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*J Immunol* 2008; 180:6010-6017; doi: 10.4049/jimmunol.180.9.6010

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Inorganic Arsenic Activates Reduced NADPH Oxidase in Human Primary Macrophages through a Rho Kinase/p38 Kinase Pathway

Anthony Lemarie,* Emilie Bourdonnay,* Claudie Morzadec,* Olivier Fardel,*† and Laurent Vernhet2*

Inorganic arsenic is an immunotoxic environmental contaminant to which millions of humans are chronically exposed. We recently demonstrated that human primary macrophages constituted a critical target for arsenic trioxide (As$_2$O$_3$), an inorganic trivalent form. To specify the effects of arsenic on macrophage phenotype, we investigated in the present study whether As$_2$O$_3$ could regulate the activity of NADPH oxidase, a major superoxide-generating enzymatic system in human phagocytes. Our results show that superoxide levels were significantly increased in a time-dependent manner in blood monocyte-derived macrophages treated with 1 μM As$_2$O$_3$ for 72 h. Concomitantly, As$_2$O$_3$ induced phosphorylation enzymatic system in human phagocytes. Our results show that superoxide levels were significantly increased in a time-dependent manner in blood monocyte-derived macrophages treated with 1 μM As$_2$O$_3$ for 72 h. Concomitantly, As$_2$O$_3$ induced phosphorylation and membrane translocation of the NADPH oxidase subunit p47$^\text{phox}$ and it also increased translocation of Rac1 and p67$^\text{phox}$. Apocynin, a selective inhibitor of NADPH oxidases, prevented both p47$^\text{phox}$ translocation and superoxide production. NADPH oxidase activation was preceded by phosphorylation of p38-kinase in As$_2$O$_3$-treated macrophages. The p38-kinase inhibitor SB-203580 prevented phosphorylation and translocation of p47$^\text{phox}$ and subsequent superoxide production. Pretreatment of macrophages with the Rho-kinase inhibitor Y-27632 was found to mimic inhibitory effects of SB-203580 and to prevent As$_2$O$_3$-induced phosphorylation of p38 kinase. Treatment with As$_2$O$_3$ also resulted in an increased secretion of the proinflammatory chemokine CCL18 that was fully inhibited by both apocynin and SB-203580. Taken together, our results demonstrate that As$_2$O$_3$ induced a marked activation of NADPH oxidase in human macrophages, likely through stimulation of a Rho-kinase/p38-kinase pathway, and which may contribute to some of the deleterious effects of inorganic arsenic on macrophage phenotype.

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1 Institut National de la Santé et de la Recherche Médicale, Unité 620, Détoxication et Réparation Tissulaire, Université de Rennes 1 and *Département Hématologie, Immunologie et Thérapie cellulaire, Centre Hospitalier Universitaire Pontchaillou, Rennes, France.

2 Address correspondence and reprint requests to Dr. Laurent Vernhet, Institut National de la Santé et de la Recherche Médicale, Unité 620, Détoxication et Réparation Tissulaire, Université de Rennes 1, 2 avenue du Pr. Léon Bernard, 35043 Rennes, France. E-mail address: Laurent.Vernhet@rennes.inserm.fr

*This work was supported by grants from Association pour la Recherche sur le cancer et Ligue Contre le Cancer (Comité d’Ille-et-Vilaine). A.L. and E.B. are recipients of a fellowship from Ligue Nationale Contre le Cancer and Ligue Contre le Cancer (Comité d’Ille-et-Vilaine), respectively.

†Address correspondence and reprint requests to Dr. Laurent Vernhet, Institut National de la Santé et de la Recherche Médicale, Unité 620, Détoxication et Réparation Tissulaire, Université de Rennes 1, 2 avenue du Pr. Léon Bernard, 35043 Rennes, France. E-mail address: Laurent.Vernhet@rennes.inserm.fr

1 Abbreviations used in this paper: iAs, inorganic arsenic; ROCK, Rho kinase; ROS, reactive oxygen species; SOD, superoxide dismutase; DAPI, 4′,6-diamidino-2-phenylindole.

