ''Toxicité des contaminants de l'environnement'', 2 avenue du Pr. Léon Bernard, 35043 Rennes, France*

[2]*UPRES-EA SeRAIC ''Signalisations et Réponses aux Agents Infectieux et chimiques'', IFR140, Université Rennes 1, 2 av Pr. Léon Bernard, 35043 Rennes, France*

[3]*Département HITC, Centre Hospitalier Universitaire (CHU) Rennes, 2 rue Henri Le Guilloux, 35033 Rennes, France*

ABSTRACT

Inorganic arsenic is an environmental contaminant toxic for key immune cells. We recently reported that low micromolar concentrations of arsenic trioxide (As_2O_3) alter functions and differentiation gene program of human macrophages. Particularly, prolonged treatment with As_2O_3 concomitantly reverses expression of a macrophage-specific gene subset and triggers reactive oxygen species (ROS) production, suggesting a possible role of cell stress in As_2O_3 gene effects. This study was thus designed to determine whether redox-sensitive signaling pathways could mediate gene expression in metalloid-exposed macrophages. Our results show that As_2O_3-dependent alterations of stress (HMOX1 and GCLM) and macrophage-specific (MMP9, CCL22, and CXCL2) gene expression are not mediated by ROS or related signaling pathways. Notably, As_2O_3 alters neither activity of the redox-sensitive transcription factor Sp1 nor that of AP-1 or NF-κB. In contrast, *N*-acetylcysteine, a potent cysteine reductive compound, significantly prevents up-regulation of HMOX1, GCLM, and CXCL2 genes, and repression of MMP9 and CCL22 genes induced by As_2O_3. In addition, we demonstrate that As_2O_3 markedly alters nuclear levels of Nrf2 and Bach1, two redox-sensitive regulators of stress genes, and represses expression of the transcription factor EGR2 which is involved in mouse macrophage differentiation; such effects are reduced by *N*-acetylcysteine. Finally, we report that genetic invalidation of EGR2 gene partially mimics metalloid effects; it significantly represses CCL22 gene expression and weakly induces that of CXCL2. In conclusion, our results demonstrate that As_2O_3 alters macrophage gene expression through redox-sensitive signaling pathways unrelated to ROS production and reveal the transcription factor EGR2 as a new molecular target of arsenic. J. Cell. Biochem. 107: 537–547, 2009.

KEY WORDS: INORGANIC ARSENIC; MACROPHAGE; DIFFERENTIATION; GENE; STRESS

Inorganic arsenic (iAs) is an environmental contaminant to which millions of humans are exposed worldwide [Yoshida et al., 2004]. Epidemiologic studies have shown that chronic exposure to iAs is associated with increased incidence of atherosclerosis, diabetes and cancer [Shi et al., 2004; Simeonova and Luster, 2004; Navas-Acien et al., 2008]. Molecular and cellular mechanisms mediating these chronic diseases are complex and likely multifactorial. Some recent works suggest that such toxic effects of iAs can, in part, be related to immunotoxic properties.

It is now well demonstrated that iAs can alter physiology of various key immune cells. iAs decreases in vivo and in vitro proliferation and activation of T-lymphocytes in response to mitogenic agents [Galicia et al., 2003; Soto-Pena et al., 2006]. It alters migration, adhesion and degranulation of human neutrophils [Binet and Girard, 2008]. This metalloid also impairs differentiation status and activity of macrophages which play a major role in host defense and immune response [Bishayi and Sengupta, 2003; Lemarie et al., 2006]. We have notably demonstrated that low micromolar concentrations of As_2O_3, in the range of iAs blood levels detected in plasma of environmentally exposed subjects, reduces endocytosis and phagocytosis, some key functions of human mature macrophages [Lemarie et al., 2006]. In addition, by establishing transcriptome of As_2O_3-treated macrophages, we recently demonstrated that metalloid reverses expression of a large subset of genes specifically regulated during differentiation process [Bourdonnay et al., 2009]. Indeed, differentiation of monocytes into functional

Additional Supporting Information may be found in the online version of this article.

Grant sponsor: Ligue Contre le Contre Cancer (comité d'Ille et Vilaine).

*Correspondence to: Prof. Laurent Vernhet, UPRES EA SeRAIC, Université Rennes 1, 2 av Pr. Léon Bernard, 35043 Rennes, France. E-mail: laurent.vernhet@rennes.inserm.fr

Received 21 November 2008; Accepted 2 March 2009 • DOI 10.1002/jcb.22155 • 2009 Wiley-Liss, Inc.
Published online 6 April 2009 in Wiley InterScience (www.interscience.wiley.com).

macrophages is controlled by a complex program of gene induction and repression [Hashimoto et al., 1999]. Particularly, we found that As_2O_3 rapidly represses expressions of genes coding for the metalloproteinase MMP9 and for the cytokine CCL22, which constitute hallmarks of mature macrophages, whereas it induces that of CXCL2 gene, normally decreased during differentiation [Hashimoto et al., 1999; Ishii et al., 2000]. In order to specify molecular mechanisms mediating alterations of macrophage-specific gene expression, we analyzed levels of PU.1, a master transcription factor involved in macrophage differentiation. As_2O_3 significantly decreases PU.1 gene expression at both mRNA and protein levels, but only in macrophages exposed for 72 h; consequently, this effect is too late to initiate alteration of MMP9, CCL22 or CXCL2 gene expression. Molecular mechanisms mediating As_2O_3-dependent reversion of macrophage differentiation gene program remain thus unclear.

iAs is a major pro-oxidant metalloid that stimulates production of reactive oxygen species (ROS) in several cell types [Shi et al., 2004; Kumagai and Sumi, 2007]. ROS modulate redox-sensitive signaling pathways by oxidizing key signaling proteins and control some effects of iAs, especially those towards gene expression; several reports have indeed demonstrated that iAs induces expression of stress genes, such as HMOX1 and GADD45, through an increase of intracellular ROS levels [Bower et al., 2006; Cooper et al., 2007]. Interestingly, we previously showed that prolonged treatment of human macrophages with As_2O_3 concomitantly activates the ROS generator NADPH oxidase, up-regulates classical stress genes and alters macrophage-specific gene expressions [Lemarie et al., 2008; Bourdonnay et al., 2009]. Altogether, these results suggest that macrophage stress may control, at least in part, metalloid effects on differentiation gene program. The present study was thus designed to assess this hypothesis, notably by specifying the roles of ROS and redox-sensitive signaling pathways.

Our results demonstrate that As_2O_3 effects on expressions of stress- (HMOX1 and GCLM) and macrophage-specific (MMP9, CCL22, and CXCL2) genes are not mediated by ROS-dependent signaling pathway but inhibited by the thiol-reductive agent *N*-acetylcysteine (NAC). This compound was moreover found to prevent As_2O_3-induced alteration of nuclear levels of nuclear factor (erythroid-derived 2)-like 2 (also name Nrf2) and Bach1, two redox-sensitive regulator of stress gene; it also prevents repression of EGR2, a transcription factor likely involved in regulation of macrophage CCL22 gene expression.

MATERIALS AND METHODS

CELL CULTURE

Cells were first isolated from bloody buffy coats of healthy donors through Ficoll gradient centrifugation. Monocytes were then prepared by a 1 h-adhesion step, which routinely obtained >90% of adherent CD14-positive cells as assessed by immunostaining. Monocytic cells were next cultured for 6 days in RPMI medium supplemented with 10% fetal calf serum, antibiotics and 400 UI/ml GM-CSF, as previously reported [Lemarie et al., 2006]. Once differentiated, macrophages were cultured in GM-CSF-free medium and then treated with 1 μM As_2O_3 for indicated time intervals. In some experiments, macrophages were first pre-treated with superoxide dismutase (SOD, 1,000 UI/ml), catalase (2,000 UI/ml, SIGMA), cycloheximide (CXH), actinomycin D or NAC (10 mM). All these chemicals were from Sigma–Aldrich.

