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Original Contribution

Enhanced ROS production and redox signaling with combined arsenite and UVA exposure: Contribution of NADPH oxidase

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A B S T R A C T

Solar ultraviolet radiation (UVR) is the major etiological factor in skin carcinogenesis. However, in vivo studies demonstrate that mice exposed to arsenic and UVR exhibit significantly more tumors and oxidative DNA damage than animals treated with either agent alone. Interactions between arsenite and UVR in the production of reactive oxygen species (ROS) and stress-associated signaling may provide a basis for the enhanced carcinogenicity. In this study keratinocytes were pretreated with arsenite (3 μM) and then exposed to UVA (10 kJ/m^2). We report that exposure to UVA after arsenite pretreatment enhanced ROS production, p38 MAP kinase activation, and induction of a redox-sensitive gene product, heme oxygenase-1, compared to either stimulus alone. UVR exposure resulted in rapid and transient NADPH oxidase activation, whereas the response to arsenite was more pronounced and persistent. Inhibition of NADPH oxidase decreased ROS production in arsenite-treated cells but had little impact on UVA-exposed cells. Furthermore, arsenite-induced, but not UVA-induced, p38 activation and HO-1 expression were dependent upon NADPH oxidase activity. These findings indicate differences in the mechanisms of ROS production by arsenite and UVA that may provide an underlying basis for the observed enhancement of redox-related cellular responses upon combined UVA and arsenite exposure.

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Skin cancer is linked to ultraviolet radiation (UVR) via sun exposure [1]. UVA (320–400 nm) and UVB (280–320 nm) comprise the wavelengths of primary concern in human skin cancers [2]. A majority of the studies on UVR-induced skin cancers have focused on UVB-induced DNA damage. Pyrimidine photoproducts are induced by UVB, but UVR also causes oxidative DNA lesions due to the production of reactive oxygen species (ROS) [3]. ROS are generated by the reaction of UVR with endogenous photosensitizers, blocking of antioxidant activity, and inflammation in the dermis. UVA is primarily associated with elevated levels of ROS, but both UVA and UVB are reported to induce ROS production in keratinocytes [4].

Arsenic is also a skin carcinogen, and chronic exposure has been linked with increased incidence of basal cell carcinoma and squamous cell carcinoma [5–7]. Arsenic enhances tumor development when combined with other carcinogens [8–10], with chronic overexpression of growth factors [11], and with UVR [12,13]. For example, mice chronically exposed to arsenite in drinking water develop significantly more skin tumors than mice exposed to arsenite or UVR alone [12,13]. Additionally, increased oxidative DNA damage was detected in the skin and tumors of the dually treated animals [14]. Therefore, arsenic may be better characterized as a cocarcinogen capable of potentiating the actions of a carcinogenic partner, such as UVR.

Arsenite exposure elevates ROS levels, leading to increased cellular oxidative stress [15,16] resulting in oxidative DNA and protein damage [17–19]. ROS production is, therefore, a shared consequence of UVR and arsenic exposure. However, DNA damage is not the only result of excess ROS production. ROS and arsenite also cause damage to proteins and lipids and alterations in intracellular signaling pathways [4]. Altered signaling due to ROS can ultimately lead to activation of transcription factor complexes, such as AP-1 or NF-κB. Activation of these transcription factors is crucial in the stimulation of the complex transcriptional alterations required for keratinocyte transformation [20,21] and are likely to be wavelength dependent [22]. Both UVA and UVB are known to activate mitogen-activated protein kinase (MAP kinase) signaling and stimulate gene transcription in human and mouse models of UVR-exposed tissues. UVA induces phosphorylation and activation of extracellular signal-regulated kinases (ERKs), c-*jun* N-terminal kinases (JNKs), and p38 kinases [23,24]. Arsenic, typically as arsenite (As(III)), also stimulates MAP kinase cascades including ERK and the stress-activated kinases JNK and p38 [25–29]. Recently we have shown that arsenite acts through at least two distinct pathways; ERK activation is epidermal growth factor (EGF) receptor dependent, but p38 activation is independent of EGF receptor activation [26]. Further

