Oxidative DNA Adducts and DNA–Protein Cross-Links Are the Major DNA Lesions Induced by Arsenite

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Arsenic is recognized to be a nonmutagenic carcinogen because it induces DNA damage only at very high concentrations. However, many more DNA strand breaks could be detected by digesting the DNA of arsenite-treated cells with endonuclease III, formamidopyrimidine–DNA glycosylase, and proteinase K. By doing so, arsenite could be shown to induce DNA damage in human cells within a pathologically meaningful concentration range. Oxidized guanine products were detected in all arsenite-treated human cells examined. DNA–protein cross-links were also detected in arsenite-treated NB4 and HL60 cells. In human umbilical vein endothelial cells, the induction of oxidized guanine products by arsenite was sensitive to inhibitors of nitric oxide (NO) synthase but not to oxidant modulators, whereas the opposite result was obtained in vascular smooth muscle cells. On the other hand, the arsenite-induced oxidized guanine products and DNA–protein cross-links in NB4 and HL60 cells were sensitive to modulators of calcium, NO synthase, oxidant, and myeloperoxidase. Therefore, although oxidized guanine products were detected in all the human cells treated with arsenite, the pathways could be different in different cell types. Because the sensitivity and the mechanism of arsenic intoxication are cell specific, it is important that target tissues and target cells are used for investigations. It is also important that pathologically or pharmacologically meaningful concentrations of arsenic are used. This is because in most cases we are dealing with the chronic effect rather than acute toxicity. *Key words:* **arsenic, comet assay, DNA damage, hypochlorous acid, nitric oxide, peroxynitrite, thymine glycol, reactive oxygen species.** *Environ Health Perspect* **110(suppl 5):753–756 (2002).**
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Arsenic Can Induce DNA Damage at Pathologically Meaningful Concentrations

Arsenic is recognized to be a nonmutagenic carcinogen because previous studies have indicated that arsenite induces DNA damage only at very high concentrations that are not pathologically meaningful. However, recent studies have shown that formamidopyrimidine–DNA glycosylase (Fpg) and proteinase K (PK) markedly increase the number of DNA strand breaks (DSB) in arsenite-treated cells (*1*). Using Fpg-incorporated comet assay, oxidative DNA damage was detected in human vascular smooth muscle cells treated with 1 μM arsenite for 4 hr (*2*) and in human lymphocytes treated with 10 μM arsenite for 2 hr (*3*). By incorporating Fpg and PK into the comet assay, DSB were detected in the human leukemia cell line HL60 treated with 0.25 μM arsenite for 4 hr (*1*). These concentrations of arsenic are comparable with the report that individuals in Inner Mongolia, China, who continued drinking well water containing high concentrations of inorganic arsenic (0.41 μg/mL) have a mean blood concentration of total arsenic of 42.1 ng/mL (about 0.56 μM) (*4*). The findings that Fpg and PK can markedly increase DSB in arsenite-treated cells indicate that arsenite induces DNA adducts rather than DSB directly. During the cellular DNA repair process, because DSB appear only temporarily on the incision of adducts and are rejoined immediately, the level of DSB will be low at any given time point. This is most likely the reason that low levels of DSB were detected in previous studies. Thus, detection of arsenite-induced DSB using the standard comet assay or alkaline elution without enzyme digestion is inadequate. To reveal the maximum level of DNA adducts with enzyme digestion, it is desirable to sample the cells immediately after arsenite treatment. This is to minimize cellular excision activity and maintain the true level of DNA adducts.