TOTAL RNA ISOLATION ASSAY AND REVERSE TRANSCRIPTION-QUANTITATIVE POLYMERASE CHAIN REACTION (RT-qPCR) ANALYSIS

Following cell treatments, macrophages were scrapped in RPMI medium and centrifugated. Pellets were washed once with sterile PBS and total mRNAs were extracted from macrophages using the TRIzol method (Invitrogen Life Technologies); ARN were then subjected to RT-qPCR analysis using the fluorescent SYBR Green methodology, specific gene primers (QuantiTect Primer Assay, Qiagen) and an ABI Prism 7000 detector (Applied Biosystems), as previously described [Lemarie et al., 2006]. Specificity of each gene amplification was checked up at the end of qPCR through analysis of dissociation curves of the PCR products. The 18S ribosomal RNA subunit was used as the internal reference for all analyses. Differences in transcript levels were determined using the cycle threshold method as described by the manufacturer.

DETERMINATION OF INTRACELLULAR ROS LEVELS

For measurement of ROS production, cells were first treated with 1 μM As_2O_3 for indicated intervals. Then, macrophages were incubated for 30 min at 37°C with 1 μM 2′,7′-dichloro-dihydro-fluorescein diacetate (DCHF-DA), a redox-sensitive fluorescent dye detecting hydrogen peroxide (H_2O_2) [Lemarie et al., 2004]. After treatment, cells were collected, centrifuged, washed with PBS and analyzed using a FC500 flow cytometer (Beckman). Fluorescence emission from oxidized dye was detected at 525 nm.

WESTERN BLOT IMMUNOASSAYS

For whole cell lysates, macrophages were harvested and lysed for 20 min on ice in RIPA buffer supplemented with 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, 0.5 μg/ml aprotinin, 0.5 mM dithiothreitol and 1 mM orthovanadate. Cells were then centrifugated at 13,000 rpm for 15 min at 4°C. The resulting supernatants were collected and frozen at −80°C or used immediately. For nuclear cell extracts, macrophages were lysed and nuclear proteins were obtained using the Nuclear Extract Kit (Active Motif) according to manufacturer's instructions. 50 μg of each whole cell lysate or 10 μg of nuclear cell extract were heated for 5 min at 100°C, analyzed by 10% SDS–PAGE, and then electro-blotted overnight onto nitrocellulose membranes (Bio-Rad). After blocking, membranes were hybridized with primary antibodies overnight at 4°C and incubated with appropriate horseradish peroxidase-conjugated secondary antidodies. Immunolabeled proteins were visualized by chemiluminescence. Primary antibodies directed against Bach1, EGR2, Nrf2 (H-300), Hsc70 and p38-kinase were from Santa Cruz whereas HO-1 antibody was from Stressgene Biotechnologies.

MEASUREMENT OF TRANSCRIPTION FACTOR DNA BINDING ACTIVITIES

p65 NF-κB, c-jun (AP-1) and Sp1 DNA binding activities were analyzed using specific ELISA-based TransAM™ kits from Active

Motif, as previously described [Lemarie et al., 2004]. In brief, nuclear cell extracts (10 μg) were incubated for 1 h in a 96-well plate to which oligonucleotides, containing a p65, Sp1 or AP-1 (c-jun) consensus binding site, have been immobilized. After washing, plate was incubated for 1 h with appropriate primary antibodies (1:1,000) which specifically detected an epitope accessible only when p65, Sp1 or AP-1 is activated and bound to its cognate oligonucleotide. The plate was then washed and incubated with horseradish peroxidase-conjugated secondary antibody (1:1,000) for 1 h at room temperature. After washing, colorimetric readout was quantified by spectrophotometry at 450 nm. In order to monitor specificity of this assay, wild-type and mutated consensus oligonucleotides were used as competitors for p65, Sp1 and c-jun binding in different experiments.

TRANSFECTION OF siRNA

Two sets of siRNA from Dharmacon (Perbio Sciences) were used: control siRNA, 5′-UAGCGACUAAACACAUCAAtt-3′, corresponding to non-targeting siRNA and the siGENOME SMARTpools EGR2. Transfection of siRNAs into macrophages was performed using the Amaxa nucleofection technology, as previously described [Monteiro et al., 2007].

STATISTICAL ANALYSIS

All independent experiments were repeated at least three times with similar results. Data are expressed as means ± SD. Significant differences were evaluated using the Student's *t*-test or the multirange Dunett's *t*-test when multiple comparisons were studied.

RESULTS

We recently reported global gene expression in human macrophages exposed to As_2O_3 [Bourdonnay et al., 2009]. In this previous study, we found that As_2O_3-altered expression of macrophage-specific genes is associated with induction of several genes classically regulated by cell stress. Particularly, our microarray data show that mRNA levels of HMOX1 and GCLM, two major anti-oxidant genes coding for heme oxygenase-1 and glutamate cysteine ligase modifier respectively, were increased by at least sixfold in macrophages treated for 72 h with 1 μM As_2O_3 [Bourdonnay et al., 2009]. In the present study, using RT-qPCR assays, we confirm potent induction of HMOX1 and GCLM gene expression by the metalloid (Fig. 1A). Figure 1B demonstrates that 1 μM As_2O_3 increases HMOX1 and GCLM mRNA levels as soon as 4 h, although significant differences were only observed at 8 h. This result indicates that alteration of stress gene expression precedes that of macrophage-specific genes. Indeed, we previously reported that repression of MMP9 and CCL22 genes, and up-regulation of CXCL2 gene, could only be detected in macrophages exposed for at least 8 h (see Fig. 2A in Bourdonnay et al., 2009). In vitro, iAs is known to induce stress gene expression mainly through ROS production [Pi et al., 2003; Cooper et al., 2007]. To gain insights onto stress response of metalloid-exposed macrophages, we thus determined intracellular ROS levels using the redox-sensitive fluorescent probe DCHF-DA. Figure 1C indicates that ROS production is not modified in macrophages treated with As_2O_3 for 4 or 8 h; in contrast, H_2O_2, used as a positive control, markedly increases oxidation of DCHF and the resulting fluorescence intensity. This result is concordant with our recent study in which we demonstrated that ROS levels, detected by two other redox-sensitive fluorescent probes (i.e., hydroethidium and dihydrorhodamine), were only increased in macrophages treated for at least 16 h with 1 μM As_2O_3 (see Fig. 1A in Lemarie et al., 2008). In these experiments, pre-treatment with the antioxidants SOD + catalase was found to totally inhibit ROS production. In the present study, Figure 1D indicates that SOD + catalase pre-treatment does not block up-regulation of HMOX1 and GCLM genes. In addition, as shown in Figure 1E, these compounds prevent neither down-regulation of MMP9 or CCL22 genes nor induction of CXCL2 gene. Altogether, our results suggest that ROS could not initiate alteration of stress and macrophage-specific gene expressions in response to metalloid.

Besides ROS, it may be hypothesized that metalloid could modify gene expression by directly oxidizing key cysteine residues in critical proteins [Chakraborti et al., 1992; Spuches et al., 2005]. To test this hypothesis, we used NAC, a potent thiol reductive agent demonstrated to prevent stress-related toxicity of iAs in leukemia cells [Dai et al., 1999]. As shown in Figure 2A, NAC markedly reduces HMOX1 and GCLM mRNA levels in cells treated with As_2O_3 for 16 h; it also blocks induction of HMOX1 mRNA and protein levels in macrophages treated for 48 h (Fig. 2B). Moreover, Figure 2C demonstrates that macrophage pre-treatment with NAC significantly reduces effects of As_2O_3 on MMP9, CCL22 and CXCL2 gene mRNA (Fig. 2C). Consequently, alteration of stress and macrophage-specific gene expressions likely results, at least in part, from activation of redox-sensitive signaling pathways involving oxidation of cysteine residues.