Abbreviations: UVR, ultraviolet radiation; ROS, reactive oxygen species; DHE, dihydroethidium; DCFDA, 2′,7′-dichlorofluorescein diacetate; DPI, diphenyleneiodonium; ERK, extracellular signal-regulated kinase; MAP kinase, mitogen-activated protein kinase; JNK, c-*jun* N-terminal kinases; PBS, phosphate-buffered saline.

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research demonstrated that in human keratinocytes ROS, specifically superoxide, are upstream of p38 activation by arsenite and lead to robust expression of heme oxygenase (HO)-1 with concurrent EGF receptor activation contributing to the persistence and magnitude of the response [30].

There are many potential sources of increased ROS, including mitochondrial activity and activation of enzymes, such as NADPH oxidase [31–33]. Arsenite has been shown to increase the activity of NADPH oxidase via upregulation and phosphorylation of key subunits [34] and NADPH oxidase has been implicated in arsenite-induced oxidative DNA damage [35]. NADPH oxidase has been reported to be a major target involved in the production of ROS after arsenite exposure [36]. However, the mechanism of UVR-induced ROS production remains controversial.

Although mouse models demonstrate cocarcinogenic activity with arsenite and UVR [12,13], the underlying mechanisms remain unknown. The similarities of action between arsenite and UVR in the production of ROS and activation of MAP kinases may provide a basis for understanding the cooperative actions in skin carcinogenesis and are further investigated here. In this study we report that exposure to UVA after arsenite pretreatment results in an additive increase in ROS production. Furthermore, NADPH oxidase was required for arsenite-induced ROS, but was only partially involved in UVA-induced ROS. The combination of arsenite and UVR led to enhanced p38 MAP kinase activation and induction of a redox-sensitive gene product, HO-1, compared to either stimulus alone. Together these data demonstrate cooperation between arsenite and UVR in signaling pathways implicated in skin carcinogenesis.

Materials and methods

Reagents and antibodies

Bovine serum albumin (BSA), cell culture reagents, dimethyl sulfoxide (DMSO), nitroblue tetrazolium, dihydroethidium (DHE), diphenyleneiodonium (DPI), lucigenin, tiron, diethyldithiocarbamate, NADPH, apocynin, rotenone, and sodium arsenite were purchased from Sigma (St. Louis, MO, USA). Newborn calf serum was acquired from Life Technologies (Gaithersburg, MD, USA). Atpenin and MnTMPyP were purchased from CalBiochem (San Diego, CA, USA) and were dissolved in DMSO. Phosphospecific phospho-p44/42 MAP kinase (Thr202/Tyr204) and phospho-p38 MAP kinase (Thr180/Tyr182) antibodies and pan-ERK and total p38 antibodies were purchased from Cell Signaling (Beverly, MA, USA). HO-1, $p22^{phox}$, $p67^{phox}$, and β-tubulin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Phosphospecific phospho-serine antibodies were purchased from Abcam. (Cambridge, MA, USA). Peroxidase-conjugated anti-rabbit IgG and anti-mouse IgG were purchased from Promega (Madison, WI, USA). SuperSignal chemiluminescence detection system was purchased from Pierce (Rockford, IL, USA). Caution: Inorganic arsenic is toxic and classified as a human carcinogen. It must be handled with appropriate care.

UV source

UVR exposures were performed using an Oriel 1000-W solar ultraviolet simulator (Oriel Corp., Stratford, CT, USA). This solar simulator produces a high-intensity UVR beam in both the UVA (320–400 nm) and the UVB (280–320 nm) spectrum. Clear glass plates were used to reduce the amount of UVB in the beam. The proportion of UVA was measured using a radiospectrometer (Optronics Laboratories, Orlando, FL, USA) and exposure times were calculated to give the desired doses. Filtration resulted in a final UVR spectrum containing approximately 97% UVA and 3% UVB (data not shown).