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catalytically active NADPH oxidase complex (18). In phagocytes, relocation of p47\(^{phox}\)/p67\(^{phox}\) to the membrane is generally driven by phosphorylation of several serine residues in p47\(^{phox}\) (18). Inactivation of the *NCF1* gene, which encodes p47\(^{phox}\), or inhibition of serine p47\(^{phox}\) phosphorylation, correlates with reduced superoxide production (19, 20). Several kinases have been demonstrated to increase phosphorylation of p47\(^{phox}\) serine residues, including the ERK (ERK1/2) and p38 kinase (18, 21). Once produced, superoxides are released either outside or inside the cells and then rapidly converted into hydrogen peroxide (H\(_2\)O\(_2\)) (17). Besides their antimicrobial role, it is established that NADPH oxidase-derived superoxides can modify many cellular functions of macrophages, such as adhesion or gene expression (17, 18). This suggests that NADPH oxidase may be a major target of iAs in macrophages, putatively accounting for some of its deleterious effects on macrophage phenotype. The present study was therefore designed to investigate effects of As\(_2\)O\(_3\) on NADPH-oxidase expression and activity in human primary macrophages. Our results demonstrate that low micromolar concentrations of As\(_2\)O\(_3\) (0.5–1 \(\mu\)M), in the range of iAs blood levels measured in chronically exposed humans (10–60 \(\mu\)g/l) (22, 23), induced phosphorylation and membrane translocation of p47\(^{phox}\) and subsequent ROS formation through a ROCK/p38-kinase pathway. In addition, we show that NADPH oxidase-derived ROS were likely involved in metalloid-induced secretion of the chemokine CCL18, but not in As\(_2\)O\(_3\)-triggered morphological changes due to actin cytoskeleton reorganization.

### Materials and Methods

#### Chemicals, reagents, and Abs

As\(_2\)O\(_3\), NaAsO\(_2\), superoxide dismutase (SOD), catalase, and phalloidin-FITC were purchased from Sigma-Aldrich. Dihydrorhodamine 123 and dihydroethidium were obtained from Invitrogen-Molecular Probes (Interchim). Apocynin, PD-98059, SB-202474, NSC-23766, and Y-27632 were obtained from Calbiochem (VWR). GM-CSF (sp. act. 1.2 \(\times\) 10\(^3\) IU/mg) was obtained from Schering Plough. The c-Jun N-terminal kinase inhibitor 1 n-stereoisomer (D-JNKI1) was purchased from Alexis Biochemicals. Rabbit polyclonal Ab against Rac1 and mouse mAbs against p47\(^{phox}\) and \(\beta\)-catenin were obtained from Santa Cruz Biotechnology (Tebu-bio). Mouse mAb against p67\(^{phox}\) was obtained from BD Pharmingen (BD Biosciences). Rabbit polyclonal Abs against phospho- and total p38 kinase were purchased from Cell Signaling Technology (Ozyme). Detection of phosphoserine residues was performed with a specific rabbit polyclonal Ab obtained from Zymed Laboratories (Clinscience).

#### Preparation of human macrophages

PBMC were first isolated from bloody buffy coats of healthy donors through Ficoll gradient centrifugation. Human monocytes were then prepared by a 2-h adhesion step, which routinely obtained \(>90\%\) of adherent CD14-positive cells as assessed by immunostaining. To obtain macrophages, monocytoid cells were next cultured for 6 days with 800 IU/ml GM-CSF, as previously reported (13). Differentiated macrophages were then treated with As\(_2\)O\(_3\) in GM-CSF-free RPMI 1640 medium for the indicated time intervals. In some experiments, macrophages were first pre-treated with various inhibitors for 2 h and then exposed to As\(_2\)O\(_3\). Apocynin, PD-98059, NSC-23766, SB-203580, and SB-202474 were used as stock solution in DMSO. Final concentrations of solvent in culture medium did not exceed 0.2% (v/v); control cultures received the same dose of solvent as for their treated counterparts. Y-27632, SOD, and catalase were dissolved in distilled water. For flow cytometric studies, macrophages were collected after a 15-min incubation at 37°C in PBS supplemented with 100 \(\mu\)M EDTA.