In order to specify these molecular mechanisms, we next studied different redox-sensitive transcription factors. We first analyzed effects of As_2O_3 on nuclear levels of Nrf2, a stress-inducible protein controlling expression of several anti-oxidant genes, notably in iAs-treated cells [Pi et al., 2003; He et al., 2006; Reichard et al., 2008]. Our results demonstrate that 1 μM As_2O_3 rapidly increases nuclear levels of Nrf2 in macrophages exposed for 6, 24, or 72 h (Fig. 3A). In the same time, As_2O_3 markedly decreases nuclear levels of Bach1, a stress sensor and a well-known repressor of HMOX1 gene expression [Ishikawa et al., 2005; Reichard et al., 2008]. Interestingly, Figure 3B shows that macrophage pre-treatment with NAC totally prevents increase of Nrf2 nuclear levels; it also partially reduce the decrease of Bach1nuclear levels. We also studied activities of Sp1, AP-1 (c-jun) and p65 NF-κB, which are key targets of iAs in many cell types [Kumagai and Sumi, 2007]; moreover, these three redox-sensitive transcription factors are known to be involved in regulation of MMP9, CCL22 or CXCL2 gene expression [Shattuck et al., 1994; Ghadially et al., 2005; Huang et al., 2007]. Figure 3C indicates that, whatever time exposure, As_2O_3 alters neither DNA binding activity of Sp1 nor that of AP-1; moreover, it weakly, but not significantly, increases that of p65 NF-κB. Thus, none of these transcriptions factors are likely to mediate As_2O_3 effects towards macrophage-specific gene expression.

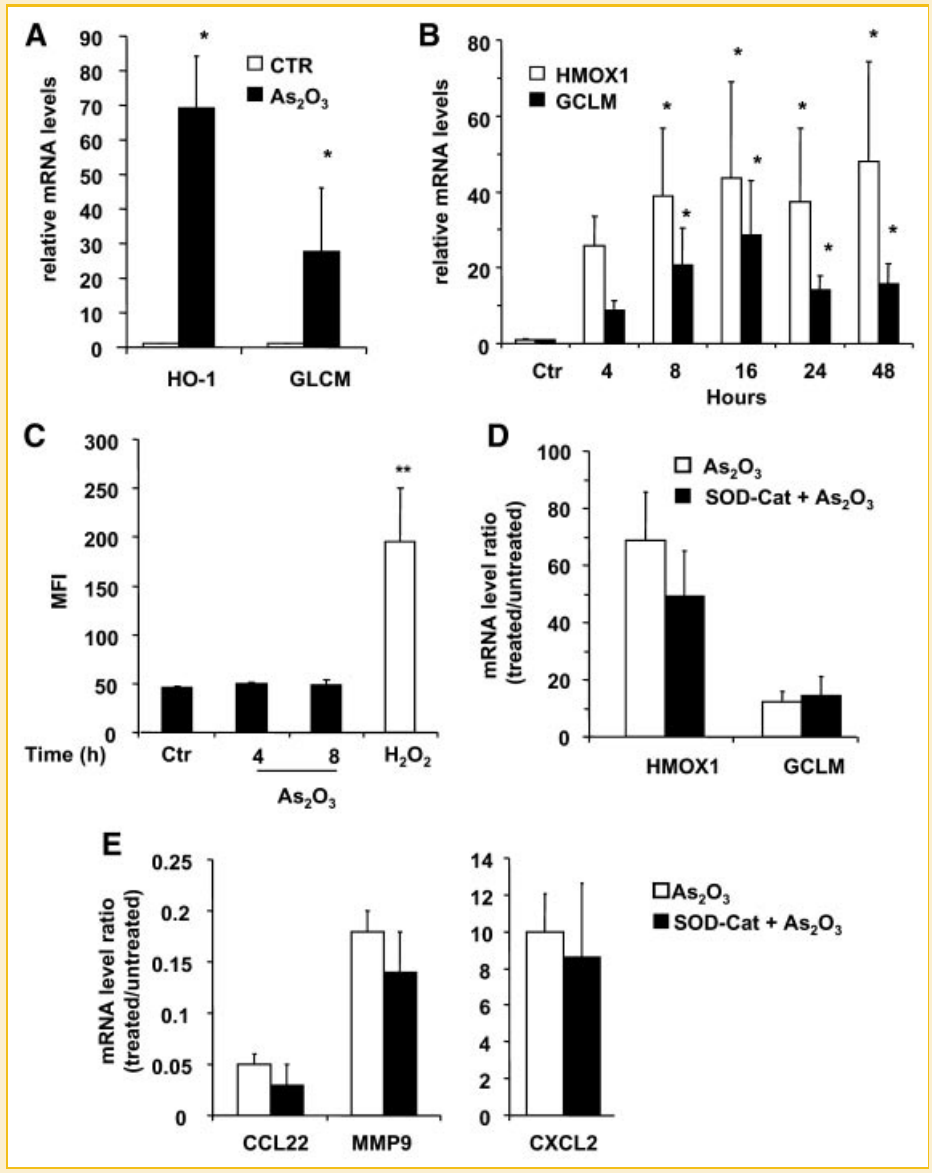

Fig. 1. ROS do not mediate As_2O_3 effects on gene expression. Macrophages were pre-treated or not with SOD + catalase and then exposed to As_2O_3 for 48 h (D,E), 72 h (A) or indicated time intervals (B,C). mRNA levels were determined by RT-qPCR assays; data are expressed relatively to mRNA levels found in untreated (Ctr) macrophages arbitrarily set at the value of 1 (A,B,D,E). In (C), at the end of macrophages treatment, cells were incubated for 30 min with the redox-sensitive fluorescent dye DCHF-DA to quantify intracellular ROS levels. Cells were then collected, centrifuged, washed with PBS and analyzed using a FC500 flow cytometer. Results are expressed as mean ± SD of at least four independent experiments. $^*P < 0.05$, versus untreated macrophages. MFI: mean fluorescence intensity.

Our microarray data previously revealed that metalloid represses macrophage expression of EGR2 gene (see Table I in Bourdonnay et al., 2009), a member of the early growth response transcriptional regulator (EGR) family. Interestingly, high EGR2 protein levels appear to be critical for macrophage differentiation program in mouse [Laslo et al., 2006]. We consequently analyzed expression of EGR2 gene in macrophages treated or not with the metalloid and determined its role in CCL22, MMP9 and CXCL2 expression. Figure 4A indicates that EGR2 mRNA levels are up-regulated in mature macrophages when compared to those detected in fresh monocytes; as expected, As_2O_3 blocks induction of EGR2 gene expression. In addition, as previously observed for macrophage-specific genes such as CLL22 [Bourdonnay et al., 2009], EGR2 down-regulation was significantly reversed, after metalloid withdrawal from culture medium, and re-addition of GM-CSF to macrophage cultures (Fig. 4B). In order to determine if down-regulation of this transcription factor could mimic As_2O_3 effects towards macrophage-specific gene expressions, we then invalided EGR2 expression by siRNA technology. Interestingly, Figure 4C indicates that partial EGR2 protein silencing is associated with a marked and significant reduction of CCL22 mRNA levels and a small increase of CXCL2 gene expression; however, in contrast to As_2O_3 treatment (Fig. 1E), EGR2 invalidation does no alter MMP9 gene expression (data not shown). If EGR2 down-regulation is involved in