Recent signal transduction research has indicated that arsenic might act on signaling pathways to regulate cell proliferation (*5–7*). These results point to the possibility that arsenic may induce cancer via an epigenetic mechanism. However, the significance of these results may be questioned, as the data were derived from experiments using very high concentrations of arsenic. Many experiments were performed using arsenite concentrations ranging from 10 μM to several hundred μM, and nontarget cells were used. Under our experimental conditions, among the various cell types listed in Table 1, NB4 cells are most sensitive to arsenite in terms of cytotoxicity. A 4-hr 0.25-μM arsenite treatment induces DNA damage but does not affect cell viability (*1*). A 72-hr, 0.25-μM arsenite treatment does not reduce cell survival, whereas a 72-hr, 2-μM arsenite treatment does. This suggests that arsenite can induce DNA damage at noncytotoxic concentrations. On the other hand, arsenic does not appear to induce significant mutagenesis at endogenous loci at high levels of cell survival (*8,9*). One of the main types of DNA adduct digested by Fpg is 8-oxoguanine, which induces G:C→T:A transversions (*10*). We still cannot explain why arsenite can induce oxidized bases when it is not very mutagenic. More experiments are therefore needed to demonstrate that a genetic or epigenetic mechanism, or both, are involved in arsenic-associated carcinogenesis.

Oxidative DNA Adducts Are Predominant and Prevalent

Fpg protein cleaves oxidative bases such as 8-oxoguanine, 5-hydroxycytosine, 5-hydroxyuracil, 2,6-diamino-4-hydroxy-5-*N*-methylformamidopyrimidine, and 4,6-diamino-5-formamidopyrimidne (*11*). Endonuclease III (EnIII) can also cleave many pyrimidine derivatives, including thymine glycol, 5,6-dihydrothymine, 5-hydroxy dihydrothymine, 5-hydroxycytosine, 5-hydroxyuracil, and uracil glycol (*12*). These two enzymes revealed higher levels of DSB than did PK (*13*). The results presented in Table 1 further indicate that Fpg digestion enhanced DSB production in all arsenite-treated human cells examined, whereas PK enhanced DSB production in arsenite-treated leukemia cells HL60 and NB4 and Chinese hamster ovary cells but not in human umbilical vein endothelial cells, human vascular smooth muscle cells, or human skin fibroblasts. Because oxidative DNA adducts were predominant in terms of relative quantity and prevalence in different cell types, we may conclude that oxidative DNA damage is the predominant type of DNA damage in arsenite-treated human cells. Alternatively, the

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DNA damage revealed by Fpg and EnIII digestion may come from arsenite inhibition of the excision of endogenous oxidative DNA damage (*14*). However, this notion is not supported by the observation that sequential digestion of untreated cells with EnIII, Fpg, and PK did not give significant amounts of DSB (*15*).

Arsenite Induces Oxidized Guanine Products via Nitric Oxide and/or Reactive Oxygen Species

The X-ray–hypersensitive Chinese hamster ovary cells XRS5, which are deficient in antioxidant enzymes such as catalase and glutathione peroxidase, are also highly sensitive to arsenite in terms of micronucleus induction (*16*). Moreover, catalase and glutathione peroxidase decrease, whereas their inhibitors increase, micronuclei induction in arsenite-treated XRS5 cells. These results suggest that reactive oxygen species (ROS) are involved in arsenite genotoxicity in XRS5 cells. However, in normal Chinese hamster ovary cells, the arsenite-induced micronuclei, DSB, poly(adenosine diphosphate-ribosylation), and nicotinamide adenine dinucleotide depletion are suppressible by superoxide dismutase and inhibitors of nitric oxide (NO) synthase, suggesting the involvement of NO and/or peroxynitrite in arsenite genotoxicity (*17,18*). This notion is also supported by the observation that in bovine aortic endothelial cells, treatment with arsenite increases nitrite production, whereas treatment with cadmium increases cellular hydrogen peroxide (H_2O_2) levels (*19*). The arsenite-induced DSB can be decreased by NO synthase inhibitors, superoxide scavengers, and peroxynitrite scavengers, and increased by superoxide generators and NO generators. On the other hand, the cadmium-induced DSB can be modulated by various oxidant modulators but not by NO synthase inhibitors. These results suggest that whereas cadmium induces DSB via the superoxice anion radical ($O_2^{\bullet-}$), H_2O_2, and $^{\bullet}OH$, arsenite seems to increase NO, which then reacts with $O_2^{\bullet-}$ to produce peroxynitrite and cause DNA damage. Thus, although ROS are involved in arsenite-induced DSB in XRS5 cells, NO and/or peroxynitrite seem to be involved in arsenite-induced DSB in normal Chinese hamster ovary cells and bovine aortic endothelial cells. To confirm this notion further, we tested the effect of NO synthase inhibitors and ROS modulators on arsenite-induced DSB in a variety of mammalian cells. The results presented in Table 2 indicate that the arsenite-induced DSB were suppressible by NO synthase inhibitors in human umbilical vein endothelial cells, human leukemia cell lines HL60 and NB4, and bovine aortic endothelial cells but not in human vascular smooth muscle cells. On the other hand, the arsenite-induced DSB were sensitive to ROS modulators in human vascular smooth muscle cells, human leukemia cell lines HL60 and NB4 but not in human umbilical vein endothelial cells and bovine aortic endothelial cells. These results confirm that the arsenite-induced DSB can come from either NO or ROS, or both, depending on the cell type examined.