#### Preparation of mouse macrophages

Male Swiss mice (CERJ) were injected with 0.5 ml of 4% sterile thioglycollate (i.p.) for 4 days; then, peritoneal macrophages were harvested and cultured, as previously described (24). Briefly, thioglycollate-elicted macrophages were prepared by a 2-h adhesion step, which routinely obtained \(>85\%\) of adherent CD11b-positive cells as assessed by immunostaining.

#### Detection of ROS by flow cytometry

Detection of ROS was performed using the nonspecific dihydrodihydroxidine (25). Macrophages were next cultured for 48 h in the absence or presence of iAs and analyzed for ROS production. In some experiments, mice were cotreated for 4 days with thioglycollate and 0.9% NaCl (i.p.) or 8 mg/kg/day (i.p.) NaAsO\(_2\). After isolation, thioglycollate-elicted macrophages were directly used for flow cytometry analysis.

#### Measurement of ROS by flow cytometry

Detection of ROS was performed using the nonspecific dihydrodihydroxidine 123 dye and the superoxide-sensitive dihydrodihydroxidine dye (26). Dihydroidrodihyamine 123 can be oxidized into fluorescent rhodamine 123 (25). Macrophages were next cultured for 10 h in the absence or presence of iAs and analyzed for ROS production. In some experiments, mice were cotreated for 4 days with thioglycollate and 0.9% NaCl (i.p.) or 8 mg/kg/day (i.p.) NaAsO\(_2\). After isolation, thioglycollate-elicted macrophages were directly used for flow cytometry analysis.

#### Detection with specific probes (25). Macrophages were next cultured for 48 h in the absence or presence of iAs and analyzed for ROS production. In some experiments, mice were cotreated for 4 days with thioglycollate and 0.9% NaCl (i.p.) or 8 mg/kg/day (i.p.) NaAsO\(_2\). After isolation, thioglycollate-elicted macrophages were directly used for flow cytometry analysis.

#### Measurement of ROS by flow cytometry

Detection of ROS was performed using the nonspecific dihydrodihydroxidine 123 dye and the superoxide-sensitive dihydrodihydroxidine dye. Dihydroidrodihyamine 123 can be oxidized into fluorescent rhodamine 123 by H\(_2\)O\(_2\) and peroxynitrite, the anion formed when NO reacts with superoxide. Dihydrodihydroxidine, also called hydroethidium, is selectively oxidized by superoxides into fluorescent ethidium. After treatment with iAs in the absence or presence of inhibitors, macrophages were collected, washed, and incubated with 10 \(\mu\)M dihydrodihydroxidine or 5 \(\mu\)M dihydrodihydroxidine for 30 min at 37°C and then analyzed using a FACScalibur flow cytometer (BD Biosciences). Fluorescence emission from oxidized dye was detected at 525 nm (FL1-H) and 605 nm (FL2-H) for rhododihydroxidine and dihydrodihydroxidine, respectively. Each measurement was conducted on 10,000 events and analyzed on Cell Quest software (BD Biosciences). In each experiment, a positive control for rhodamine 123 and ethidium detection was performed.
by incubating macrophages with H2O2 (1 mM) and menadione (50 μM), respectively. Superposition of control and H2O2 or menadione graphs allowed defining a gate for calculating the percentage of rhodamine- or ethidium-positive cells (27).