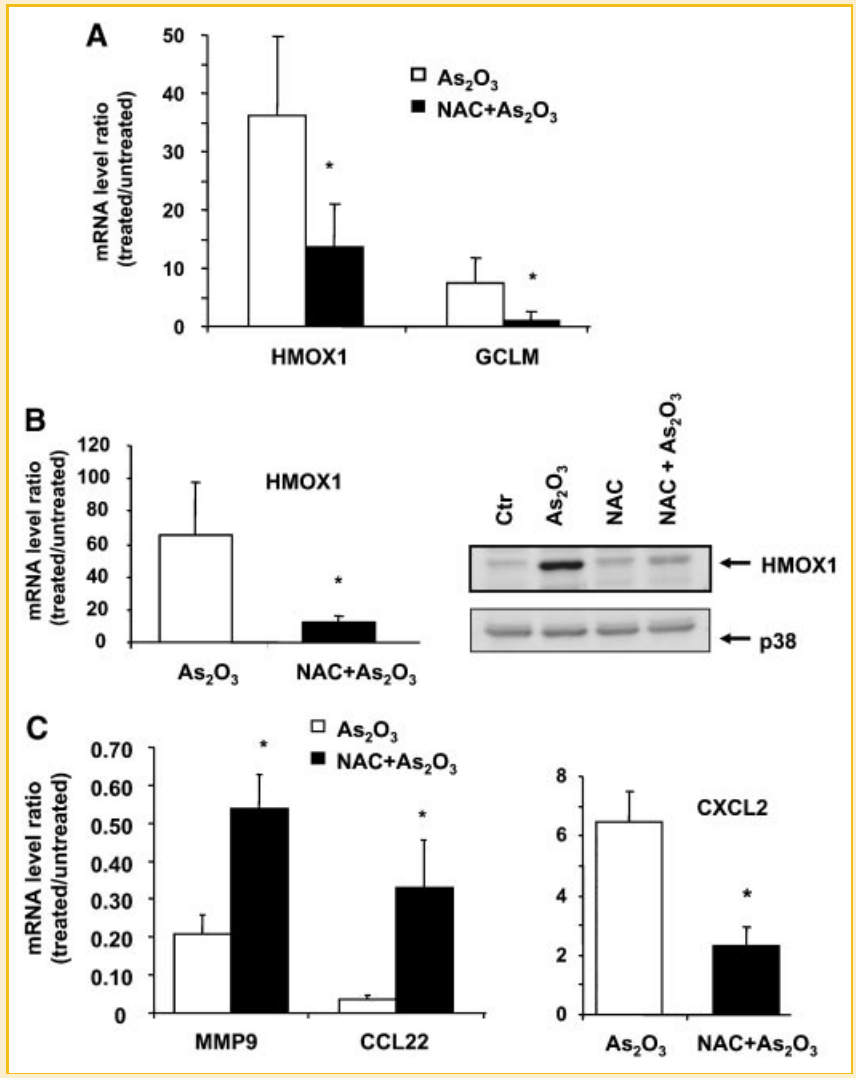

Fig. 2. NAC prevents As_2O_3 effects on macrophage gene expression. Macrophages were pre-treated or not with NAC and then exposed to As_2O_3 for 16 h (A) or 48 h (B,C). mRNA levels were determined by RT-qPCR assays; data are expressed relatively to mRNA levels found in untreated macrophages arbitrarily set at the value of 1. Results shown are the mean ± SD of at least four independent experiments. $^*P < 0.05$, versus As_2O_3-treated macrophages. In (B), levels of HMOX1 in whole cell lysates were determined by Western blot. Equal gel loading and transfer efficiency were checked by protein hybridization with an antibody directed against p38-kinase (p38). Data are representative of three independent experiments.

metalloid-induced repression of CCL22 gene, it should precede it. As shown in Figure 5A, repression of EGR2 mRNA levels is very rapid and significant as soon as 2 h after macrophage treatment with the metalloid; moreover, EGR2 protein levels are also rapidly decreased in whole cell lysates and nuclei (Fig. 4B,C). Figure 4D shows that such effects were dose-dependent and significant at 0.3 μM. Taken together these results indicate that down-regulation of EGR2 gene expression precedes that of CCL22 gene and thus support a role for this transcription factor in mediating some effects of the metalloid in human macrophages. Then, to gain insight on molecular mechanisms controlling decrease of EGR2 mRNA levels, we next assessed CHX, a potent protein synthesis inhibitor; indeed, CHX is known to rapidly increase EGR2 mRNA levels in NIH 3T3 fibroblats [Cortner and Farnham, 1990], which suggests that a repressor protein can control EGR2 expression in these cells. Figure 6A clearly demonstrates that CHX also markedly increases EGR2 mRNA levels in human macrophages; however CHX pre-treatment could not prevent EGR2 down-regulation in metalloid-exposed macrophages, indicating that As_2O_3 is unlikely to inhibit EGR2 expression through induction of a putative repressor. We also determined whether metalloid can decrease EGR2 mRNA levels by altering their stability. To this purpose, we first pre-treated macrophages with actinomycin D, a well-known transcription inhibitor, and then exposed cells to the metalloid for different time intervals. When transcription is blocked, kinetic of degradation of EGR2 mRNA is quite similar in untreated and metalloid-treated macrophages (Fig. 6B); thus, As_2O_3 does not seem to affect EGR2 mRNA stability but rather to act at the transcriptional level. We finally determined preventive effect of NAC. Interestingly, we observed that NAC pre-treatment partially reduces the decreases of EGR2 mRNA and protein levels in As_2O_3-

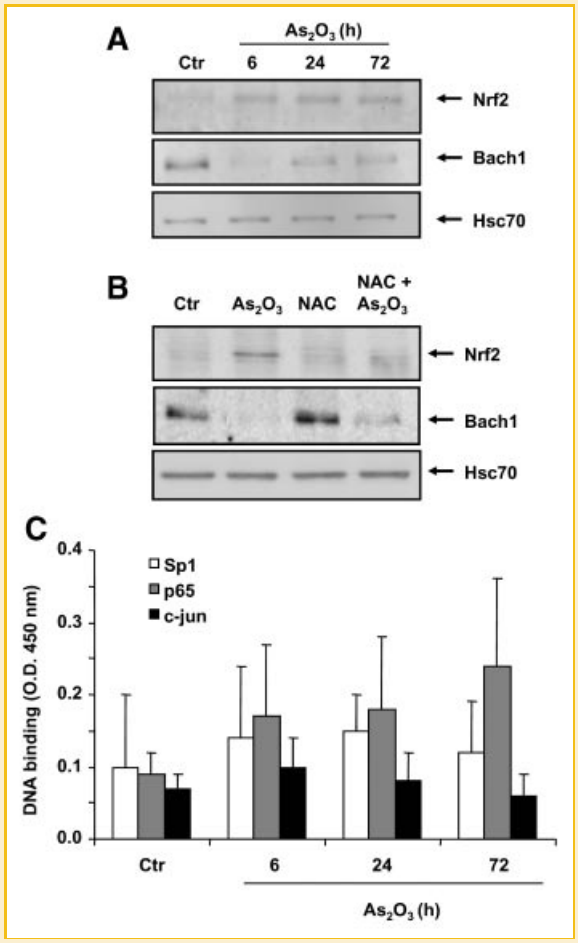

Fig. 3. As_2O_3 alters nuclear levels of Nrf2 and Bach1 in human macrophages. Cells were pre-treated or not with NAC and then exposed to As_2O_3 for 6 h (B) or indicated time intervals (A,C). Nuclear Nrf2 and Bach1 levels were determined by Western blot. Equal gel loading and transfer efficiency were checked by protein hybridization with antibodies directed against Hsc70 or p38-kinase (p38). Data are representative of four independent experiments. In (C), DNA-binding activity of indicated transcription factors was measured on nuclear extracts as described in Materials and Methods Section. Results are expressed as mean ± SD of four independent experiments.