Interestingly, although arsenite and 3-morpholinosydnonimine (a peroxynitrite-generating agent) induce DNA damage via NO and/or peroxynitrite, and cadmium and H_2O_2 induce DSB via oxygen radicals, treatment with all of these three agents induces Fpg-digestible adducts (*19*). These results suggest that NO, peroxynitrite, and H_2O_2 are all capable of inducing oxidized guanine products. This notion is further supported by the observation that in human umbilical vein endothelial cells, the level of arsenite-induced oxidized guanine products was reduced by NO synthase inhibitors but not by catalase. On the other hand, in human vascular smooth muscle cells the level of arsenite-induced oxidized guanine products was reduced by catalase but not by NO synthase inhibitors, and in HL60 cells the level of arsenite-induced oxidized guanine products was reduced by both catalase and NO synthase inhibitors (Figure 1).

Arsenite May Induce DNA Damage via Hypochlorous Acid

In vascular smooth muscle cells, arsenite seems to activate NADH oxidase to produce

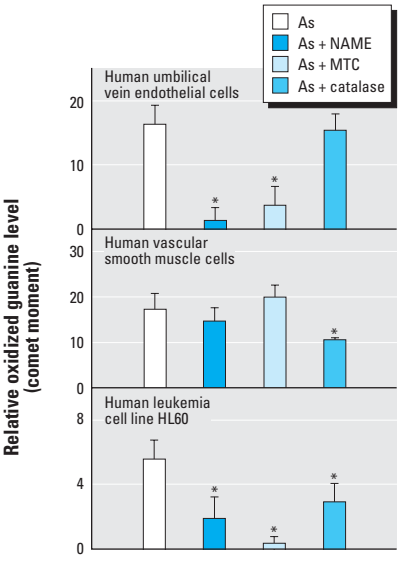

Figure 1. Effects of NO synthase inhibitors and ROS modulators on arsenite-induced oxidized guanine products in human cells. Human umbilical vein endothelial cells, human vascular smooth muscle cellss and cells of the human leukemia cell line HL60 were treated with 2 μM sodium arsenite for 4 hr, or sodium arsenite plus 10 μM NAME, 40 μM MTC, or 800 U/mL catalase for 4 hr. The DSB were analyzed by comet assay with or without Fpg digestion. The comet moments of Fpg digested were subtracted by the comet moments without enzyme digestion and presented as relative level of oxidized guanine products. *$p < 0.05$, with modulators versus without modulators.

Table 1. Effects of Fpg and proteinase K digestion on arsenite-induced DNA strand breaks.