**Membrane isolation**

As2O3-treated macrophages were harvested, sonicated on ice in a buffer containing 100 mM KCl, 3 mM NaCl, 3.5 mM MgCl2, 10 mM HEPES, 1 mM EGTA, 10 μg/ml pepstatin, 10 μg/ml leupeptin, 0.5 mM PMSF, 1 mM sodium orthovanadate, and 1% protease inhibitor mixture. Lysates were then centrifuged twice at 13,000 g for 10 min at 4°C to remove nuclei and unbroken cells. The supernatant was then ultracentrifuged at 100,000 × g for 1 h at 4°C. Membranes were washed in the same buffer, quantified (28), and resuspended in Laemmli sample buffer, before Western blot analysis.

**Western blot immunoassays**

After treatment, macrophages were harvested, centrifuged, washed with cold PBS, and lysed, as previously described (13). A total of 50 μg of each sample were heated for 5 min at 100°C, analyzed by 12% SDS-PAGE and electroblotted overnight onto nitrocellulose membranes (Bio-Rad). After blocking, membranes were hybridized with primary Ab overnight at 4°C, washed, and incubated with appropriate HRP-conjugated secondary Ab (DakoCytomation). Immunolabeled proteins were visualized by chemiluminescence. Densitometry data analysis was performed using Bio-Profil software.

**Immunoprecipitation**

After treatment, macrophages were collected, washed in cold PBS, and lysed for 30 min on ice in a buffer containing 1 × TBS (pH 7.6), 1% Triton X-100, 50 mM NaF, 1 mM PMSF, 1 mM sodium orthovanadate, and 1% protease inhibitor mixture. Lysates were then centrifuged twice at 13,000 × g for 10 min at 4°C. Preclearing was achieved on the resulting supernatants with 50 μl of Sepharose 6B (Sigma-Aldrich) for 2 h at 4°C. Then, after protein quantification (28), 1 mg of each sample was incubated overnight at 4°C in the presence of 5 μg of anti-p47phox Ab. The resulting immunocomplexes were precipitated with 50 μl of agarose G plus beads (Sigma-Aldrich) for 2 h at 4°C. After washings, phosphoserine levels were quantified by fluorescence microscopy (magnification, ×400). Scale bar, 10 μm. Data are representative of at least three independent experiments. C, After fixation and permeabilization, cells were stained with anti-p47phox Ab and DAPI to detect nuclei and were subsequently viewed by fluorescence microscopy (magnification, ×400). Scale bar, 10 μm. Data are representative of at least three independent experiments. D, Cells were lysed and immunoprecipitated with anti-p47phox Ab. Levels of phosphoserine (p-serine) and p47phox in immunocomplexes were analyzed by Western blot. Intensity of phosphoserine signals was quantified using BioID software and normalized to p47phox; values in C are means ± SEM of three independent results; *, p < 0.05 vs control.

**Immunolocalization**

Monocytes were first differentiated with GM-CSF into macrophages on glass coverslips for 6 days and then treated with As2O3. After washings, cells were fixed on coverslips with 3% paraformaldehyde in PBS for 30 min at 4°C and washed three times with PBS. Fixed cells were subsequently incubated with a blocking and permeabilizing solution (PBS, 2% BSA, 0.2 mg/ml saponin) for 1 h at room temperature. Cells were incubated with mouse anti-p47phox Ab (BD Pharmingen) in a blocking-permeabilizing solution for 2 h at room temperature, washed in PBS, and then stained with rhodamine Red-X-conjugated anti-mouse Ab (Invitrogen) in the same solution for 1 h at room temperature. In some experiments, to detect F-actin filaments, cells were incubated with FITC-phalloidin (1.5 μM) in the same blocking and permeabilizing solution (13). Thereafter, cells were costained by a 15-min incubation in a blocking solution containing 0.25 μg/ml 4′,6-diamidino-2-phenylindole (DAPI), a fluorescent dye specific for DNA. After washing, coverslips were mounted with PBS-glycerol-DABCO (Sigma-Aldrich). Fluorescent-labeled cells were captured with a DMRXA2 Leica microscope.