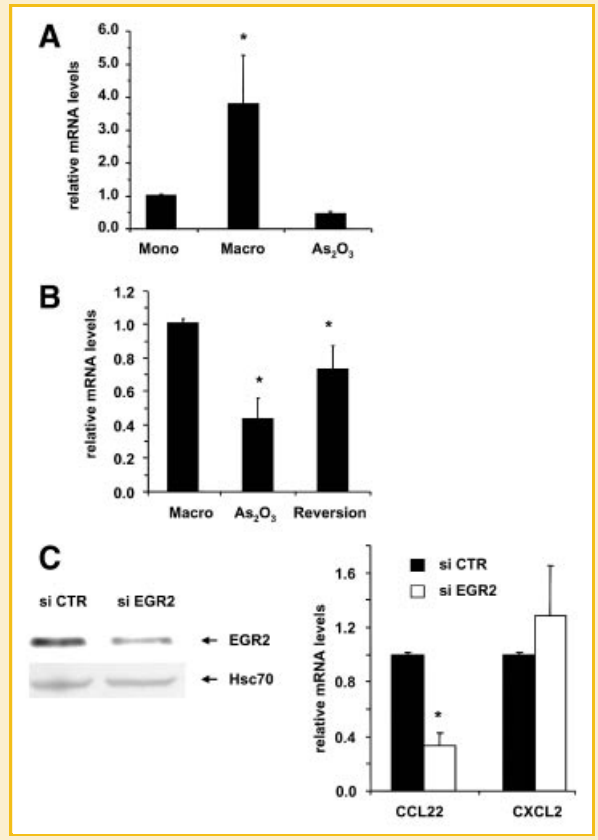

Fig. 4. Expression of EGR2 gene in macrophages. A,B: EGR2 mRNA levels were determined by RT-qPCR assays in fresh monocytes (mono), mature macrophages (macro) and macrophages treated with 1 μM As_2O_3 for 72 h (As_2O_3). In (B), after As_2O_3 treatment, metalloid was withdrawn from culture medium and macrophages were cultured for six other days in the presence of GM-CSF (reversion). Data are expressed relatively to mRNA levels found in fresh blood monocytes (A) or in untreated macrophages (macro) (B), and arbitrarily set at the value of 1. Results are expressed as mean ± SD of at least four independent experiments. $^*P < 0.05$, versus mono (A) and versus macro (B). In (C), EGR2 protein (left) and mRNA levels of indicated genes (right) were analyzed in macrophages transfected with either negative control siRNA (si CTR) or EGR2 siRNA (si EGR2). EGR2 protein levels were determined by Western blot. Equal gel loading and transfer efficiency were checked by protein hybridization with an antibody directed against Hsc70. mRNA levels were determined by RT-qPCR assays; data are expressed relatively to mRNA levels found in si CTR-transfected macrophages, arbitrarily set at the value of 1. Results are expressed as mean ± SD of five independent experiments. $^*P < 0.05$, versus Si CTR-transfected macrophages.

treated macrophages; this suggests that As_2O_3-mediated repression of EGR2 expression is in part sensitive to redox-sensitive pathways (Fig. 6C,D).

DISCUSSION

In present study, we report several arguments supporting the idea that As_2O_3 regulates expression of a macrophage gene subset through redox-sensitive signaling pathways which do not require ROS production or activation of classical ROS-sensitive transcription factors.

First, As_2O_3 markedly increases expression of HMOX1 and GCLM, two genes classically up-regulated in response to cell stress [Suh et al., 2004; Cooper et al., 2007]. HMOX1 and GCLM gene induction is rapid and precedes alteration of MMP9, CCL22 and CXCL2 gene expression in As_2O_3-treated macrophages. Several studies have demonstrated that, in iAs-treated cells, expression of stress genes, including HMOX1, is generally induced by an increased production of ROS and blocked by ROS scavengers such as SOD or catalase [Lee et al., 2005; Cooper et al., 2007]. In contrast, we show here that up-regulations of HMOX1 and GCLM genes in macrophages are not initiated by ROS formation. Indeed, our result indicate that metalloid is unable to increase ROS levels during a short 8-h treatment whereas, at this time, it significantly increases mRNA levels of both HMOX1 and GCLM genes; we previously reported that prolonged

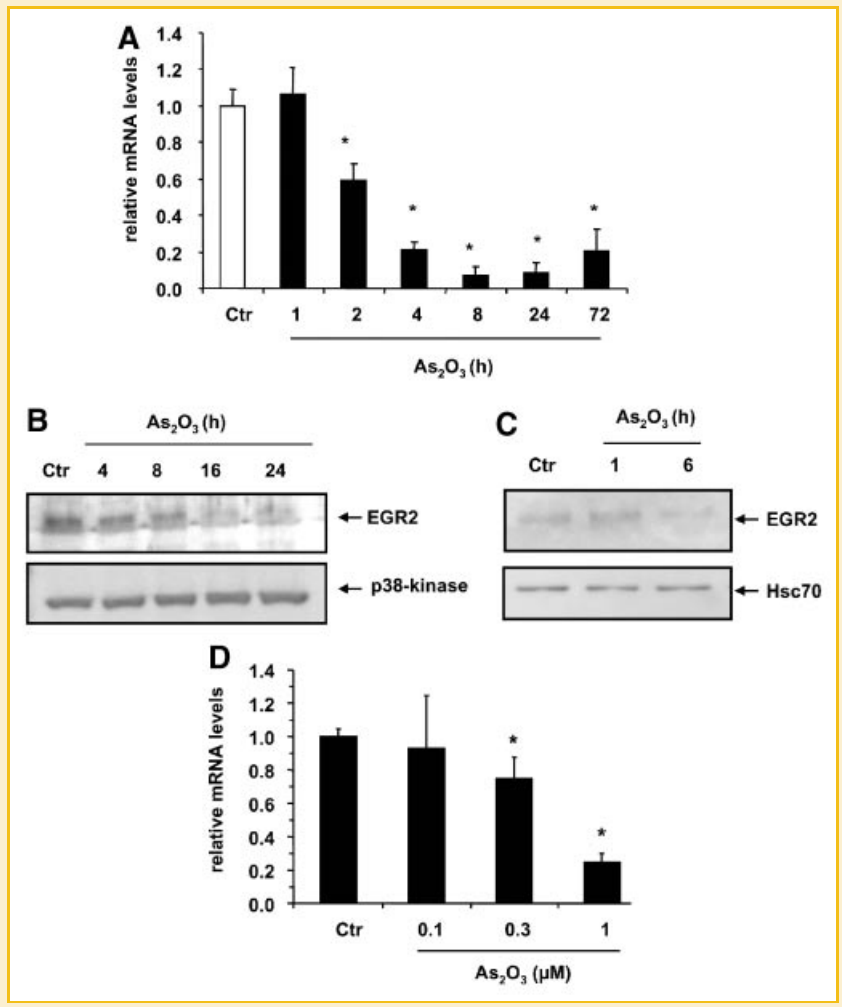

Fig. 5. Characterization of As_2O_3-induced repression of EGR2 gene expression. Macrophages were untreated (Ctr) or treated for indicated time intervals (A–C) or for 24 h (D) with 1 μM As_2O_3 (A–C) or indicated concentrations (D). EGR2 mRNA levels were determined by RT-qPCR (A,D); data are expressed relatively to mRNA levels found in untreated macrophages, and arbitrarily set at the value of 1. Results are expressed as mean ± SD of four independent experiments. $^*P < 0.05$, versus Ctr. In (B) and (C), total and nuclear EGR2 protein levels were determined by Western blot. Equal gel loading and transfer efficiency were checked by protein hybridization with an antibody directed against p38-kinase or Hcs70. Data are representative of four independent experiments.

treatment (16–72 h) of human macrophages with As_2O_3 can trigger activation of NADPH oxidase and formation of ROS (see Fig. S1 which summarizes metalloid effects in macrophages). However, macrophage pre-treatment with SOD + catalase, which prevents such a late ROS formation, inhibits neither induction of stress genes (HMOX1 and GCLM) nor alteration of macrophage-specific gene expression (CCL22, MMP9, CXCL2). In contrast, we demonstrate that NAC, a potent thiol-reductive compound known to prevent cytotoxicity of iAs on leukaemia cells [Dai et al., 1999], markedly reduces both up-regulation of HMOX1, GCLM CXCL2 genes, and repression of MMP9 and CCL22 genes. NAC is known to reduce oxidized thiol group of key cysteine residues in various proteins such as receptor or kinases [Garant et al., 1999; Laragione et al., 2003; Krasnowska et al., 2008]. Our results thus suggest that iAs could induced HMOX1 and GCLM expression by direct oxidation of cysteine residues in critical signaling proteins. Indeed, iAs is thought to bind to reduced thiol group and form stable complexes, particularly in vicinal cysteine residues of several enzymes [Kapahi et al., 2000; Spuches et al., 2005].

