NADH Oxidase Activation Is Involved in Arsenite-Induced Oxidative DNA Damage in Human Vascular Smooth Muscle Cells

Shugene Lynn, Jia-Ran Gurr, Hsien-Tsung Lai, Kun-Yan Jan

Abstract—Arsenic is atherogenic, carcinogenic, and genotoxic. Because atherosclerotic plaque has been considered a benign smooth muscle cell tumor, we have studied the effects of arsenite on DNA integrity of human vascular smooth muscle cells. By using single-cell alkaline electrophoresis, apparent DNA strand breaks were detected in a 4-hour treatment with arsenite at a concentration above 1 μmol/L. DNA strand breaks of arsenite-treated cells were increased by Escherichia coli formamidopyrimidine–DNA glycosylase and decreased by diphenylene iodinium, superoxide dismutase, catalase, pyruvate, DMSO, or d-mannitol. Extract from arsenite-treated cells showed increased capacity for producing superoxide when NADH was included in the reaction mixture; however, addition of arsenite to extract from untreated cells did not increase superoxide production. The superoxide-producing ability of arsenite-treated cells was also suppressed by diphenylene iodinium, 4,5-dihydroxy-1,2-benzenedisulfonic acid disodium salt (Tiron), or superoxide dismutase. Superoxide production and DNA strand breaks in arsenite-treated cells were also suppressed by transfecting antisense oligonucleotides of p22phox, an essential component of NADH oxidase. Treatment with arsenite also increased the mRNA level of p22phox. These results suggest that arsenite activates NADH oxidase to produce superoxide, which then causes oxidative DNA damage. The result that arsenite at low concentrations increases oxidant levels and causes oxidative DNA damage in vascular smooth muscle cells may be important in arsenic-induced atherosclerosis. (Circ Res. 2000;86:514-519.)

Key Words: arsenite ■ oxidative DNA damage ■ NADH oxidase ■ atherosclerosis

Arsenic, an element present in the earth’s crust, comes to the surface through mining and utilization of groundwater. Serious contamination by inorganic arsenic occurs through long-term ingestion of high concentrations of arsenic in drinking water. Chronic exposure to arsenic has been related to increased incidences of skin, lung, bladder, liver, and kidney cancers.1,2 Arsenic exposure is also associated with various vascular disorders, including angiosarcomas,3 atherosclerotic plaques,4 and hypertension in humans.5

The epidemiological evidence for shared risk factors for cancer and atherosclerosis has been reviewed by Hansen.6 Exposure to carcinogenic environmental agents is associated with an increased risk of atherosclerosis. Therefore, somatic mutation and cell proliferation may play a role in the pathogenesis of atherosclerotic plaques. The predominant cell type in plaques is the vascular smooth muscle cell (VSMC). Proliferation of VSMCs is essential for plaque formation and development. By examining the isoenzymes of glucose-6-phosphate dehydrogenase, human atherosclerotic plaques were shown to be monoclonal in origin.7 Furthermore, DNA samples from human coronary artery plaques were demonstrated to transform NIH3T3 cells.8 These observations suggest that atherosclerotic plaques are presumably benign smooth muscle cell tumors and that somatic mutation of VSMCs may play a role in the pathogenesis of atherosclerotic plaque.

In humans, exposure to arsenic has been shown to be associated with increased frequency of chromosomal aberrations9 and sister chromatid exchange.10 Arsenic has also been shown to induce gene mutation in human fibroblasts.11 Therefore, genotoxic effects may be the common etiology in arsenic-induced carcinogenesis and atherogenesis. Although arsenite (20 to 80 μmol/L)12 and methylated arsenic (at mmol/L level)13 have been reported to induce DNA strand breaks in cultured mammalian cells, the involvement of arsenic-induced DNA damage in human disorders is somewhat unconvincing, because the concentrations of arsenic used were beyond the range of normal exposure. In Blackfoot disease–affected areas of Taiwan, arsenic concentrations in drinking water and in red blood cells of residents14 can reach 15 and 1.2 μmol/L, respectively. Therefore, we examined the effect of arsenite at 1 to 10 μmol/L levels on DNA integrity of human VSMCs. The results show that arsenite at this concentration range can increase NADH oxidase activity and induce DNA strand breaks.