**Determination of cytokine levels**

Levels of TNF-α, IL-8, and CCL18 in the supernatants of macrophage cultures were quantified using Duoset ELISA development system kits obtained from R&D Systems, as previously described (13).
Statistical analysis

The results are presented as means ± SEM. Significant differences were evaluated with the multirange Dunnett’s t test for experiments in which multiple comparisons were studied. Other differences were evaluated with the Student t test. Criterion of significance of the difference between means was $p < 0.05$.

Results

$As_{2}O_{3}$ increases ROS production in human macrophages

We first investigated the effects on ROS levels of $As_{2}O_{3}$ using the nonspecific dye dihydroethidium 123. Fig. 1A shows that during prolonged time cultures, the amount of rhodamine-positive cells in untreated macrophages was only slightly and not significantly altered. In contrast, 1 μM $As_{2}O_{3}$, a nontoxic concentration (13), markedly increased the percentage of positive cells in a time-dependent manner. 1As effect was detected at 16 h and was maximal after 72 h (42.9% of rhodamine-positive cells). To specify the nature of ROS, we next used the superoxide-specific dye dihydorhodamine 123. As observed with dihydroethidium 123, 1 μM $As_{2}O_{3}$ significantly increased the percentage of ethidium-positive cells during a 3-day treatment. A total of 25.1% and 34.5% of positive cells were detected in macrophages exposed to 1As for 16 and 72 h, respectively (Fig. 1B). Fig. 2A demonstrates that the $As_{2}O_{3}$ effect was concentration dependent. To confirm alteration of superoxide levels by 1As, we then tested different antioxidants. Pretreatment of macrophages with SOD alone or SOD plus catalase totally prevented 1As effects (Fig. 2B); interestingly, apocynin, an inhibitor of NADPH oxidases (29) and NSC-23766, a Rac1 inhibitor (30), also markedly reduced the percentage of ethidium-positive cells in $As_{2}O_{3}$-treated cultures; thus, these results suggest that NADPH oxidase may be involved in ROS production in human macrophages exposed to $As_{2}O_{3}$.

We also determined whether ex vivo or in vivo treatment with iAs could alter ROS production in mouse macrophages. Our results indicate that, in contrast to human macrophages, ex vivo treatment of mouse primary macrophages with non- or subtoxic concentrations (<4 μM) of $As_{2}O_{3}$ or NaAsO$_2$ did not modify percentage of ethidium-positive macrophages (data not shown); in addition, treatment of mice for 4 days with NaAsO$_2$, at the genotoxic dose of 8 mg/kg/day i.p. (31), did not seem to modify ROS production in peritoneal macrophages. 

$As_{2}O_{3}$ activates NADPH oxidase in human macrophages

To study NADPH oxidase activation in $As_{2}O_{3}$-treated macrophages, we next analyzed membrane translocation of p47$^{phox}$, p67$^{phox}$, and Rac1 by immunoblotting. Fig. 3A shows that membrane levels of these proteins were markedly increased in $As_{2}O_{3}$-treated macrophages. Total expression of p47$^{phox}$ in whole cell lysates was however not modified by 1As (Fig. 3B). Translocation of p47$^{phox}$ from cytoplasm to plasma membrane was further assessed by immunolocalization. In untreated macrophages, expression of this protein appears to be weak and highly diffused, as generally observed in nonactivated cells (Fig. 3C). Treatment of

![Image](http://www.jimmunol.org/10.4049/jimmunol.6013.6013)
macrophages with 1 μM As$_2$O$_3$ increased p47$^{phox}$ membrane expression in a time-dependent manner; its relocalization was detectable at 16 h and was maximal after 72 h. As reported in our previous study (13), this figure also shows that As$_2$O$_3$ induced macrophage rounding; this effect was maximal at 72 h. Treatment of macrophages with apocynin prevented As$_2$O$_3$-induced p47$^{phox}$ translocation (Fig. 3C). We then determined levels of phospho-serine p47$^{phox}$ by immunoprecipitation. Fig. 3D shows that As$_2$O$_3$ significantly increased the phosphorylation level of serine residues in cells treated for 72 h.