In order to specify redox-sensitive signaling proteins altered by iAs, we next studied nuclear levels or DNA binding activities of various transcription factors. Our results show that metalloid rapidly increases nuclear levels of Nrf2, a key stress-related transcription factor controlling inducible expression of stress genes such as GCLM and HMOX. In naïve cells, Nrf2 protein is tightly associated with Keap1 in cytoplasm and rapidly targeted for ubiquitination and proteosomal degradation [Kensler et al., 2007]. Activity of Keap1 is highly regulated by intracellular redox status of its cysteine residues. Particularly, electrophilic inducers, such as ter-butylhydroquinone, can oxidize C151 residue in Keap1 which reduces its affinity for Nrf2 and promote Nrf2 translocation in nuclei [Zhang and Hannink, 2003]. Nuclear Nrf2 heterodimerizes with Maf proteins and binds to anti-oxidant responsive elements (ARE) in GCLM or HMOX1 gene to induce their transcriptional activity

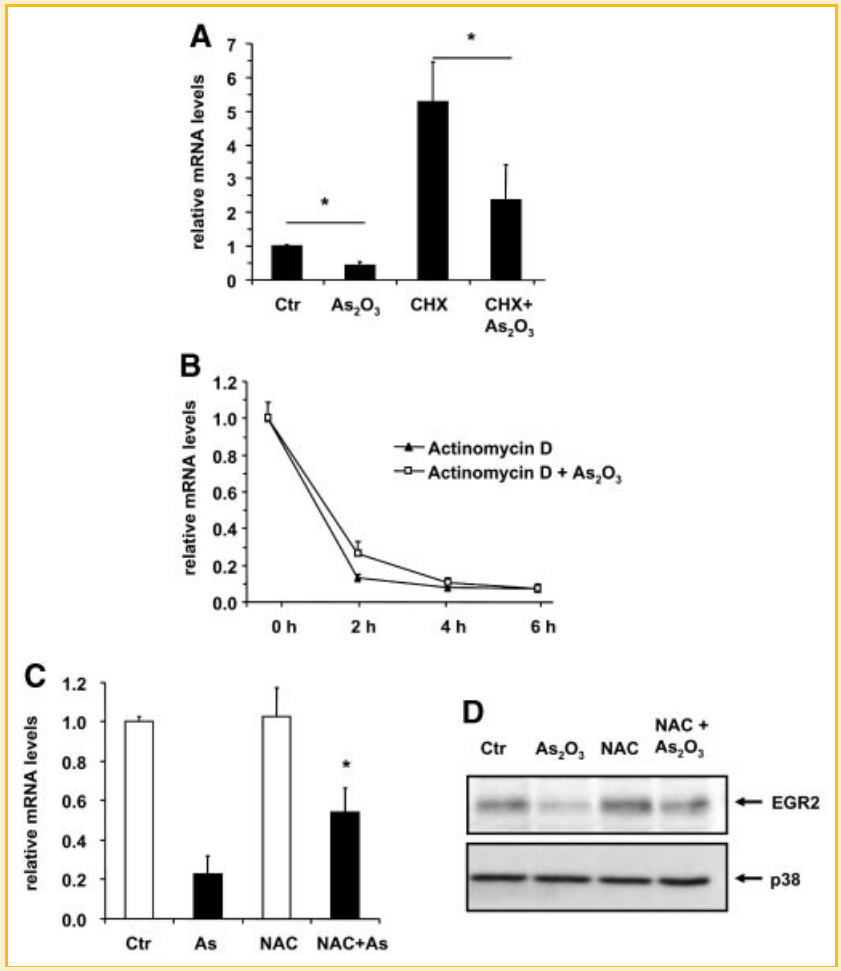

Fig. 6. Effects of CHX, actinomycin D and NAC on EGR2 gene expression. Macrophages were untreated (Ctr) or pre-treated with 5 μg/ml CHX (1 h) (A), 3 μg/ml actinomycin D (1 h) (B) or 10 mM NAC (2 h) (C,D) and then exposed or not to 1 μM As_2O_3 for 6 h (A,B) or 16 h (C,D). EGR2 mRNA levels were determined by RT-qPCR (A–C); data are expressed relatively to mRNA levels found in untreated macrophages (Ctr) (A,C) or macrophages pre-treated with actinomycin D for 1 h (0 h), and arbitrarily set at the value of 1. Results are expressed as mean ± SD of at least three independent experiments. $^*P < 0.05$, versus Ctr or CHX (A), and As_2O_3-treated cells (C). In (D), EGR2 protein levels in whole cell lysates were determined by Western blot. Equal gel loading and transfer efficiency were checked by protein hybridization with an antibody directed against p38-kinase. Data are representative of four independent experiments.

[Kensler et al., 2007]. Molecular mechanisms controlling Nrf2-dependent gene expression in response to iAs are not fully understood and, surprisingly, do not seem to involve C151 residue oxidation [Wang et al., 2008]. However, we report here that pre-treatment of macrophages with the thiol-reductive compound NAC totally prevents increase of Nrf2 nuclear levels in As_2O_3-exposed macrophages. This result thus suggests that metalloid likely activates Nrf2 pathway through direct oxidation of cysteine residues in signaling proteins. In addition, we found that As_2O_3 rapidly decreases nuclear levels of Bach1, a specific redox-sensitive repressor of HMOX1 [Reichard et al., 2008]. Disappearance of nuclear Bach1, due to cytoplasmic translocation, is known to promote HMOX1 gene expression [Reichard et al., 2008]. Indeed, nuclear Bach1 specifically represses HMOX1 promoter activity through its binding to repeated ARE sequences recognized by Nrf2 [Sun et al., 2004]. Loss of nuclear Bach1 allows interaction of Nrf2 with ARE sequences and subsequent induction of HMOX1 gene. Nuclear function of Bach1 is thought to be sensitive to oxidative stress [Ishikawa et al., 2005]. Indeed, Bach1 contains several cysteine residues, two of which control Bach1 inactivation by oxidants and its nuclear export [Ishikawa et al., 2005]. iAs induces cytoplasmic translocation of Bach1 in another cell type [Reichard et al., 2008], but its ability to directly oxidize cysteine Bach1 remains unknown. The fact that NAC increases Bach1 nuclear levels and prevents HMOX1 gene induction in As_2O_3-treated macrophages supports however this possibility.

In order to further characterize molecular mechanisms controlling macrophage-specific gene expression, we studied DNA binding activities of Sp1, AP-1, and p65 NF-κB, some redox-sensitive transcription factors and key targets of iAs [Kumagai and Sumi, 2007]. Sp1, AP-1 and p65 NF-κB control cell survival pathways, expression of several pro-inflammatory genes and regulate activity of MMP9 promoter [Chandrasekar et al., 2006; Huang et al., 2007]; p65 NF-κB is also involved in control of CCL22 and CXCL2 gene

expressions [Shattuck et al., 1994; Ghadially et al., 2005]. However, As_2O_3 modifies neither DNA binding activity of Sp1 nor that of AP-1 in macrophages; in addition, it slightly, but not significantly, increases p65 NF-κB activity. Different reports indicate that iAs mainly alters DNA binding of these three transcription factors through ROS production. Particularly, in NB4 leukaemia cells, As_2O_3-induced ROS oxidize cysteine residue in Sp1 which in turn blocks its ability to bind DNA and decreases expression of several Sp1-regulated genes [Chou et al., 2005]. At low micromolar concentrations (0.5–1 μM), arsenite can activate NF-κB activity by increasing intracellular oxidant levels in vascular endothelial and mesencephalic cells [Barchowsky et al., 1996; Felix et al., 2005]; however, it should be noted that the role of ROS is complex since they can activate or inhibit NF-κB through different post-translational modifications of upstream signaling proteins [Bubici et al., 2006]. Inability of As_2O_3 to alter DNA binding activities of Sp1, NF-κB, or AP-1 in macrophages may thus results from the absence of significant variation of intracellular ROS levels during short treatment. Nevertheless, a direct effect of metalloid on transcriptional activity of these transcription factors could not been ruled out.

