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DNA Damage Induced by Methylated Trivalent Arsenicals Is Mediated by Reactive Oxygen Species†

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Arsenic is a human carcinogen; however, the mechanisms of arsenic's induction of carcinogenic effects have not been identified clearly. We have shown previously that monomethylarsonous acid (MMA^{III}) and dimethylarsinous acid (DMA^{III}) are genotoxic and can damage supercoiled ϕX174 DNA and the DNA in peripheral human lymphocytes in culture. These trivalent arsenicals are biomethylated forms of inorganic arsenic and have been detected in the urine of subjects exposed to arsenite and arsenate. We show here by molecular, chemical, and physical methods that reactive oxygen species (ROS) are intermediates in the DNA-damaging activities of MMA^{III} and DMA^{III}. Using the ϕX174 DNA nicking assay we found that the ROS inhibitors Tiron, melatonin, and the vitamin E analogue Trolox inhibited the DNA-nicking activities of both MMA^{III} and DMA^{III} at low micromolar concentrations. The spin trap agent 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) also was effective at preventing the DNA nicking induced by MMA^{III} and DMA^{III}. ESR spectroscopy studies using DMPO identified a radical as a ROS intermediate in the DNA incubations with DMA^{III}. This radical adduct was assigned to the DMPO-hydroxyl free radical adduct on the basis of comparison of the observed hyperfine splitting constants and line widths with those reported in the literature. The formation of the DMPO-hydroxyl free radical adduct was dependent on time and the presence of DMA^{III} and was completely inhibited by Tiron and Trolox and partially inhibited by DMSO. Using electrospray mass spectrometry, micromolar concentrations of DMA^{V} were detected in the DNA incubation mixtures with DMA^{III}. These data are consistent with the conclusions that the DNA-damaging activity of DMA^{III} is an indirect genotoxic effect mediated by ROS-formed concomitantly with the oxidation of DMA^{III} to DMA^{V}.

Introduction

Exposure to arsenic is worldwide due to both natural and man-made processes (*1*−*3*). Arsenic-associated human diseases include lung, liver, bladder and skin cancer, cardiovascular and vascular disorders, neurological disorders, skin diseases, and liver disorders. Premalignant skin lesions such as Bowen's disease also have been associated with arsenic exposure (*4*). Inorganic arsenic can exist in drinking water in two major forms: arsenite (iAs^{III})[1] and arsenate (iAs^{V}) (*5*). In mammals, inorganic arsenic can be biomethylated to monomethylarsonic acid (MMA^{V}), monomethylarsonous acid (MMA^{III}), dimethylarsinic acid (DMA^{V}), dimethylarsinous acid (DMA^{III}), and trimethylarsine oxide. Levels of many of these organometallic forms of arsenic have been found in the urine of humans exposed to arsenic in their drinking water (*6*, *7*). Although biomethylation of inorganic arsenic was thought initially to be a detoxification process, this notion has been questioned because the methylated trivalent arsenic metabolites MMA^{III} and DMA^{III} have been shown to be toxic to mammalian liver, skin, urinary bladder, and lung cells in culture (*8*, *9*).

Although arsenic exposure has been strongly associated with human neoplasia, the mode(s) of carcinogenic action of the different forms of arsenic have been subjects of intense study. Because arsenic exerts pleiotropic effects, several hypotheses have emerged to explain its carcinogenic action (*10*, *11*). Many toxicological effects of arsenic have been ascribed to iAs^{III}, and a large body of literature has been amassed suggesting the role of iAs^{III} in human disease (*5*). iAs^{III} has been shown in both in vitro and in vivo studies to break chromosomes and cause extensive damage to DNA in a variety of human tissues (*12*). The chromosomal effects and DNA damage were postulated to be critical events in the initiation and progression of human cancer.

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[1] Abbreviations: iAs^{III}, arsenite; iAs^{V}, arsenate; MMA^{III}, monomethylarsonous acid; MMA^{V}, monomethylarsonic acid; DMA^{III}, dimethylarsinous acid; DMA^{V}, dimethylarsinic acid; DMPO, 5,5-dimethyl-1-pyrroline-*N*-oxide; ROS, reactive oxygen species; TE, Tris-EDTA.

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Our laboratory has recently reported that the trivalent methylated forms of arsenic, MMA^{III} and DMA^{III}, are genotoxic to supercoiled phage ϕX174 DNA and DNA in cultured human lymphocytes using a series of techniques that measure DNA damage (*13*). We further reported that methylated forms of arsenic were more genotoxic than the inorganic forms regardless of valence state, with DMA^{III} being the most potent genotoxin. These results suggested that biomethylation was a toxification step in arsenic-induced genotoxicity and possibly in the induction of cancer. Using a similar electrophoretic DNA fragmentation analyses with pBR322 plasmid DNA, Ahmad et al. (*14*) also showed that DMA^{III} damaged the plasmid DNA.

In this report, we describe studies aimed at understanding the mode of action of DNA damage induced by the methylated trivalent arsenicals MMA^{III} and DMA^{III}. We identify reactive oxygen species (ROS) as critical intermediates involved in the DNA damage process by a combination of molecular, chemical, and physical methods.