As$_2$O$_3$-induced NADPH oxidase activation involves p38 kinase

We next analyzed signaling pathways controlling activation of NADPH oxidase in As$_2$O$_3$-treated macrophages by studying involvement of MAPKs which can regulate NADPH oxidase activity in phagocytes (21). Neither the ERK pathway inhibitor PD98059 nor the JNK inhibitor D-JNKI1 could prevent superoxide production in macrophages treated with iAs for 72 h (data not shown). In contrast, increasing concentrations of the specific p38-kinase inhibitor SB-203580 significantly reduced the percentage of ethidium-positive macrophages (Fig. 4A); the inactive structural analog SB-202474, had however no effect in As$_2$O$_3$-treated cells. We also analyzed phosphorylation of p38 kinase which is necessary for its activation. As shown in Fig. 4B, As$_2$O$_3$ increased phosphorylated p38-kinase levels in a time-dependent manner; this increase was detectable at 8 h and was maximal after 72 h. No variation was observed for shorter time exposure (data not shown). We further determined involvement of p38 kinase in NADPH oxidase activation by studying its role in phosphorylation and membrane translocation of p47$^{phox}$. Our results demonstrate that pretreatment with SB-203580 totally inhibited phosphorylation (Fig. 4C) and membrane relocalization (Fig. 4D) of p47$^{phox}$, in macrophages treated with 1 μM As$_2$O$_3$ for 72 h.

Inhibition of ROCK prevents As$_2$O$_3$-induced activation of NADPH oxidase

We previously reported that rounding of As$_2$O$_3$-treated macrophages was regulated by the ROCK (13). We thus determined whether this kinase could also be involved in NADPH oxidase activation in As$_2$O$_3$-treated macrophages. Pretreatment of cells with the ROCK inhibitor Y-27632 (32) markedly reduced p47$^{phox}$ phosphorylation (Fig. 5A), p47$^{phox}$ membrane translocation (Fig. 5B), and superoxide production (Fig. 5C). Interestingly, Fig. 5D shows that Y-27632 also inhibited As$_2$O$_3$-induced phosphorylation of p38 kinase which thus suggests that p38 kinase and NADPH oxidase could be downstream targets of ROCK in iAs-exposed macrophages.

Apocynin and SB-203580 do not prevent cytoskeleton reorganization but inhibit As$_2$O$_3$-induced CCL18 secretion

We next investigated potential involvement of p38 kinase and NADPH oxidase in cell rounding and cytoskeleton reorganization occurring in As$_2$O$_3$-treated macrophages. Cytoskeleton reorganization was evaluated by immunolocalization of F-actin. As expected, Fig. 6A indicates that As$_2$O$_3$ (1 μM, 72 h) induced cell

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**FIGURE 5.** The ROCK inhibitor Y-27632 prevents p38-kinase and NADPH oxidase activation. Macrophages were pretreated or not with the selective ROCK inhibitor Y-27632 (20 μM), and then cultured in the absence or presence of 1 μM As$_2$O$_3$ for 72 h. A, Cells were lysed and immunoprecipitated with anti-p47$^{phox}$ Ab. Levels of phosphoserine (p-serine) and p47$^{phox}$ in immunocomplexes were analyzed by Western blot. Intensity of phosphoserine signals were quantified using Bio1D software and normalized to p47$^{phox}$. B, After fixation and permeabilization, cells were costained with anti-p47$^{phox}$ Ab and DAPI to detect nuclei and subsequently viewed by fluorescence microscopy (magnification ×100). Scale bar, 10 μm. C, After treatments, macrophages were incubated with dihydroethidium for 30 min. Fluorescence related to superoxide production was measured using a FACS Calibur flow cytometer. D, Levels of phospho-p38 kinase (p-p38 kinase) in whole cell lysates were analyzed by Western blot. Equal gel loading and transfer efficiency were checked by protein hybridization with anti-p38 kinase Ab. Results in A and C are expressed as means ± SEM of at least three independent experiments; *, p < 0.05. Data in B and D are representative of at least three independent experiments.
rounding and formation of actin ring. Neither apocynin nor SB-203580 could prevent metalloid effect; pretreatment of macrophages with SOD and catalase was also ineffective (data not shown). Thus, these results indicate that NADPH oxidase and ROS are not involved in morphological effects of As$_2$O$_3$. One major feature of macrophages is secretion of various cytokines (33). To determine whether iAs could alter this function, we then analyzed its effect on secretion of TNF-$\alpha_{9251}$, IL-8, and the proinflammatory chemokine CCL18. As$_2$O$_3$ altered neither TNF-$\alpha_{9251}$ (Fig. 6B) nor IL-8 (data not shown) secretions. In contrast, after a 72 h-treatment, it significantly increased that of CCL18. Interestingly, both apocynin and SB-203580 totally prevented iAs effects (Fig. 6C); a basal level of CCL18 secretion was by contrast not modified by these inhibitors.