Our results suggest that As_2O_3 can alter expression of CCL22 gene, and may be that of CXCL2 gene, by down-regulating intracellular levels of the transcription factor EGR2. Indeed, (i) metalloid rapidly and strongly decreases mRNA and protein EGR2 levels in macrophages, (ii) this effect precedes As_2O_3-induced repression of CCL22 gene, (iii) NAC pre-treatment significantly, although partially, reduces EGR2 gene repression, and (iv) EGR2 invalidation by siRNA technology markedly represses CCL22 expression and weakly increases that of CXCL2. To our knowledge, it is the first time that iAs effects towards EGR2 expression are reported. EGR2 regulates a specific gene subset during differentiation of mouse macrophage and represses differentiation of neutrophils [Laslo et al., 2006]; similar functions in human may be hypothesized, although they remain to be determined. It is noteworthy that EGR2 invalidation only partially mimics As_2O_3 effects towards expression of MMP9, CCL22 and CXCL2 genes, which represent hallmarks of human mature macrophages [Hashimoto et al., 1999]; particularly, it has no effect on MMP9 expression. Thus, EGR2 may not be considered as a master transcription factor for differentiation program of human macrophage. Interestingly EGR2 expression is markedly induced in mature macrophages when compared to that found in fresh monocytes. However, its full expression may be prevented by constitutive expression of a gene repressor; indeed, as described in mouse fibroblasts [Cortner and Farnham, 1990], inhibition of macrophage protein synthesis with CHX markedly and rapidly increases EGR2 mRNA levels. As_2O_3 is unlikely to repress EGR2 gene by increasing expression of this putative repressor since CXH pre-treatment does not prevent metalloid effects. In addition, the fact that, in actinomycin D-treated macrophages, As_2O_3 could not accelerate degradation of EGR2 mRNA demonstrates that metalloid does not alter mRNA stability; it rather suggests that As_2O_3 represses EGR2 gene expression at transcriptional level.

Besides macrophages, recent studies demonstrated that EGR2 activates expression of genes involved in negative regulation of T cell proliferation and controls expansion of self-reactive T cells and development of autoimmune disease in mouse [Zhu et al., 2008]. EGR2 also promotes M-CSF-mediated osteoclast survival downstream of the MEK-ERK1/2 pathway [Bradley et al., 2008]. Interestingly, ERK1/2 are redox-sensitive mitogen activated kinases which can be either activated or inhibited by iAs depending on cell types and experimental parameters [Kumagai and Sumi, 2007]. In the present study, inhibition of the MEK-ERK1/2 pathway by U0126 or PD98059 only slightly reduces EGR2 expression in macrophages and does not prevent further EGR2 gene repression by As_2O_3 [Bourdonnay et al., unpublished work]. Thus, ERK1/2 are unlikely to mediate As_2O_3 effects in our cell model. Above all, it is clearly demonstrated that normal expression of EGR2 is required for development of hindbrain, adequate myelination of peripheral nerves and maturation of Schwann cells [O'Donovan et al., 1999]. Since iAs is known to exert peripheral neurotoxicity in human [Vahidnia et al., 2006], its effect on EGR2 expression in neuronal cells should deserve attention.

In summary, our results demonstrated that As_2O_3 alters expression of stress and macrophage-specific genes by a redox-sensitive signaling pathway(s) likely independent of ROS production and related transcription factors. Moreover, they highlight the transcription factor EGR2, which plays major functions in immune and neural cells, as a new molecular target of iAs.

ACKNOWLEDGMENTS

This work was supported by Ligue Contre le Cancer (comité d'Ille et Vilaine). E.B. is recipient of a fellowship form Ligue Contre le Cancer (comité d'Ille et Vilaine).

REFERENCES

Barchowsky A, Dudek EJ, Treadwell MD, Wetterhahn KE. 1996. Arsenic induces oxidant stress and NF-kappa B activation in cultured aortic endothelial cells. Free Radic Biol Med 21:783–790.

Binet F, Girard D. 2008. Novel human neutrophil agonistic properties of arsenic trioxide: Involvement of p38 mitogen-activated protein kinase and/or c-jun NH2-terminal MAPK but not extracellular signal-regulated kinases-1/2. J Leukoc Biol 84:1613–1622.

Bishayi B, Sengupta M. 2003. Intracellular survival of Staphylococcus aureus due to alteration of cellular activity in arsenic and lead intoxicated mature Swiss albino mice. Toxicology 184:31–39.

Bourdonnay E, Morzadec C, Sparfel L, Galibert MD, Jouneau S, Martin-Chouly C, Fardel O, Vernhet L. 2009. Global effects of inorganic arsenic on gene expression profile in human macrophages. Mol Immunol 46:649–656.

Bower JJ, Leonard SS, Chen F, Shi X. 2006. As(III) transcriptionally activates the gadd45a gene via the formation of H_2O_2. Free Radic Biol Med 41:285–294.

Bradley EW, Ruan MM, Oursler MJ. 2008. Novel pro-survival functions of the Kruppel-like transcription factor Egr2 in promotion of macrophage colony-stimulating factor-mediated osteoclast survival downstream of the MEK/ERK pathway. J Biol Chem 283:8055–8064.

Bubici C, Papa S, Dean K, Franzoso G. 2006. Mutual cross-talk between reactive oxygen species and nuclear factor-kappa B: Molecular basis and biological significance. Oncogene 25:6731–6748.

Chakraborti PK, Garabedian MJ, Yamamoto KR, Simons SS, Jr. 1992. Role of cysteines 640, 656 and 661 in steroid binding to rat glucocorticoid receptors. J Biol Chem 267:11366–11373.

Chandrasekar B, Mummidi S, Mahimainathan L, Patel DN, Bailey SR, Imam SZ, Greene WC, Valente AJ. 2006. Interleukin-18-induced human coronary artery smooth muscle cell migration is dependent on NF-kappaB- and AP-1-mediated matrix metalloproteinase-9 expression and is inhibited by atorvastatin. J Biol Chem 281:15099–15109.

Chou WC, Chen HY, Yu SL, Cheng L, Yang PC, Dang CV. 2005. Arsenic suppresses gene expression in promyelocytic leukemia cells partly through Sp1 oxidation. Blood 106:304–310.

Cooper KL, Liu KJ, Hudson LG. 2007. Contributions of reactive oxygen species and mitogen-activated protein kinase signaling in arsenite-stimulated hemeoxygenase-1 production. Toxicol Appl Pharmacol 218:119–127.

Cortner J, Farnham PJ. 1990. Identification of the serum-responsive transcription initiation site of the zinc finger gene Krox-20. Mol Cell Biol 10:3788–3791.

Dai J, Weinberg RS, Waxman S, Jing Y. 1999. Malignant cells can be sensitized to undergo growth inhibition and apoptosis by arsenic trioxide through modulation of the glutathione redox system. Blood 93:268–277.

Felix K, Manna SK, Wise K, Barr J, Ramesh GT. 2005. Low levels of arsenite activates nuclear factor-kappaB and activator protein-1 in immortalized mesencephalic cells. J Biochem Mol Toxicol 19:67–77.

Galicia G, Leyva R, Tenorio EP, Ostrosky-Wegman P, Saavedra R. 2003. Sodium arsenite retards proliferation of PHA-activated T cells by delaying the production and secretion of IL-2. Int Immunopharmacol 3:671–682.

Garant MJ, Kole S, Maksimova EM, Bernier M. 1999. Reversible change in thiol redox status of the insulin receptor alpha-subunit in intact cells. Biochemistry 38:5896–5904.

Ghadially H, Ross XL, Kerst C, Dong J, Reske-Kunz AB, Ross R. 2005. Differential regulation of CCL22 gene expression in murine dendritic cells and B cells. J Immunol 174:5620–5629.

