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ENHANCED ROS PRODUCTION AND REDOX SIGNALING WITH COMBINED ARSENITE AND UVA EXPOSURES: CONTRIBUTIONS OF NADPH OXIDASE

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Abstract

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 μ M) then exposed to UVA (10 kJ/m²). We report that exposure to UVA following 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-, but not UVA-, induced p38 activation and HO-1 expression were dependent upon NADPH oxidase activity. These findings indicate differences in 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 exposures.

Keywords

ultraviolet radiation; reactive oxygen species; p38 MAP kinase; Heme oxygenase-1; NADPH oxidase; keratinocytes

Introduction

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 is generated by

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the reaction of UVR with endogenous photo-sensitizers, 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 (BCC) and squamous cell carcinoma (SCC) [5-7]. Arsenic enhances tumor development when combined with other carcinogens [8-10], with chronic overexpression of growth factors [11], and when combined 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 co-carcinogen 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- κ B. 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 (AsIII), 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 research demonstrated that in human keratinocytes ROS, specifically superoxide, is upstream of p38 activation by arsenite and leads 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 for 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 following arsenite exposure [36]. However, the mechanism of UVR-induced ROS production remains controversial.

Although mouse models demonstrate co-carcinogenic activity with arsenite and UVR [12, 13], the underlying mechanisms remain as yet 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 following 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, dimethylsulfoxide (DMSO), nitroblue tetrazolium (NBT), dihydroethidium (DHE), diphenyliodonium (DPI), lucigenin, tiron, diethyldithiocarbamate, NADPH, apocynin, rotenone and sodium arsenite were purchased from Sigma (St. Louis, MO). Newborn calf serum was acquired from Life Technologies (Gaithersburg, MD). Atpenin and MnTMPyP were purchased from CalBiochem (San Diego, CA) and were dissolved in DMSO. Phospho-specific 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, Inc. (Beverly, MA). HO-1, p22phox, p67^{phox}, and β -tubulin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Phospho-specific phospho-serine antibodies were purchased from Abcam, Inc. (Cambridge, MA). Peroxidase-conjugated anti-rabbit-IgG and anti-mouse-IgG were purchased from Promega (Madison, WI). SuperSignal chemiluminescent detection system was purchased from Pierce (Rockford, IL). 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 Watt Solar Ultraviolet Simulator (Oriel Corp., Stratford, CT). This solar simulator produces a high intensity UVR beam in both the UVA (320-400 nm) and UVB (280-320nm) 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, Inc.; Orlando, FL) and exposure times 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, non-tumorigenic human keratinocyte cell line [37], and were generously provided by Dr. Mitch Denning (Loyola University Medical Center, Maywood, IL). HaCaT cells were maintained in a 1:1 mixture of Dulbecco's Modified Eagle's Medium F:12 HAM (DMEM:F12) supplemented with 10% newborn calf serum, 4X final concentration of MEM amino acids, and antibiotics (penicillin, 100U/ml and streptomycin, 50 μ g/ml). Cells were cultured at 37°C in 95% air / 5% CO₂ 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% bovine serum albumin (BSA)) overnight prior to treatments. For all experiments, cellular viability was assessed by employing the Promega CellTiter 96 Aqueous Non-Radioactive 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 then original media was replaced and cells 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/m²) following 24h pretreatment with arsenite (3 μ M). For inhibitor experiments cells were treated as described above following 30 min. pretreatment with the appropriate inhibitor. NADPH oxidase was inhibited with DPI (10 μ M) or apocynin (10 μ M), the electron transport chain complexes I and II with Rotenone (200 nM) and Atpenin A5 (50 nM), respectively and MnTMPyP (5 μ M) a cell permeable 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 media and treated with arsenite (3 μ M), UVA (10 kJ/m²) or both for the times indicated in the figure legends. For the combined treatments, the cells were exposed to UVA following a 24h pretreatment with arsenite. Thirty minutes prior to cell fixation, DHE (5 μ M) 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 to glass slides with Vecta-Shield (Vector Labs. Inc., Burlingame, CA). Images were collected with an Olympus IX70 fluorescence microscope fitted with an Olympus America camera and MagnaFire 2.1 software.

When measuring relative fluorescence, 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 3 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 Krebs Ringer buffer (20mM HEPES, 10mM dextrose, 127mM NaCl, 5.5mM KCl, 1mM CaCl₂, 2mM MgSO₄, pH 7.4) then frozen at -80°C overnight. Plates were thawed for at least 2h at room temperature, stained with Hoechst dye (10 μ g/ml bis Benzimide) overnight and fluorescence determined using a Tecan plate reader equipped with 350nm excitation and 460nm 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 graphed using Microsoft Excel 2003 and statistical significance determined using GraphPad QuickCalcs.