Discussion

In this study, we demonstrate for the first time that noncytotoxic concentrations of As$_2$O$_3$ induce a marked activation of NADPH oxidase in human primary macrophages likely through a ROCK/p38-kinase-dependent pathway.

Our results show that As$_2$O$_3$ induced a marked increase of superoxide levels in human blood monocyte-derived macrophages. Several arguments support the conclusion that such ROS may originate from NADPH oxidase: first, As$_2$O$_3$ induced membrane translocation of p47$^{phox}$, p67$^{phox}$, and Rac1, a key step in activating the NADPH oxidase complex in macrophages (17, 18); second, translocation of p47$^{phox}$ was totally prevented by apocynin; this plant-derived phenol is an inhibitor of NADPH oxidases which blocks enzymatic complex assembly in leukocytes (29); third, superoxide formation was totally inhibited by apocynin and NSC-23766, a Rac1 inhibitor. Finally, kinetics of ROS production and p47$^{phox}$ translocation were very similar. Unfortunately, such results could not be extended to mouse primary macrophages. Indeed, in contrast to human macrophages, mouse primary macrophages did not respond to similar ex vivo treatment with iAs. In addition, in vivo treatment of mice with iAs did not increase ROS production in macrophages. These results indicate that the mouse model may not be adequate to assess metalloid effects on NADPH oxidase-derived ROS, and thus suggest that interspecies diversity can influence iAs response. This also indirectly strengthens the interest in human primary cultures of macrophages as a model for studying NADPH oxidase, although in vitro findings do not necessarily reflect what occurs in vivo.

Besides phagocytic NADPH oxidase, iAs can also stimulate nonphagocytic NADPH oxidase in endothelial cells (16, 34), vascular smooth muscle cells (35), and in the human promyelocytic NB4 cell line (15); however, molecular mechanisms mediating its activation remain poorly understood. Chou et al. (15) have shown that prolonged treatment of NB4 cells (9 days) with 0.75 $\mu$M As$_2$O$_3$ markedly up-regulate mRNA levels and membrane expression of p47$^{phox}$. In contrast, our results show that p47$^{phox}$ protein levels were similar in whole cell lysates of untreated and As$_2$O$_3$-treated cells; consequently, they suggest that iAs does not alter global NCF1 gene expression in macrophages. Qian et al. (16)
As$_2$O$_3$ INDUCES NADPH OXIDASE IN HUMAN MACROPHAGES

We have reported that iAs can induce serine phosphorylation of p47$_{phox}$ in vascular endothelial cells but they did not determine which kinase was involved. Our study demonstrates that As$_2$O$_3$ significantly increased the level of serine p47$_{phox}$ phosphorylation in human macrophages likely through p38 kinase. In fact, As$_2$O$_3$ markedly increased levels of phosphorylated p38 kinase. Its effect was detected as soon as 8 h and thus preceded p47$_{phox}$ translocation. In addition, the p38-kinase inhibitor SB-203580 totally inhibited phosphorylation and translocation of p47$_{phox}$ and superoxide production. The demonstration that, in vitro, p38 kinase can respond to As$_2$O$_3$ (13), we investigated the potential role of controlled macrophage rounding and F-actin reorganization in re-
cell adhesion (43). As we previously reported that RhoA/ROCK and can subsequently modify many cellular functions, including
result in necrotic or apoptotic cell death (42). In contrast, interme-
tion of NADPH oxidase activity (41).