Hashimoto S, Suzuki T, Dong HY, Yamazaki N, Matsushima K. 1999. Serial analysis of gene expression in human monocytes and macrophages. Blood 94:837–844.

He X, Chen MG, Lin GX, Ma Q. 2006. Arsenic induces NAD(P)H-quinone oxidoreductase I by disrupting the Nrf2 x Keap1 x Cul3 complex and recruiting Nrf2 x Maf to the antioxidant response element enhancer. J Biol Chem 281:23620–23631.

Huang CS, Fan YE, Lin CY, Hu ML. 2007. Lycopene inhibits matrix metalloproteinase-9 expression and down-regulates the binding activity of nuclear factor-kappa B and stimulatory protein-1. J Nutr Biochem 18:449–456.

Ishii M, Hashimoto S, Tsutsumi S, Wada Y, Matsushima K, Kodama T, Aburatani H. 2000. Direct comparison of GeneChip and SAGE on the quantitative accuracy in transcript profiling analysis. Genomics 68:136–143.

Ishikawa M, Numazawa S, Yoshida T. 2005. Redox regulation of the transcriptional repressor Bach1. Free Radic Biol Med 38:1344–1352.

Kapahi P, Takahashi T, Natoli G, Adams SR, Chen Y, Tsien RY, Karin M. 2000. Inhibition of NF-kappa B activation by arsenite through reaction with a critical cysteine in the activation loop of Ikappa B kinase. J Biol Chem 275:36062–36066.

Kensler TW, Wakabayashi N, Biswal S. 2007. Cell survival responses to environmental stresses via the Keap1-Nrf2-ARE pathway. Annu Rev Pharmacol Toxicol 47:89–116.

Krasnowska EK, Pittaluga E, Brunati AM, Brunelli R, Costa G, De Spirito M, Serafino A, Ursini F, Parasassi T. 2008. N-acetyl-l-cysteine fosters inactivation and transfer to endolysosomes of c-Src. Free Radic Biol Med 45:1566–1572.

Kumagai Y, Sumi D. 2007. Arsenic: Signal transduction, transcription factor, and biotransformation involved in cellular response and toxicity. Annu Rev Pharmacol Toxicol 47:243–262.

Laragione T, Bonetto V, Casoni F, Massignan T, Bianchi G, Gianazza E, Ghezzi P. 2003. Redox regulation of surface protein thiols: Identification of integrin alpha-4 as a molecular target by using redox proteomics. Proc Natl Acad Sci USA 100:14737–14741.

Laslo P, Spooner CJ, Warmflash A, Lancki DW, Lee HJ, Sciammas R, Gantner BN, Dinner AR, Singh H. 2006. Multilineage transcriptional priming and determination of alternate hematopoietic cell fates. Cell 126:755–766.

Lee PC, Ho IC, Lee TC. 2005. Oxidative stress mediates sodium arsenite-induced expression of heme oxygenase-1, monocyte chemoattractant protein-1, and interleukin-6 in vascular smooth muscle cells. Toxicol Sci 85:541–550.

Lemarie A, Lagadic-Gossmann D, Morzadec C, Allain N, Fardel O, Vernhet L. 2004. Cadmium induces caspase-independent apoptosis in liver Hep3B cells: Role for calcium in signaling oxidative stress-related impairment of mitochondria and relocation of endonuclease G and apoptosis-inducing factor. Free Radic Biol Med 36:1517–1531.

Lemarie A, Morzadec C, Bourdonnay E, Fardel O, Vernhet L. 2006. Human macrophages constitute targets for immunotoxic inorganic arsenic. J Immunol 177:3019–3027.

Lemarie A, Bourdonnay E, Morzadec C, Fardel O, Vernhet L. 2008. Inorganic arsenic activates reduced NADPH oxidase in human primary macrophages through a Rho kinase/p38 kinase pathway. J Immunol 180:6010–6017.

Monteiro P, Gilot D, Le Ferrec E, Lecureur V, N'Diaye M, Le Vee M, Podechard N, Pouponnot C, Fardel O. 2007. AhR- and c-maf-dependent induction of beta7-integrin expression in human macrophages in response to environmental polycyclic aromatic hydrocarbons. Biochem Biophys Res Commun 358:442–448.

Navas-Acien A, Silbergeld EK, Pastor-Barriuso R, Guallar E. 2008. Arsenic exposure and prevalence of type 2 diabetes in US adults. JAMA 300:814–822.

O'Donovan KJ, Tourtellotte WG, Millbrandt J, Baraban JM. 1999. The EGR family of transcription-regulatory factors: Progress at the interface of molecular and systems neuroscience. Trends Neurosci 22:167–173.

Pi J, Qu W, Reece JM, Kumagai Y, Waalkes MP. 2003. Transcription factor Nrf2 activation by inorganic arsenic in cultured keratinocytes: Involvement of hydrogen peroxide. Exp Cell Res 290:234–245.

Reichard JF, Sartor MA, Puga A. 2008. BACH1 is a specific repressor of HMOX1 that is inactivated by arsenite. J Biol Chem 283:22363–22370.

Shattuck RL, Wood LD, Jaffe GJ, Richmond A. 1994. MGSA/GRO transcription is differentially regulated in normal retinal pigment epithelial and melanoma cells. Mol Cell Biol 14:791–802.

Shi H, Shi X, Liu KJ. 2004. Oxidative mechanism of arsenic toxicity and carcinogenesis. Mol Cell Biochem 255:67–78.

Simeonova PP, Luster MI. 2004. Arsenic and atherosclerosis. Toxicol Appl Pharmacol 198:444–449.

Soto-Pena GA, Luna AL, Acosta-Saavedra L, Conde P, Lopez-Carrillo L, Cebrian ME, Bastida M, Calderon-Aranda ES, Vega L. 2006. Assessment of lymphocyte subpopulations and cytokine secretion in children exposed to arsenic. FASEB J 20:779–781.

Spuches AM, Kruszyna HG, Rich AM, Wilcox DE. 2005. Thermodynamics of the As(III)-thiol interaction: Arsenite and monomethylarsenite complexes with glutathione, dihydrolipoic acid, and other thiol ligands. Inorg Chem 44:2964–2972.

Suh JH, Shenvi SV, Dixon BM, Liu H, Jaiswal AK, Liu RM, Hagen TM. 2004. Decline in transcriptional activity of Nrf2 causes age-related loss of glutathione synthesis, which is reversible with lipoic acid. Proc Natl Acad Sci USA 101:3381–3386.

Sun J, Brand M, Zenke Y, Tashiro S, Groudine M, Igarashi K. 2004. Heme regulates the dynamic exchange of Bach1 and NF-E2-related factors in the Maf transcription factor network. Proc Natl Acad Sci USA 101:1461–1466.

Vahidnia A, Romijn F, Tiller M, van der Voet GB, de Wolff FA. 2006. Arsenic-induced toxicity: Effect on protein composition in sciatic nerve. Hum Exp Toxicol 25:667–674.

Wang XJ, Sun Z, Chen W, Li Y, Villeneuve NF, Zhang DD. 2008. Activation of Nrf2 by arsenite and monomethylarsonous acid is independent of Keap1-C151: Enhanced Keap1-Cul3 interaction. Toxicol Appl Pharmacol 230:383–389.

Yoshida T, Yamauchi H, Fan Sun G. 2004. Chronic health effects in people exposed to arsenic via the drinking water: Dose-response relationships in review. Toxicol Appl Pharmacol 198:243–252.

Zhang DD, Hannink M. 2003. Distinct cysteine residues in Keap1 are required for Keap1-dependent ubiquitination of Nrf2 and for stabilization of Nrf2 by chemopreventive agents and oxidative stress. Mol Cell Biol 23:8137–8151.

Zhu B, Symonds AL, Martin JE, Kioussis D, Wraith DC, Li S, Wang P. 2008. Early growth response gene 2 (Egr-2) controls the self-tolerance of T cells and prevents the development of lupuslike autoimmune disease. J Exp Med 205:2295–2307.