Material and Methods

Chemicals. Caution: *Arsenic compounds are toxic and potentially carcinogenic. Handle these compounds with appropriate safety precautions*.

iAs^{III}, boric acid, catalase, 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO), DMSO, EDTA, ethidium bromide, melatonin, superoxide dismutase, Tris, and Tiron were obtained from Sigma Chemical Co. (St Louis, MO) and Trolox from BIOMOL Research Laboratories (Plymouth Meeting, PA). DMA^{V} (cacodylic acid) (> 99% pure) was obtained from Fluka Chemical Co. (St Louis, MO). Agarose was obtained from Invitrogen (Carlsbad, CA). Both monomethlyarsonous oxide (99%) and iododimethylarsine (99%) were synthesized by previously reported methods (*13*). The source of MMA^{III} was monomethylarsonous oxide, and that of DMA^{III} was iododimethylarsine (*15*, *16*).

DNA Nicking Assay. ϕX174 RF (replicative form) I DNA (double-stranded circular DNA) was obtained from Amersham/Pharmacia Biotech (Piscataway, NJ), Promega (Madison, WI), and New England Biolabs (Beverly, MA). The arsenicals were dissolved in double-distilled water and incubated in 30-μL reaction volumes, each containing 100 ng of ϕX174 DNA buffered with varying amounts of 10 × Tris-EDTA (TE) (0.1 M Tris-HCl, 10 mM EDTA, pH 7.9). The pH in each reaction was adjusted to pH 7.5−7.7 with 10 × TE to a final concentration range of 10−15 mM Tris, 1−1.5 mM EDTA. This was done to prevent DNA nicking caused by the acidic pH of the arsenicals. The pH of the reaction was determined by test spotting on colorPHast strips (EM-Reagents, Gibbstown, NJ). The complete reaction mixtures were incubated for 22−24 h at 37 °C. Fifteen microliters of the reaction mixtures were loaded onto 0.8% agarose gels that contained 50 μg of ethidium bromide/100 mL of agarose. Electrophoresis was performed in 0.5 × Tris-borate EDTA buffer (pH 8) at 120 V for 90−120 min. The gels were photographed on a UV light box with both Polaroid type 667 film and a Kodak DC290 digital camera. Either the digital photographs or digital scans of the Polaroid photographs were used with the KODAK EDAS system to analyze the intensity of the DNA bands. The preparation of ϕX174 RFI DNA as certified by the manufacturer contained 85% RFI (closed supercoiled form) and ~15% nicked (relaxed) form, each migrating as a distinct band. The determination of nicking was the comparison of the faster-migrating RFI form of ϕX174 DNA with the nicked form with lower electrophoretic mobility. The data in Figures 1-4 presented here are representative of replicate assays performed on different days. For the MMA^{III} studies, melatonin and Trolox were dissolved in ethanol. For the DMA^{III} studies melatonin and Trolox were dissolved in 15 mM Tris buffer pH 7.9 containing 1.5 mM EDTA. Catalase was suspended in phosphate buffer, pH 7.0. Tiron, superoxide dismutase, and DMSO were dissolved in water. All ROS inhibitor solutions were freshly prepared before being added to the reaction tubes prior to the addition of DNA.

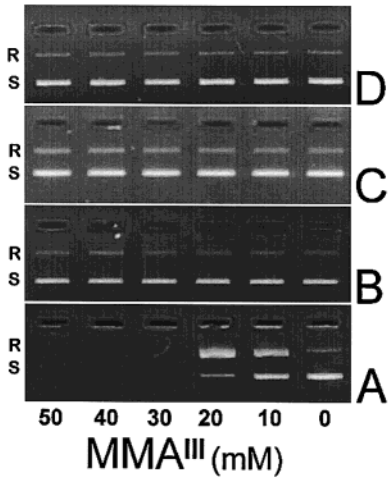

Figure 1. The effects of ROS inhibitors on the nicking of supercoiled ϕX174 DNA by 0−50 mM MMA^{III} after a 24-h incubation: (A) MMA^{III} alone; (B) Trolox, 1 mM; (C) melatonin, 3 mM; (D) Tiron, 10 mM. S, supercoiled form; R, relaxed form.

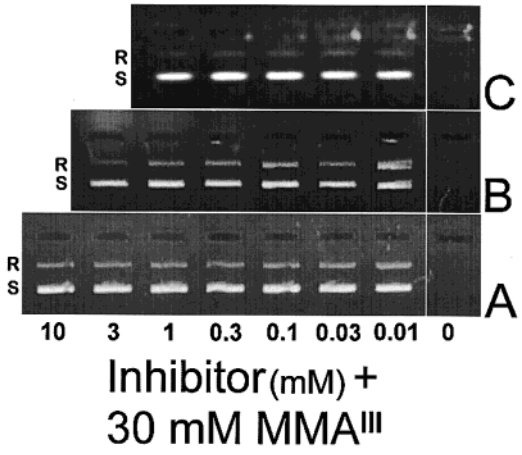

Figure 2. Concentration-dependent effects of ROS inhibitors on the nicking of supercoiled ϕX174 DNA by 30 mM MMA^{III} after a 24-h incubation. All incubation mixtures contained ϕX174 DNA, TE buffer, and 30 mM MMA^{III}. (A) Tiron and 30 mM MMA^{III}. (B) melatonin and 30 mM MMA^{III}. (C) Trolox and 30 mM MMA^{III}. The panels on the extreme right are ϕX174 DNA incubated with TE buffer and 30 mM MMA^{III}. S, supercoiled form; R, relaxed form.

All reaction preparations were performed under HEPA-filtered, laminar-flow, 100% exhaust, biological safety cabinets. Pipet tips and tubes used in reactions were certified by the manufacturer to be nuclease-free, and all preparations for the assays described above were performed wearing nitrile or latex gloves as standard operating procedures to prevent contamination from nucleases that might be contained in fingerprints. In addition, we tested the double-distilled water and methylated trivalent arsenicals for the presence of DNA-degrading activities before and after they had been passed through centrifugal filtration membranes (Millipore, Boston, MA), and no evidence for nuclease-associated DNA-degrading activities was detected.