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Materials and Methods

Cell Culture

Human aorta VSMCs (American Type Culture Collection, CRL-1999) were grown in F12K medium with 2 mmol/L L-glutamine; 1.5 g/L sodium bicarbonate; 10% FCS; 10 ng/mL sodium selenite; 100 U/mL penicillin; and (in μg/mL) endothelial cell growth supplement 20, transferrin 10, insulin 10, and streptomycin 100.

DNA Strand Breaks

DNA strand breaks were analyzed by single-cell alkaline electrophoresis (comet assay) as described previously.18

Determination of Superoxide-Producing Activity in Cell Extract

Cells suspended in 0.2 mL lysis buffer (in mmol/L) monobasic potassium phosphate [pH 7.0] 20, EGTA 1, and PMSF 0.5, as well as [in μg/mL] aprotinin 10, leupeptin 0.5, and pepstatin 0.7) were sonicated for 3 minutes with a 9-s pulse and 1-s off mode. Superoxide was measured by monitoring the chemiluminescence every 10 s for 5 minutes in 50 mmol/L phosphate buffer (pH 7.0), containing 10 μg cell extract, 1 mmol/L EGTA, 150 mmol/L sucrose, 500 μmol/L lucigenin, and either NADH, NADP, or xanthine (100 μmol/L each) as the electron donor (final volume, 0.5 mL). Using standard curves generated from xanthine and xanthine oxidase, the chemiluminescence was converted to the amount of superoxide.19 Each determination was based on 3 to 5 samples.

Transfecting With p22phox Antisense and Sense Oligonucleotides

Cells were transfected with 1 μmol/L oligonucleotides with the aid of SuperFect transfection reagent (Qiagen). The oligonucleotides were antisense or sense of p22phox, the α subunit of cytochrome b-558. The sequences17 were as follows: antisense p22phox, 5′-GATCTGGCCCCATCGGTGAGGACC-3′, and sense p22phox, 5′-GTCTCACCACATGGGCGACATC-3′.

Reverse Transcription–Polymerase Chain Reaction (RT-PCR)

RT-PCR was performed by a RNA PCR kit (Perkin Elmer). Briefly, 1 μg RNA was mixed in 1× PCR buffer, 5 mmol/L MgCl2, 1 mmol/L dNTPs, 1 U/μL RNase inhibitor, 2.5 mmol/L oligo(dT)12, and 2.5 U/μL Moloney murine leukemia virus reverse transcriptase. The RT was performed at 42°C for 15 minutes, followed by denaturation at 95°C for 5 minutes. PCRs were performed in duplicates in a total volume of 50 μL, each containing 10 μL of RT reaction buffer, 2 mmol/L MgCl2, 1× PCR buffer, 0.025 U/μL AmpliTaq DNA polymerase, and 1.5 μmol/L each of sense and antisense primers. The cycle profile included an initial step at 95°C for 10 s, melting at 95°C for 15 s, annealing and extending at 60°C for 30 s, and holding at 72°C for 30 s. Thirty-five cycles of melting, annealing, and extending were performed to amplify p22phox and α-actin. The sequence of primers for p22phox were 5′-GAGTTGGGGCATTGATGGCGACGAA-3′ and 5′-GGATGGTGCGCAGCAGGAAAG-3′17 and those for α-actin were 5′-ACTGGGACAGTGAAAG-3′ and 5′-GAAGAATAGCCAGCTCAG-3′.18 The sizes of products for p22phox and α-actin were 314 and 386 bp, respectively. PCR amplification of RT reactions without reverse transcriptase revealed no PCR product, thus eliminating the possibility of amplifying contaminated DNA (data not shown).

Statistical Analysis

Results are expressed as mean±SE. Statistical analyses were performed with the Student 2-tailed paired t test and ANOVA when more than 2 treatments were compared. * and ** indicate P<0.05 and P<0.01, respectively.

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Figure 1. Effect of treatment with arsenite (As) on DNA integrity of VSMCs. A, Cells were treated with 10 μmol/L arsenite for various lengths of time. B, Cells were treated with various concentrations of arsenite for 4 hours. DNA strand breaks were analyzed by comet assay. Pictures are nuclear images of untreated cells (C) and 4-hour 10 μmol/L arsenite-treated cells (D). Results are from 3 independent experiments. **P<0.01, samples with vs samples without arsenite.