ROS elicit a wide spectrum of responses that notably depend upon the magnitude of their doses. High doses of ROS rapidly result in necrotic or apoptotic cell death (42). In contrast, interme-
diate and low doses of ROS directly modulate signaling cascades and can subsequently modify many cellular functions, including cell adhesion (43). As we previously reported that Rhoa/ROCK controlled macrophage rounding and F-actin reorganization in re-
sponse to As$_2$O$_3$ (13), we investigated the potential role of NADPH oxidase-derived superoxides in these phenotypic alter-
ations; our results indicate that neither apocynin nor catalase/SOD could prevent actin ring formation in iAs$_2$O$_3$-treated macrophages. Thus, the present study does not support a role for ROS in mor-
phological effects induced by iAs; it rather indicates that the Rhoa/ROCK pathway, activated by iAs, likely mediates unrelated down-
stream effects such as NADPH oxidase activation and actin filament reorganization.

ROS regulate expression of several genes, notably proinflam-
matory genes (17, 44). Although iAs can increase levels of various cytokines in vivo (45), our results clearly demonstrate that As$_2$O$_3$ altered neither TNF-α production nor that of IL-8 in human mac-
rophages. However, we show for the first time that iAs significa-
cantly increased secretion of CCL18, a recently described chemo-
kine (46); moreover, both apocynin and SB-203580 inhibited As$_2$O$_3$-induced chemokine production which suggests involve-
ment of NADPH oxidase in this effect. CCL18 is preferentially secreted by human APCs and primarily targets lymphocytes (46). Its production can be increased by allergens and LPS but signaling pathways controlling CCL18 up-regulation remain poorly under-
stood (47). Like ROS, iAs generally regulates gene expression by altering activity of redox-sensitive transcription factors such as AP-1, NF-κB, or Sp1 (4, 14). Interestingly, the proximal promoter of CCL18 contains two putative AP-1 regulatory elements (48); thus, a role for AP-1 in As$_2$O$_3$-induced CCL18 expression could not be excluded.

Increased ROS production in iAs-treated macrophages may con-
tribute to metalloid chronic effects, notably atherosclerosis, cancer, or immunotoxicity. Indeed, macrophages play a major role in de-
velopment of atherogenesis through low-density lipoprotein oxida-
tion and such effects are, at least in part, mediated by NADPH oxidase-derived superoxides (18, 49). In addition, during chronic inflammation, production of superoxides by recruited macrophages may drive carcinogenesis by damaging neighboring epithelial or stromal cells (50, 51). Moreover, ROS can induce cell proliferation and/or modify DNA methylation (51); these cellular and molecular effects could also be triggered by iAs (52, 53). Finally, up-reg-
ulation of CCL18 may contribute to iAs-mediated immunotoxic-
ity, owing to the role played by this chemokine in immunity and allergy (47).

In conclusion, the present study demonstrates that iAs activates human macrophagic NADPH oxidase, a key ROS-generating sys-
tem, which may contribute to chronic toxicity of this environmen-
tal contaminant.

Acknowledgments

We are grateful to Etablissement Français du Sang (Rennes, France) for providing us with blood buffy coats, and express thanks to the Institut Fédératif de Recherche 140 microscopy platform in Rennes. We thank Justin Monnier for helpful advice on manuscript copyediting.

Disclosures

The authors have no financial conflict of interest.

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age differentiation through nuclear factor-κB-related survival pathway down-