Electron Spin Resonance Measurements. The generation of free radicals from the complete system containing ϕX174 DNA (2.4 μg), DMA^{III} (1 mM), and TE buffer was investigated by ESR utilizing DMPO (10 mM) as a spin trap agent. ϕX174 DNA and various reactants were mixed in a final volume of 720 μL. ESR

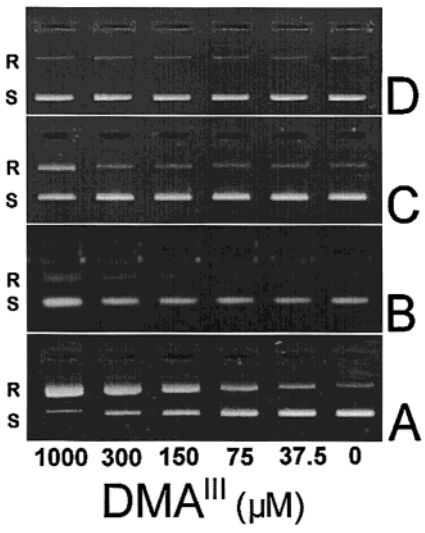

Figure 3. Effects of ROS inhibitors on the nicking of supercoiled ϕX174 DNA by 0−1000 μM DMA^{III} after a 24 h incubation: (A) DMA^{III} alone. (B) Trolox, 1 mM. (C) melatonin, 3 mM. (D) Tiron, 10 mM. S, supercoiled form; R, relaxed form.

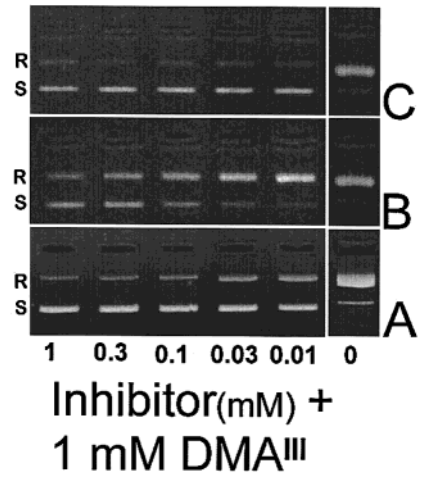

Figure 4. Concentration-related effects of ROS inhibitors on the nicking of supercoiled ϕX174 DNA by 1 mM DMA^{III} after a 24-h incubation. All incubation mixtures contained ϕX174 DNA, TE buffer, and 1 mM DMA^{III}. (A) Tiron and 1 mM DMA^{III}. (B) melatonin and 1 mM DMA^{III}. (C) Trolox and 1 mM DMA^{III}. S, supercoiled form; R, relaxed form. The panels on the extreme right are ϕX174 DNA incubated with TE buffer and 1 mM DMA^{III}.

recording was performed at room temperature using a quartz flat cell and a Bruker EMX spectrometer equipped with a Super High Q cavity. The instrumental conditions are indicated in the figure legends. Spectra were recorded via an IBM computer interfaced to the spectrometer. Hyperfine coupling constants were determined with a spectral simulation program that is available from NIEHS/NIH on the Internet (http://epr.niehs.nih.gov, last accessed Nov 2002).

Mass Spectrometry. Mass spectra were obtained on a Agilent Technologies 1100 LC/MS system with an electrospray mass selective detector. Spectra were scanned simultaneously in both positive and negative mode over a mass range of 100−300 amu. Ten microliters of each sample was injected and chromatographed on a Phenomenex Luna Phenyl-hexyl column (2 × 250 mm, 5 μm particle size) (Phenomenex, Torrance, CA) using an isocratic HPLC-grade water (pH 5.0):methanol (99:1) mobile phase at a flow rate of 0.2 mL/min. A calibration curve was obtained using negative mode extracted ion spectra using dilutions of a solution of 3.45 mg of DMA^{V} in TE buffer.

Results

The supercoiled ϕX174 DNA nicking assay was used to study the effects of ROS inhibitors on MMA^{III}- and DMA^{III}-induced DNA damage using a modification of the reported method (*13*). The published procedure was modified by incorporating strict pH control and extending the incubation time from 2 to 24 h. This was done because the higher arsenical concentrations were found to overwhelm the TE buffering capacity. This resulted in the DNA damage arising from both the arsenical and the low pH. Using the revised procedure, MMA^{III}- and DMA^{III}-induced DNA damages were observed after 2 h of incubation. However, the 24-h incubation time gave greater DNA damage which was needed to observe the effects of the ROS inhibitors. The ϕX174 DNA studies with iAs^{III} were also reexamined using the 24-h incubation period. As reported earlier, iAs^{III} did not damage ϕX174 DNA after 24 h of incubation over a concentration range of 10 μM to 30 mM using log increments (data not shown).

As shown previously, MMA^{III} generated from the precursor, monomethylarsenic oxide, completely fragmented the ϕX174 DNA at concentrations of 30−50 mM (Figure 1A). At the 20 mM concentration, both the supercoiled (S) and relaxed (R) open forms of ϕX174 DNA were detected. Background levels (ca. 15%) of DNA nicking were observed in buffer-treated ϕX174 DNA. The effects of the ROS inhibitors Trolox (Figure 1B), melatonin (Figure 1C), and Tiron (Figure 1D) on MMA^{III}-induced DNA nicking were studied using a single concentration of the ROS inhibitors and by varying the MMA^{III} concentration. The concentrations of the inhibitors were Trolox (1 mM), melatonin (3 mM), and Tiron (10 mM). All three agents completely inhibited the DNA-nicking activity of MMA^{III} to background levels at all concentrations of MMA^{III}. The effects of increasing inhibitor concentration on a fixed concentration of MMA^{III} (30 mM) is shown in Figure 2. This concentration was chosen to provide enough DNA damage so that the effects of inhibitors could be observed. Although MMA^{III} alone completely fragmented the DNA, significant inhibition of DNA nicking was observed with Tiron (Figure 2A), melatonin (Figure 2B), and Trolox (Figure 2C) at concentrations as low as 0.01 mM, and this inhibition continued up to the maximum concentrations tested.