Cell type	$NaAsO_2$ treatment for 4 hr (μM)	Fold of DSB[a]	
		Fpg	PK
Human umbilical vein endothelial cells	2	3.7	1.0
Human vascular smooth muscle cells	1	3.2	1.0
Human leukemia cells, HL60	0.25	3.1	7.0
Human leukemia cells, NB4	0.25	2.5	4.4
Human fibroblasts	2	1.9	1.0
Chinese hamster ovary cells	0.25	2.8	1.6

[a]Comet moment of Fpg or PK digested/comet moment of sham digested.

Table 2. Effects of modulators on arsenite-induced DBS.[a]

Cell type	Calcium modulators		NO synthase inhibitors	ROS modulators		Myeloperoxidase inhibitors
	A	B	C	D	E	F
Human umbilical vein endothelial cells	↓	↑	↓	=		
Human vascular smooth muscle cells			=	↓		
Human leukemia cells, HL60	↓	↑	↓	↓	↑	↓
Human leukemia cells, NB4	↓	↑	↓	↓	↑	↓
Bovine aortic endothelial cells			↓	=	=	

Abbreviations: ↓, decreased; ↑, increased; =, ineffective. [a]Cells were incubated for 4 hr in arsenite with or without modulator. The modulators were (A) 10 μM 1,2-bis(2-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid tetrakis(acetoxymethyl ester), and 10 μM Quin2; (B) 10 μM A23187; (C) 25 μM *S*-methyl-L-thiocitrulline (MTC) or 50 μM Nω-nitro-L-arginine methyl ester (NAME); (D) 100 μg/mL catalase; (E) 0.5 μM diethyldithiocarbamic acid; and (F) 50 μM 4-aminobenzoic acid hydrazide or 50 μM salicylhydroxamic acid.

superoxide and cause oxidative DNA damage because extract from arsenite-treated cells showed an increased capacity to produce superoxide when NADH was given, and the superoxide production as well as DSB were both suppressed by transfecting antisense oligonucleotides of $p22^{phox}$, an essential component of NADH oxidase (*2*). A well-known oxidative DNA damage pathway is that superoxide is converted to H_2O_2 by superoxide dismutase, and H_2O_2 can then react with Fenton metal ions to produce hydroxyl radicals that can cause oxidative DNA damage. However, H_2O_2 can also cause oxidative DNA damage through the production of hypochlorous acid, a less well-known pathway. In the presence of chloride ions, which are abundant in plasma, H_2O_2 and myeloperoxidase can generate hypochlorous acid, causing oxidative DNA damage (*20,21*). Because myeloperoxidase protein is actively expressed in HL60 cells (*22,23*), we tested the possibility that this pathway may exist in these cells. The results indicate that myeloperoxidase inhibitors can indeed effectively suppress arsenite-induced DSB (Table 2).

Arsenite-Induced Nitric Oxide Seems to be Calcium Dependent

In bovine aortic endothelial cells and Chinese hamster ovary cells, treatment with arsenite increases the production of NO (*18,19*). In Chinese hamster ovary cells, the arsenite-induced NO production is sensitive to calcium modulators. Because the arsenite-induced DSB were sensitive to NO synthase inhibitors and calcium modulators, it seems that arsenite may increase NO production through a calcium-dependent pathway. We still know very little about how arsenite induces NO production.

Arsenite Induces Oxidized Guanine Products and DNA–Protein Cross-Links via the Same Pathways

Digestion with PK also increases DSB substantially in arsenite-treated cells. The DSB released by PK have been referred to as DNA–protein cross-links (*24*). It seems as if these DSB had already existed and were bound by proteins. It is not known whether these DNA–protein cross-links are the same as those revealed by the technique of potassium–sodium dodecyl sulfate (K-SDS) precipitation (*25*). Although we have concluded that Fpg-digestible adducts are greater in quantity and more prevalent in different cell types than PK-digestible adducts in arsenite-treated cells, the arsenite-induced Fpg- and PK-digestible adducts were both sensitive to modulators of NO, oxidant, myeloperoxidase, and calcium in HL60 and NB4 cells (*1*). We therefore propose identical pathways for the induction of oxidative DNA and DNA–protein cross-links on treatment with arsenite (Figure 2). An immediate question raised is, if arsenite induces oxidized guanine products and DNA–protein cross-links via the same pathway, why does arsenite induce oxidized guanine products in all the cell types and DNA–protein cross-links in only some cell types?