Cell culture and treatment

HaCaT cells are a spontaneously transformed, nontumorigenic human keratinocyte cell line [37] and were generously provided by Dr. Mitch Denning (Loyola University Medical Center, Maywood, IL, USA). HaCaT cells were maintained in a 1:1 mixture of Dulbecco's modified Eagle's medium:F12 HAM (DMEM:F12) supplemented with 10% newborn calf serum, 4× final concentration of MEM amino acids, and antibiotics (100 U/ml penicillin and 50 μg/ml streptomycin). Cells were cultured at 37 °C in 95% air/5% CO_2 humidified incubators. HaCaT cells were typically grown to 50–70% of confluent density, rinsed in phosphate-buffered saline (PBS), and placed into serum-free medium (DMEM:F12 containing 0.1% BSA) overnight before treatments. For all experiments, cellular viability was assessed by employing the Promega CellTiter 96 aqueous nonradioactive cell proliferation assay to ensure treatments were not significantly inducing cell death (data not shown). For UVA exposures cells were placed in sterile PBS and kept on ice while exposed to the UVR and then the original medium was replaced and the cells were returned to the incubator. All cells were treated in an identical manner and conditions were consistently maintained for each treatment group. Cells were treated with arsenite, UVR, or both and collected as indicated in the figure legends. For combined arsenite and UVR treatment, cells were exposed to UVR (10 kJ/m^2) after 24 h pretreatment with arsenite (3 μM). For inhibitor experiments cells were treated as described above after 30 min pretreatment with the appropriate inhibitor. NADPH oxidase was inhibited with DPI (10 μM) or apocynin (10 μM), the electron transport chain complexes I and II were inhibited with rotenone (200 nM) and atpenin A5 (50 nM), respectively, and MnTMPyP (5 μM), a cell permeative superoxide dismutase mimic, was used to inhibit ROS.

ROS detection

Cells were cultured on glass coverslips in complete medium. When cells reached approximately 40% of confluent density, cultures were placed in serum-free medium and treated with arsenite (3 μM), UVA (10 kJ/m^2), or both for the times indicated in the figure legends. For the combined treatments, the cells were exposed to UVA after a 24-h pretreatment with arsenite. Thirty minutes before cell fixation, DHE (5 μM) was added as a fluorescent indicator of ROS generated in response to the described treatment. Coverslips were washed three times with PBS, fixed with paraformaldehyde (3.7%), and mounted on glass slides with VectaShield (Vector Laboratories, Burlingame, CA, USA). Images were collected with an Olympus IX70 fluorescence microscope fitted with an Olympus America camera and MagnaFire 2.1 software.

When relative fluorescence was being measured, cells were cultured on coverslips in 12-well plates to approximately 50% of confluent density and treated under the same conditions used for image collection. Relative fluorescence intensity was quantified by measuring the intensity of fluorescence emission using a Wallac Victor 2 fluorescence spectrophotometer equipped with 390 nm excitation and 410 nm emission filters. A minimum of three independent samples were analyzed per treatment and time point. Values were normalized to total DNA fluorescence as previously described [38]. Briefly, plates previously analyzed for ROS were rinsed with Kreb Ringer buffer (20 mM Hepes, 10 mM dextrose, 127 mM NaCl, 5.5 mM KCl, 1 mM $CaCl_2$, 2 mM $MgSO_4$, pH 7.4) and then frozen at −80 °C overnight. Plates were thawed for at least 2 h at room temperature and stained with Hoechst dye (10 μg/ml bis-benzimide) overnight, and fluorescence was determined using a Tecan plate reader equipped with 350 nm excitation and 460 nm emission filters. This method of fluorescence quantification was validated by comparison with data obtained using Metamorph software (version 6.3r6) as previously described [30]. Results were

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