The effects of ROS inhibitors on DMA^{III} nicking of ϕX174 DNA was evaluated. DMA^{III} generated from the precursor iododimethylarsine nicked DNA at micromolar concentrations (Figure 3A). Significant nicking was observed at 37.5 μM, and almost complete conversion of the supercoiled ϕX174 DNA to the relaxed form was recorded at 1 mM after a 24-h incubation. The effects of ROS inhibitors on DMA^{III}-induced DNA nicking were studied using a single concentration of the inhibitors and varying the DMA^{III} concentration. The concentrations of the inhibitors were Trolox (1 mM), melatonin (3 mM), and Tiron (10 mM). Trolox completely inhibited DNA nicking at DMA^{III} concentrations of 37.5 μM to 1 mM (Figure 3B). Between DMA^{III} concentrations of 37.5 and 300 μM, melatonin prevented the arsenic-induced nicking (Figure 3C). At 1 mM DMA^{III}, melatonin was partially protective. Tiron, like Trolox, was completely protective of DMA^{III}-induced DNA nicking at all DMA^{III} concentrations. To determine the effects of ROS inhibitors at lower concentrations, a study was performed with concentra-

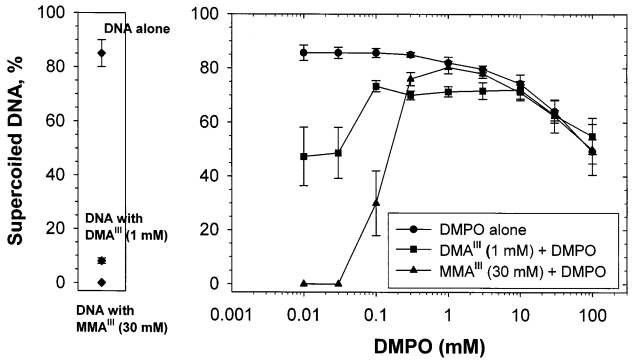

Figure 5. Effects of DMPO on nicking of ϕX174 DNA by MMA^{III} and DMA^{III} after a 24-h incubation. Left panel, incubation mixtures contained ϕX174 DNA, TE buffer, and MMA^{III} (30 mM) or DMA^{III} (1 mM). Right panel, incubation mixtures contained ϕX174 DNA, TE buffer, MMA^{III} (30 mM), or DMA^{III} (1 mM), and DMPO.

tion responses for each inhibitor (0.01 to 1 mM) using a fixed concentration of DMA^{III} (1 mM) (Figure 4). Trolox significantly inhibited the DMA^{III} -induced DNA nicking at all concentrations (Figure 4C). Tiron also significantly inhibited the DNA nicking at 0.01 mM (10 μM) with increasing inhibition at increasing concentrations (Figure 4A). Melatonin was less effective than Trolox or Tiron at the same concentrations (Figure 4B).

The inhibiting effects of DMPO, a known spin trap agent, were evaluated using both MMA^{III} and DMA^{III} (Figure 5). In the case of MMA^{III}, 30 mM MMA^{III} completely degraded the ϕX174 DNA (Figure 5, left panel), whereas addition of increasing amounts of DMPO induced a concentration-dependent inhibition of MMA^{III}-induced DNA damage back to control levels (Figure 5, right panel). In similar fashion DNA damage induced by 1 mM DMA^{III} (Figure 5, left panel) was completely inhibited by DMPO in a concentration−response manner (Figure 5 right panel). DMPO as obtained from Sigma without purification was found to damage DNA at concentrations above 1 mM. However, after purification by sublimation, the DMPO did not exert any DNA-damaging activity up to 100 mM. Furthermore, the sublimed DMPO was still capable of inhibiting the MMA^{III}- and DMA^{III}-induced DNA-damage (data not shown).

The effects of the ROS-degrading enzymes catalase and superoxide dismutase on MMA^{III}- and DMA^{III}-induced DNA nicking were investigated (data not shown). These studies could not be interpreted after the observation that heat-denatured catalase and superoxide dismutase were as effective as the native enzymes toward inhibiting the DNA damage. Even when superoxide dismutase was heat-denatured for 70 min at 100 °C, the preparation still inhibited the DNA-damaging effects of both arsenicals. It is known that trivalent arsenicals bind to proteins through thiol groups (*17*, *18*). Therefore, we surmise that the arsenicals are binding to catalase and superoxide dismutase proteins and preventing the arsenicals from inducing DNA damage.