Effects of Arsenite Treatment on DNA Integrity

In this investigation, the comet assay was done in an alkaline condition (pH 13.4). Thus, the DNA strand breaks included double and single strand breaks and alkali-labile sites. The results show that enhanced DNA migration, an indication of DNA strand breaks, was clearly detected in arsenite-treated cells. With a 4-hour treatment, the extent of DNA strand breaks increased with arsenite concentration (Figure 1). Apparent DNA strand breaks were observed in cells treated with arsenite above 1 μmol/L. Because nitric oxide12,19 and reactive oxygen species20 were shown to be involved in arsenite genotoxicity, the effects of their modulators on arsenite-induced DNA strand breaks were studied. The results show that nitric oxide synthase inhibitors, Nω-nitro-L-arginine methyl ester and S-methyl-L-thiocitrulline, had no apparent effects (Figure 2A). However, superoxide dismutase, catalase, diphenylene iodium, DMSO, p-mannitol, and pyruvate

Figure 2. Effects of nitric oxide and reactive oxygen species modulators on arsenite (As)–induced DNA strand breaks. A, Cells were untreated (Unt) or treated for 4 hours with 10 μmol/L arsenite plus various concentrations of Nω-nitro-L-arginine methyl ester (As/NAME) or S-methyl-L-thiocitrulline (As/MTC). B, Cells were untreated (Unt) or treated for 4 hours with 10 μmol/L arsenite or with 10 μmol/L arsenite plus 25 μg/mL superoxide dismutase (As/SOD), 25 μg/mL catalase (As/Cat), 10 μmol/L diphenylene iodium (As/DPI), 0.5% DMSO, 0.5 mmol/L p-mannitol (As/Man), or 1 mmol/L pyruvate (As/Py). Results are from 3 independent experiments. **P<0.01, samples with vs samples without modulator.
bases such as formamidopyrimidine and 8-oxoguanine21; dimerized oligonucleotide was induced in arsenite-treated E. coli Fpg to determine whether oxidation and treatment time (Figures 5A and 5B). However, the addition of arsenite to extract from untreated cells decreased superoxide production (Figure 5A).

Effects of arsenite on NADH Oxidase Activity
To gain further evidence that oxidative DNA damage was induced by treating VSMCs with arsenite, we determined the superoxide-producing ability in cell extract by the lucigenin-mediated chemiluminescence method. The results show that, in the presence of NADH, the extract from arsenite-treated cells generated stronger chemiluminescence than did extract from untreated cells (Figures 4A and 4B), whereas the increase of chemiluminescence in extract from arsenite-treated cells was less when NADPH was used as substrate, and xanthine was unable to support the increase of chemiluminescence in extract from untreated cells (Figures 4A and 4B). The results presented in Figure 3C indicate that oxidative DNA damage was induced by a 4-hour treatment with 1 μmol/L arsenite.

Figure 3. Effects of E. coli Fpg on DNA strand breaks in arsenite (As)-treated VSMCs. A, Cells untreated (Unt) or treated with 10 μmol/L arsenite for 4 hours (As 10) were lysed and then incubated with 0.5 U/μL Fpg for various lengths of time before electrophoresis. B, Cells treated with various concentrations of arsenite for 4 hours or with 0.5 mmol/L H2O2 for 1 hour were lysed and then incubated with 0.5 U/μL Fpg for 120 minutes before electrophoresis. H and As represent cells treated with H2O2 or arsenite but without Fpg digestion; H/Fpg and As/Fpg, cells treated with H2O2 or arsenite and with Fpg digestion. C, Cells untreated (Unt) or treated with 1 μmol/L arsenite for 4 hours without (open columns) or with (hatched columns) 0.5 U/μL Fpg digestion. Results are from 3 independent experiments.