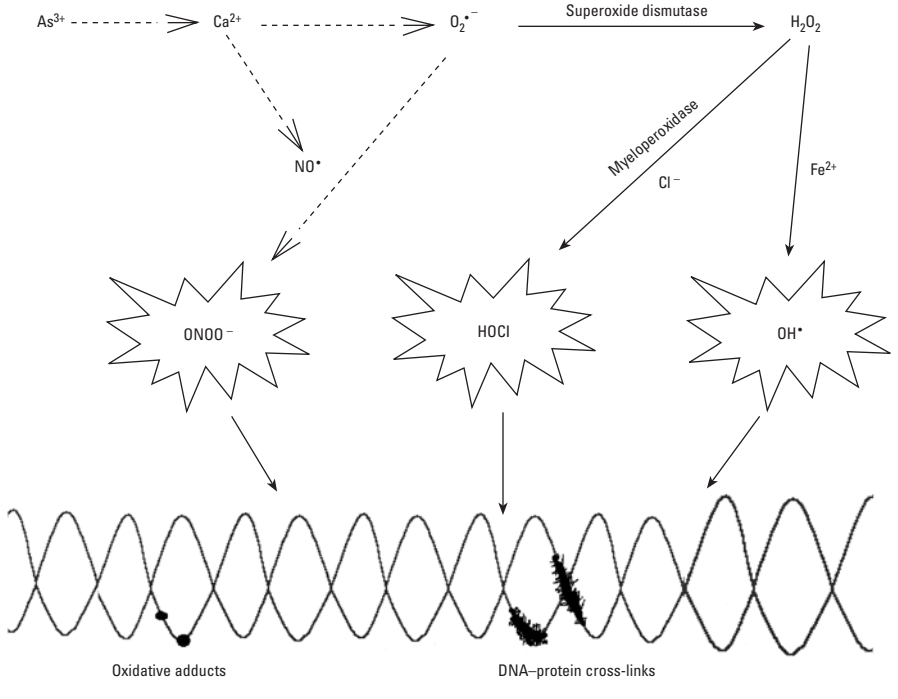

Figure 2. Hypothesis for arsenite induction of oxidative DNA adducts and DNA–protein cross-links. Solid lines indicate well-established pathways. Dotted lines indicate tentative pathways.

Arsenite-Induced Nitric Oxide and Reactive Oxygen Species May Not Attack DNA Only

In addition to oxidative DNA damage, NO, peroxynitrite, and ROS also attack macromolecules such as lipids and proteins. There is increasing evidence to support the notion that these molecules interfere with signal transduction pathways and modulate transcription factors. They have also been implicated in the multistage carcinogenic process, including carcinogen activation, DNA damage, and tumor promotion, and also in various human diseases. Therefore, arsenic-induced NO, peroxynitrite, and ROS may also cause human disorders via pathways other than oxidative DNA damage.

Arsenite Toxicology Is Cell Specific

The results presented in Table 1 indicate that the induction of DNA–protein cross-links by arsenite is not a generalized phenomenon. Moreover, although arsenite induction of oxidized guanine products was shown to be a generalized phenomenon, the pathways could be different in different cell types (Figure 1). Because the sensitivity and the mechanism of arsenic intoxication are cell specific, it is important that target tissues and target cells are used for investigations. It is also important that pathologically or pharmacologically meaningful concentrations of arsenic are used because in most cases we are dealing with a chronic effect rather than acute toxicity.

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