ESR spectrometry was used to identify the nature of the radical being trapped by DMPO in the DMA^{III} DNA-damage studies. Of the two trivalent methylated arsenicals, DMA^{III} was selected for ESR studies based on its ability to damage DNA at micromolar concentrations. The experimental conditions employed in the ESR studies were those used in the DNA-damage studies with the exception of larger volumes. The complete system containing ϕX174 DNA (2.4 μg), DMA^{III} (1 mM), DMPO (10 mM), and TE buffer gave a characteristic 4 line spectrum of a DMPO-radical adduct minutes after the reactions were initiated (Figure 6A). The ESR spectrum of this radical adduct increased in amplitude over a 24-h period with scans obtained at 2, 6, and 24 h (Figure 6, panels B−D). The computer simulation of this radical adduct was calculated using the following hyperfine coupling constants: $a^N = 15.00$ G and $a_b{}^H = 14.64$ G. This radical adduct was assigned to the DMPO-hydroxyl free radical adduct on the basis of comparison of these hyperfine splitting constants and line widths with those reported in the literature (*19*). The amplitude of the spectrum from the hydroxyl radical adduct generated in the incubations of reaction mixtures was time-dependent, indicating that radical adduct formation increased with time (Figure 6, panels A−D). To probe the dependence of the ESR signal on specific components in the incubation mixtures, a series of deletion studies was also performed. The complete system, ϕX174 DNA (2.4 μg), DMA^{III} (1 mM), DMPO (10 mM), and TE buffer after a 24-h incubation produced the DMPO-hydroxyl radical adduct (Figure 7A). Formation of the DMPO-hydroxyl radical adduct was unchanged in the absence of ϕX174 DNA (Figure 7B). When the experiment in panel A was repeated without DMA^{III}, only a residual signal of the DMPO-hydroxyl radical adduct was observed, thereby confirming the DMA^{III} dependence on the radical formation (Figure 7C). Similarly, there was only a small residual ESR signal of a radical adduct without ϕX174 DNA and DMA^{III} (Figure 7D). Addition of Trolox (10 mM, Figure 7E) or Tiron (1 mM, Figure 7F) to the complete system led to a complete

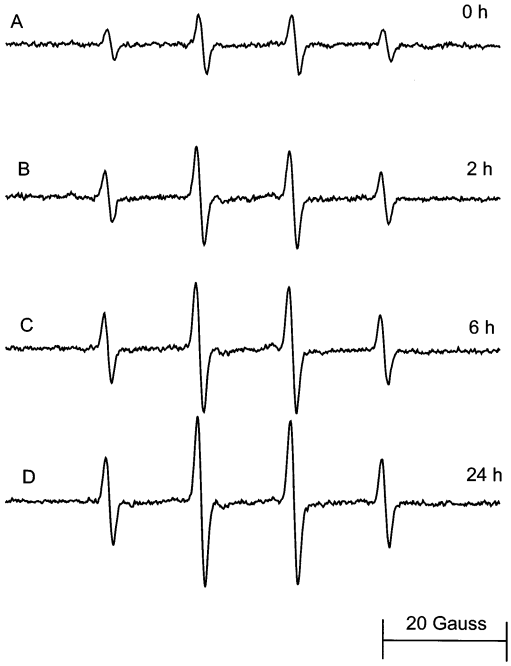

Figure 6. Time-course study of ESR spectra of the DMPO-hydroxyl radical adduct detected from incubations (720 μL) of ϕX174 DNA (2.4 μg), DMA^{III} (1 mM), and DMPO (10 mM) in TE buffer. (A) DMPO-hydroxyl radical adduct detected by ESR immediately after incubation. (B) Same as in panel A but radical adduct detected 2 h after incubation. (C) Same as in panel A but radical adduct detected 6 h after incubation. (D) Same as in panel A but radical adduct detected 24 h after incubation. Instrumental conditions: microwave power, 20 mW; modulation amplitude, 1 G; conversion time, 0.3 s; and time constant, 0.6 s.

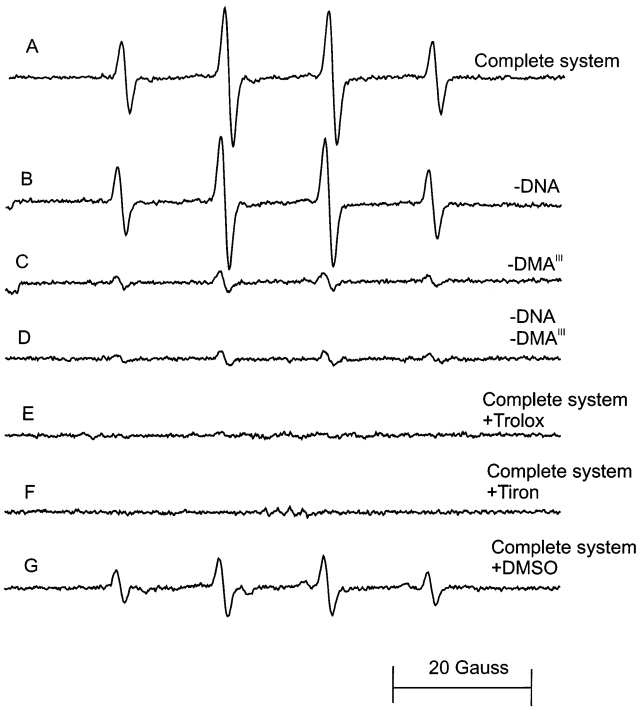

Figure 7. Studies on component requirements and the effects of ROS inhibitors on the ESR spectrum of DMA^{III}-induced radical adducts detected in incubations with ϕX174 DNA. (A) Complete system containing, ϕX174 DNA (2.4 μg), DMA^{III} (1 mM), and DMPO (10 mM) in TE buffer. Spectra were recorded 24 h after incubation. (B) Same as in panel A but without ϕX174 DNA. (C) Same as in panel A but without DMA^{III}. (D) Same as in panel A but without ϕX174 DNA and DMA^{III}. (E) Same as in panel A but with Trolox (1 mM). (F) Same as in panel A but with Tiron (10 mM). (G) Same as in panel A but with DMSO (10 mM). Instrumental conditions, microwave power, 20 mW; modulation amplitude, 1 G; conversion time, 0.3 s; and time constant, 0.6 s.

inhibition of free radical generation by DMA^{III} and DNA. In contrast, addition of the hydroxyl radical inhibitor DMSO (10 mM) resulted in partial inhibition of free radical generation by ϕX174 DNA and DMA^{III} (Figure 7G).