Figure 4. Superoxide-generating activity in extracts from arsenite-treated cells. Cell extracts (2 μg) from untreated cells (A) or from cells treated with 10 μmol/L arsenite for 4 hours (B) were incubated in 0.5 mL superoxide assay buffer containing NADH, NADPH, or xanthine (100 μmol/L each). Chemiluminescences were measured at various time points. Results of panels A and B are from 1 of 3 experiments giving similar results. C, Effects of oxidant modulators on the superoxide generation of extract from arsenite (As)-treated cells. Extracts (2 μg) from cells treated with 10 μmol/L arsenite for 4 hours were incubated in 0.5 mL superoxide assay buffer containing 100 μmol/L NADH without or with 50 μg/mL superoxide dismutase (As/SOD), 10 mmol/L Tiron (As/Tiron), 100 μmol/L diphenylene iodonium (As/DPI), 100 μmol/L allopurinol (As/Allo), or 100 μmol/L N-nitro-L-arginine methyl ester (As/NAME). Extracts from untreated cells were incubated in superoxide assay buffer containing NADH (Unt). Chemiluminescences were measured after a 5-minute incubation. Results are from 3 independent experiments. **P<0.01, samples with vs samples without modulator.

Effects of p22phox mRNA Expression on Arsenite-Induced Superoxide and DNA Strand Breaks
The molecular structure of VSMC NADH oxidase, which bears some similarity to NADPH oxidase of neutrophils, is composed of at least 7 subunits. In VSMCs, upregulation of one of the subunits, p22phox, is a pivotal mechanism for NADH oxidase.22 To confirm that NADH oxidase is activated in arsenite-treated cells, we interfered with the expression of p22phox using p22phox antisense oligonucleotides and measured the effects of the antisense sequence on the superoxide production and DNA strand breaks (Figures 5A and 5B). These results suggest that reactive oxygen species, but not nitric oxide, are involved in arsenite-induced DNA strand breaks. We then used Escherichia coli formamidopyrimidine–DNA glycosylase (Fpg) to determine whether oxidized oligonucleotide was induced in arsenite-treated VSMCs. Fpg is known to catalyze the excision of oxidized bases such as formamidopyrimidine and 8-oxoguanine23; therefore, it will convert these oxidized bases into DNA strand breaks. Fpg incubation increased the DNA strand breaks in H2O2-treated as well as in arsenite-treated cells (Figures 3A and 3B). The results presented in Figure 3C suggest that reactive oxygen species, but not nitric oxide, are involved in arsenite-induced DNA strand breaks (Figure 2B). These results suggest that reactive oxygen species, but not nitric oxide, are involved in arsenite-induced DNA strand breaks.
NADH oxidase by transfecting a p22phox antisense oligonucleotide. The results indicate that transfection with p22phox antisense oligonucleotide markedly reduced superoxide production and DNA strand breaks in arsenite-treated cells (Figures 6A and 6B), whereas transfection with p22phox sense oligonucleotide slightly reduced arsenite-increased superoxide production (Figure 6A) but did not suppress arsenite-induced DNA strand breaks (Figure 6B). We also studied the effects of arsenite treatment on the expression of p22phox mRNA by RT-PCR. The results show that arsenite treatment increased mRNA production of p22phox (Figure 7). The \( \alpha \)-actin mRNA expression was designed to serve as an internal control; however, arsenite treatment apparently also decreased the \( \alpha \)-actin mRNA expression (Figure 7). Therefore, the normalizing of p22phox mRNA expression with \( \alpha \)-actin mRNA expression is invalid.

Effects of Arsenite on Nonproliferating VSMCs
The above-described results were obtained from VSMCs that were cultured in 10% FCS plus growth factors. Therefore, it was unclear whether or not arsenite was affecting signaling systems that would be induced by these growth factors, or whether arsenite alone could cause these effects. We then repeated some of the experiments by using VSMCs that had been grown for 48 hours in medium containing only 0.1% FCS and without the addition of any other growth-promoting factors. The results indicate that the superoxide level of untreated cells was 0.44±0.03 nmol/min×mg protein\(^{-1}\). This value is similar to that for VSMCs cultured in 10% FCS plus growth factors as reported by other investigators\(^23\) and also as shown in Figure 5. Moreover, a doubling of the NADH oxidase activity was measured by a 4-hour treatment with 1 \( \mu \)mol/L arsenite (Figure 8A). A 4-hour treatment with arsenite at a concentration above 1 \( \mu \)mol/L also increased DNA strand breaks (Figure 8B). The induction of DNA strand break was even more apparent with the Fpg digestion (Figure 8B). Therefore, the increase of NADH oxidase activity by arsenite seems not to be due to the interaction of arsenite with the signals induced by growth factors.