Electrospray mass spectrometry was used to identify and quantitate the arsenic species formed during the DMA^{III} DNA-nicking studies. A DMA^{V} standard was chromatographed by reversed-phase chromatography on a phenyl-hexyl column using water−methanol and detected using electrospray mass spectrometry. DMA^{V} eluted at 6.06 min (Figure 8A). In negative mode electrospray mass spectrometry, DMA^{V} ($C_2H_7AsO_2$, MW = 137.9) gave a characteristic M − 1 molecular ion of *m*/*z* 136.9 (Figure 8B). In positive mode, the M + 1 molecular ion was *m*/*z* 138.9. Due to the interfering ions from Tris and EDTA, extracted ion spectra are shown. DMA^{V} was identified as being formed in a 30-μL incubation mixture of ϕX174 DNA (100 ng), TE buffer, and DMA^{III} (1 mM) incubated at 37 °C for 24 h. When a sample from the incubation mixture was analyzed, DMA^{V} eluted at 6.01 min (Figure 8C). Its mass spectrum revealed a molecular ion *m*/*z* 137.0 (Figure 8D). The concentrations of DMA^{V} in complete reaction mixtures of ϕX174 DNA (100 ng), TE buffer, and DMA^{III} (1 mM) were determined using the external standard method. The calibration curve for DMA^{V} is shown in Figure 8E, and an R^2 of 0.991 was obtained. Samples of triplicate incubation mixtures were taken after an incubation period of 24 h at 37 °C. Values of 136.3 ± 27.5 μM DMA^{V} (mean ± SD) were obtained. This represented a ∼14% conversion of DMA^{III} to DMA^{V}. These results are consistent with the report of Gong et al. (*15*) who examined the stability of DMA^{III} in distilled water and human urine over time at 25 °C. DMA^{III} was oxidized to DMA^{V} in both distilled water and human urine but at different rates. In distilled water complete oxidation was observed after 10 days, whereas in human urine this conversion was achieved after 90 min.

Discussion

Environmental exposure to inorganic arsenic is a major health problem in many parts of the world. Exposures can occur through the air, water, and food routes (*1*, *2*). Epidemiological studies in Taiwanese populations found associations between high levels of inorganic arsenic in the drinking water and tumors at multiple sites, including skin, bladder, liver, and lung (*20*, *21*). In mammals and mammalian cells, inorganic arsenic is biomethylated (*7*, *8*, *22*−*24*). Specific arsenic methyltransferases have been identified and characterized from rabbit liver, Chang human hepatocytes (*25*), and Rhesus monkey liver (*26*). An *S*-adenosyl-L-methionine:arsenic (III) methyltransferase was isolated recently and characterized from rat liver cytosol (*27*). This enzyme catalyzes transfer of a methyl group from *S*-adenosyl-L-methionine to trivalent arsenicals, producing mono- and dimethylated arsenicals. In rats, transferase activity was detected in lung, liver, and urinary bladder tissues, which are targets of arsenic carcinogenesis.

Urinary excretion of MMA^{III} and DMA^{III} has been measured in human populations exposed to arsenic. Mandal et al. (*6*) have reported significant amounts of both of these trivalent arsenicals in urine from groups of individuals from West Bengal, India, exposed to inorganic arsenic in their drinking water. Persons consuming drinking water with total inorganic arsenic levels of 33−248 μg of As/L had urinary concentrations of MMA^{III} of 3−30 μg/L (0.24 μM) and DMA^{III} concentrations of 8−63.8 μg/L (0.46 μM). A population from Inner Mongolia, China, that consumed drinking water containing 510−660 μg/L of inorganic arsenic had urinary levels of 32−127 μg/L of MMA^{III} and 21−38 μg/L of DMA^{III} (*7*). Groups of Romanian subjects exposed to 2.8−61.2 μg As/L in their drinking water were reported to excrete 4.8−6.9 μg/L MMA^{III} in their urine (*28*).

Although inorganic arsenic exposure has long been known to be associated with human neoplasia, experimental arsenic carcinogenesis has been problematical (*1*, *2*, *29*, *30*). More success toward inducing experimental arsenic carcinogenesis has been obtained with the methylated arsenical DMA^{V}. One of the most significant rodent cancer studies of methylated arsenicals is that of Wei et al. (*31*, *32*). In a 2-year bioassay urinary bladder carcinomas were induced in male F344 rats exposed to DMA^{V} in the drinking water at concentrations of 0, 12.5, 50, and 200 ppm. Female F344 rats exposed to 100 ppm DMA^{V} in the diet also developed urinary bladder tumors (*30*). Oral administration of DMA^{V} to rats enhanced the amounts of 8-oxo-2′-deoxyguanosine (8-oxo-dG), a biomarker of DNA oxidation in the urinary bladder, the target organ of arsenic carcinogenesis in the rat (*32*). In the same study iAs^{III} did not enhance 8-oxo-dG formation. On the basis of these and other studies, oxidative damage

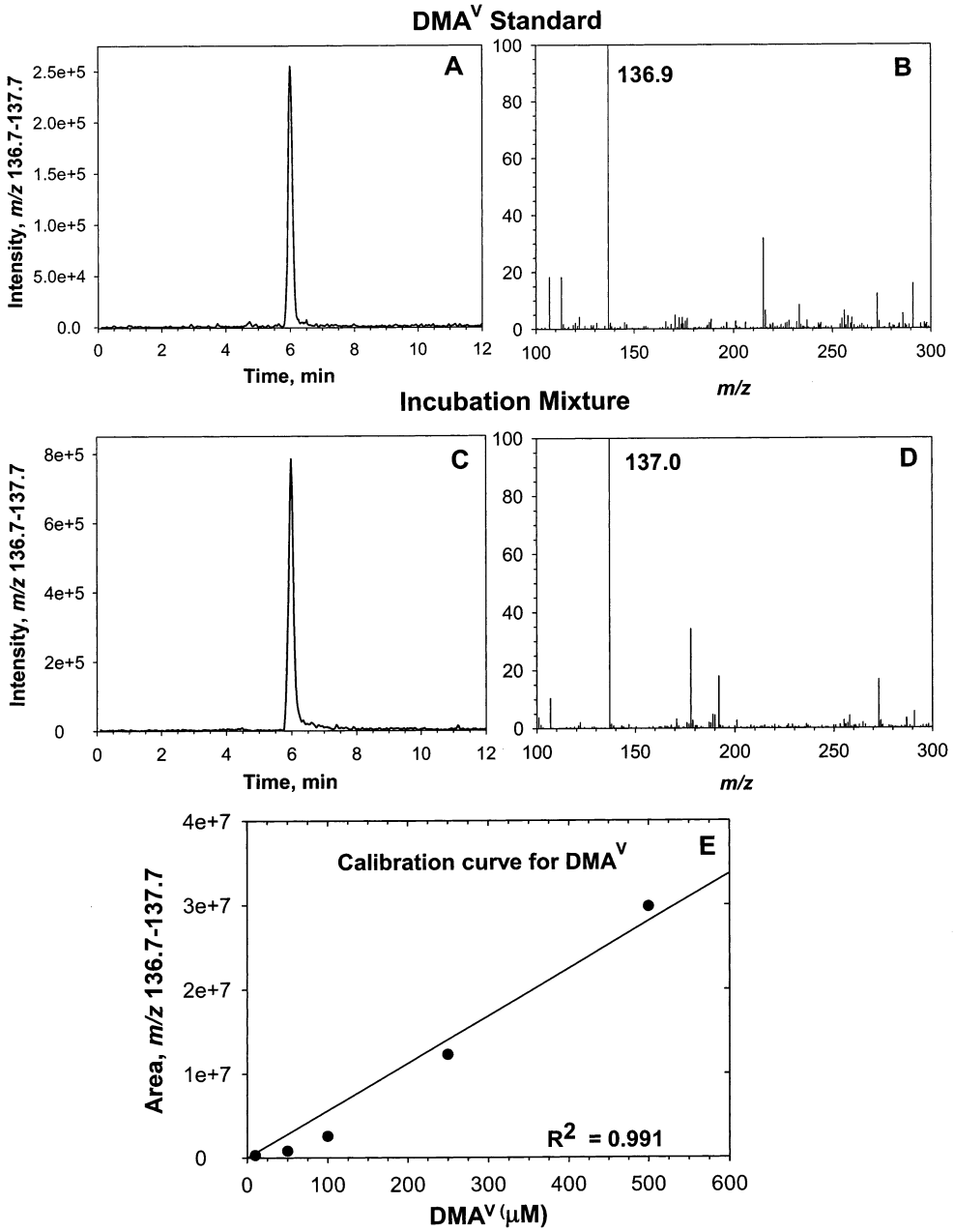

Figure 8. HPLC electrospray mass spectrometric analyses of DMA^V. (A) Extracted ion electrospray mass spectrometric HPLC chromatogram of DMA^V standard. The sample was chromatographed on a reversed-phase column using water-methanol (99:1) and the ion detected in negative mode. (B) Mass spectral fragmentation analyses of DMA^V standard eluting at 6.06 min. (C) Extracted ion electrospray mass spectrometric HPLC chromatogram of a sample from an incubation of ϕX174 DNA (100 ng), TE buffer, and DMA^{III} (1 mM) incubated at 37 °C for 24 h. (D) Mass spectral fragmentation analyses of the peak eluting at 6.01 min from a sample of an incubation mixture of ϕX174 DNA (100 ng), TE buffer, and DMA^{III} (1 mM) incubated at 37 °C for 24 h. (E) Calibration curve of the DMA^V standard.

has been proposed to play an important role in experimental arsenic carcinogenesis (*30*, *32*, *33*). Cohen et al. (*34*) reported that high doses of DMA^V (40 or 100 ppm in the diet) fed for 10 weeks to rats increased urothelial cell proliferation and cytotoxic changes, including cellular necrosis, suggesting that administration of DMA^V results in cytotoxicity with necrosis, followed by regenerative hyperplasia of the bladder epithelium. In follow-up studies, Cohen et al. (*35*) examined the urinary concentrations of DMA^{III} from rats exposed to DMA^V at 100 ppm in the diet. They found 1−5-μM concentrations of DMA^{III} being excreted. Using rat and human urothelial cells in culture, they found <1 μM concentrations of DMA^{III} to be cytotoxic. From the results of these studies they suggested that the toxic effects observed in the rat urinary bladder induced by DMA^V may be induced by DMA^{III} formed in vivo (*35*).