Discussion
The oxidation of dichlorofluorescein is widely used to monitor the intracellular oxidative status, and arsenite has been shown to oxidize dichlorofluorescein.\(^24,25\) This result suggests

Figure 7. Effects of treatment with arsenite on p22phox and \( \alpha \)-actin mRNA expression. Cells were treated with various concentrations of arsenite for 4 hours. Total RNA was then extracted, and p22phox and \( \alpha \)-actin mRNA expressions were analyzed by RT-PCR as described in Materials and Methods. Results are from 1 experiment.
that treatment with arsenite may increase intracellular peroxide levels. However, we have previously shown that the increase of dichlorofluorescein fluorescence by treatment with arsenite in Chinese hamster ovary cells is due to the induction of nitric oxide.19 In this investigation, superoxide was measured by the lucigenin-mediated chemiluminescence method. The elevation of superoxide production by treatment with arsenite was made evident by the observation that an appreciable increase of chemiluminescence was detected only when NADH or NADPH was added to the reaction mixture, and the chemiluminescence in extract from arsenite-treated cells was reduced by superoxide dismutase, Tiron, or diphenyleneiodonium, but not by nitric oxide synthase inhibitors. This notion is further supported by the observation that arsenite-induced superoxide production was suppressed by the antisense but not by the sense oligonucleotide of p22phox. Therefore, the present experiments provide strong evidence that treatment with arsenite can increase intracellular superoxide levels and that this probably resulted from activation of NADH oxidase. Although the present results also show that arsenite increased the mRNA level of p22phox, it is still premature to conclude that this is the mechanism by which arsenite activates NADH oxidase. Further research is needed to elucidate how this is done. The present results show that treatment with arsenite increases DNA strand breaks, and this is due to oxidative DNA damage, as is evident by its sensitivity to various oxidant modulators and to Fpg, but not to nitric oxide synthase inhibitors. In contrast to the present finding, arsenite has been shown to induce DNA damage12 and micronuclei19 through the generation of nitric oxide in Chinese hamster ovary cells. Therefore, it seems that different genotoxic mechanisms are involved with arsenite treatment in different types of cells.

The present results show that a 4-hour treatment with arsenite at a concentration above 1 μmol/L increases superoxide levels and oxidative DNA damage in VSMCs. This concentration is consistent with the report that the arsenic concentration in red blood cells of residents exposed to arsenic from drinking water reaches 1.2 μmol/L.14 Using a comet assay, we also examined the induction of DNA strand breaks by a 4-hour treatment with arsenite in human umbilical vein endothelial cells. The results indicate that apparent DNA strand breaks were detected only by treatment with arsenite at concentrations above 5 μmol/L (data not shown). Because data regarding the sensitivity of other types of cells in human blood vessels are not yet available, it is premature to conclude that VSMCs are the most sensitive target for arsenite. However, the demonstration that arsenite at very low concentrations can induce superoxide and oxidative DNA damage in VSMCs suggests that oxidative stress may be an important etiology in arsenic-induced vascular disorders. The DNA strand breaks detected in the present experiments may account for the chromosomal rearrangements in atherosclerotic plaques described by Casalone et al.26 These results are consistent with the view that an increase in the mutation rate may be involved in the formation of atherosclerotic plaques.27 In fact, arsenite has been shown to induce gene mutation through reactive oxygen species.28 In addition to DNA damage, VSMC-derived reactive oxygen species may also promote proatherogenic processes by affecting VSMC proliferation29 and chemotaxis30,31 through the activation of nuclear factorκB,32 oxidative modification of LDL,33,34 and induction of the immediate early genes c-myc and c-fos.35 Arsenite has also been shown to initiate gene transcription by altering signal-transduction molecules.36,37 However, much higher concentrations of arsenite were used in these studies. Therefore, the involvement of signal molecules in arsenite-induced human disorders remains to be determined.

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