ROS, including superoxide anion, hydroxyl radicals, and hydrogen peroxide have been associated with the genotoxicity of iAs^{III}. In human−hamster hybrid cells treated with iAs^{III}, ROS production from iAs^{III} was detected by ESR using the spin trap agent TEMPOL-H (*36*). The intensity of the signal was reduced by both catalase and superoxide dismutase. The iAs^{III}-induced genotoxicity in the cells was reduced by DMSO, a ROS scavenger (*37*). On the basis of these data, Liu et al. (*36*) proposed the following sequence of events in arsenic-induced genotoxicity in mammalian cells: iAs^{III} → su-

peroxide anions → hydrogen peroxide → hydroxyl radicals → genotoxicity.

The studies presented here were undertaken to probe the mechanisms of action of the ϕX174 DNA-damaging activities of MMA^{III} and DMA^{III}. As reported above for iAs^{III}, we found a strong inference of ROS mediation in the cleavage of supercoiled ϕX174 DNA to its relaxed and fragmented forms by the two trivalent methylated arsenicals. The DNA-damaging activities of both MMA^{III} and DMA^{III} were inhibited by the chemically diverse ROS inhibitors Tiron, Trolox, and melatonin and the spin trap agent DMPO. We observed the formation of a DMPO−hydroxyl radical adduct minutes after placing the iododimethylarsine into TE buffer, and the intensity of this radical increased over time. This indicated a slow but sustained formation of hydroxyl radical. The formation of the DMPO-hydroxyl radical adduct was inhibited by ROS scavengers, including DMSO, a hydroxyl radical scavenger. Concurrent with these observations was the detection of micromolar concentrations of DMA^{V} in the incubation mixtures by mass spectrometry. Also consistent with these observations were the remarkable findings that low micromolar concentrations of the ROS inhibitors could affect millimolar concentrations of methylated arsenicals. These results in toto suggested that the ROS inhibitors were not interacting directly with the methylated arsenicals but were interacting with the small quantities of ROS, including hydroxyl radical, produced by the arsenicals in the incubation mixtures concomitant with DMA^{V} formation. The complex mechanisms of the oxidation of trivalent arsenicals to their pentavalent forms and the formation of ROS are not known. There is a lack of stoichiometry between the amounts of ROS inhibitors that can prevent DMA^{III}-induced DNA damage and the amount of DMA^{V} formed in the incubation mixtures. This suggests that in the process of oxidizing DMA^{III} to DMA^{V} not all of the ROS formed are capable of damaging DNA and thus being inhibited. It is also possible that free radical chain reactions might be taking place as described for lipid peroxidation (*38*). The ROS inhibitors might be scavenging a rate limiting step early in the chain reaction process. More studies are needed to elucidate these pathways.

The identification of all of the ROS formed in the ROS cascade in the ϕX174 DNA incubations could not be ascertained. The ESR studies identified the hydroxyl radical; however, we were not able to use catalase or superoxide dismutase, diagnostic for hydrogen peroxide and superoxide anion, respectively, to identify the presence of these species. Nonetheless, Tiron has been associated with scavenging superoxide anion (*39*), Trolox has been associated with scavenging hydroxyl radical and superoxide anion (*40*), and melatonin with scavenging hydroxyl radical, hydrogen peroxide, and singlet oxygen (*41*). From these data we propose that other ROSs form in addition to hydroxyl radical in the incubation mixtures. Superoxide anion and/or hydrogen peroxide could be formed in the process of the oxidation of DMA^{III} to DMA^{V} possibly in combination with Haber−Weiss (*42*) chemistry. ROS-mediated DNA damage of ϕX174 DNA is well-established with the following ROS generating oxidation/reduction systems being shown to damage ϕX174 DNA: Cu (II)/H_2O_2 (*43*), Cu (II)/1,4-hydroquinone (*44*), gamma rays/O_2 (*45*), and Cu(II)/4-hydroxyestradiol (*46*). Several ROS can damage DNA. The hydroxyl radical is the most energetic and capable of abstracting a hydrogen radical on deoxyribose in DNA to induce DNA strand scission (*47*). Hydroperoxides (e.g., linoleic acid hydroperoxides) can also induce single strand and double strand breaks in DNA (*48*).

Like any model system, the ϕX174 DNA-nicking assay can only indicate possible mechanisms that might occur in more complex biological systems. However, confirmation of the DMA^{III}-induced DNA damage data obtained with the ϕX174 DNA-nicking assay was obtained in human lymphocytes in culture at the same concentration ranges as shown here (*13*). This suggested that the ϕX174 DNA system is a useful model to study mechanisms of action of the arsenicals. The ϕX174 DNA studies reported here revealed significant DNA damage induced by DMA^{III} at 37.5 μM, and this damage was inhibited by ROS inhibitors. This concentration of DMA^{III} is ∼7−38 times the DMA^{III} concentrations measured in the urine of rats fed carcinogenic doses of DMA^{V} (*35*).

Our demonstration of the production of ROS by methylated trivalent arsenicals and the association of these ROS with the DNA-cutting activity of these agents provide a mechanism for the genotoxicity of these arsenicals. This genotoxicity is an indirect effect via the generation of ROS. Cohen et al. (*35*) proposed that DMA^{III} formed in vivo from DMA^{V} can mediate rat bladder cell proliferation and cytotoxicity changes, including cellular necrosis. They suggested that these changes result in regenerative hyperplasia of the bladder epithelium, which is a potential mechanism for arsenic-induced neoplasia. Because DMA^{III} has been shown to damage the DNA of human lymphocytes in culture, we suggest that a possible mechanism for the DMA^{V}-induced neoplasia in rats through DMA^{III} is, in part, a result of ROS-induced DNA strand breaks.

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