Immunoblot Analyses

Control and treated cells were washed with ice cold PBS and harvested in lysis buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 50 mM dithiothreitol and 0.1% w/v bromophenol blue). Cell lysates were clarified (10,000 x g, 4°C, 10 minutes), and 10-30 μ g of total cell lysate was resolved by electrophoresis through 8-12% SDS-polyacrylamide gel. Proteins were transferred to nitrocellulose membranes (Millipore Corp., Bedford, MA) and probed with the indicated antibodies according to the vendor's instructions [26,39]. The membranes were developed using the SuperSignal chemiluminescent detection system (Pierce, Rockford, IL). Quantification of immunoblot results was performed using a Digital Science Image Station on a Kodak 440CF Imager with ID Image Analysis software. A minimum of 3 independent samples were analyzed per treatment and time point. Student's *t*-test was used to determine statistical significance of data obtained from densitometric analysis.

NADPH Oxidase Activity

HaCaT cells were placed in serum-free medium overnight and treated as described for ROS experiments. NADPH oxidase activity was detected using the Lucigenin Illumination method. Briefly, following treatment and incubation time cells were rinsed thoroughly with PBS, removed by scraping, resuspended in 500 μ l PBS and placed in eppendorf tubes. Cells were frozen at -80°C overnight to lyse cells. Samples were aliquoted (100 μ l) in quadruplicate, placed in luminometer tubes and incubated with diethyldithiocarbamate (DTC, 1M) for 20 min at 37°C to inhibit superoxide dismutase activity. Immediately before measuring, 1 μ l lucigenin (0.5 mM) was added to the sample followed by 1 μ l NADPH (10mM) and mixed. Luminiscence was measured in a TD20/20 Luminometer (Turner Designs, Sunnyvale, CA) with ten 30 sec.

counts. Sample values were integrated and average units calculated. Parallel samples were analyzed with the addition of Tiron (1M) to confirm measurement of NADPH oxidase activity.

Results

Activation of MAP kinases by arsenite and UVA

Both arsenite and UVR activate MAPK cascades [4,26,28,30,41,42] however, the effect of the combination has not been investigated. HaCaT cells were pretreated with 3 μ M arsenite for 24 hours, a concentration shown previously to elicit sub-maximal MAP kinase activity [26], then exposed to submaximal UVA (10 kJ/m²) and incubated for an additional 4 hours. Activation of the EGF receptor and MAP kinases was detected by immunoblot analysis. As expected, UVA or arsenite alone activated the EGF receptor, p38 and ERK (Fig. 1). Arsenite pretreatment followed by UVA exposure led to activation of ERK (1.9 fold) and EGF receptor (2.5 fold) which were not appreciable higher than levels observed with arsenite or UVA alone. However, activation of p38 was increased by 1.37 fold with UVA alone, 1.81 fold with arsenite alone while the combined treatment led to a 3.6 fold increase in activation (Fig. 1A&B). These data demonstrate that arsenite and UVA both stimulate MAP kinase signaling and that arsenite in combination with UVA further activates p38 suggesting that the impact of combined exposures exceeds that of single agents.

ROS production and HO-1 expression in response to arsenite and UVA

Several researchers have shown that UVR and arsenite induce production of intracellular ROS [4,17,43] and we have shown that ROS is upstream of p38 activation [30]. However, little is known about the action of combined arsenite and UVR on ROS production. Dihydroethidium (DHE) is a molecular probe specific to superoxide that stains the nucleus a bright fluorescent red when oxidized by intracellular superoxide. DHE was used to detect ROS production in order to more specifically investigate the contributions of superoxide as this species has been identified as the “parent species” of intracellular ROS [44]. Immunofluorescence quantification showed arsenite (3 mM) and UVA (10 kJ/m²) increased the production of ROS (2.43 and 1.78 fold, respectively), and the response is further increased (5.34 fold) when cells are treated with arsenite 24h prior to the UVA challenge (Fig. 2A&B). A dose dependent increase in the UVA-stimulated ROS production was detected in both UVR alone and arsenite pretreated samples at 4h post exposure (data not shown). ROS generation by both arsenite and (3 μ M) and UVA (10 kJ/m²) is maximal approximately 4h after UVA exposure and is maintained up to 24 h (Fig. 2C). ROS production was significantly elevated in the arsenite and UVA co-treated samples at each time point and maximal ROS production was evident at 2h after exposure (Fig. 2C). The cell permeable superoxide dismutase mimic, MnTMPyP, was employed to confirm that observed and measured fluorescence was indeed from superoxide. In all treatments, ROS was decreased to near control levels when MnTMPyP was included (Fig. 2A).

A sensitive indicator of arsenite exposure is heme oxygenase-1 (HO-1), a redox sensitive gene [19,45-47] We have shown previously that induction of HO-1 by arsenite is dependent on ROS and p38 activity. Inhibition of p38 activation or ROS production inhibited arsenite activation of p38 and subsequent induction of HO-1 [30]. HO-1 is upregulated in response to arsenite (Fig. 3) and HO-1 is significantly elevated with the combined exposure (Fig. 3A). Densitometry shows that HO-1 was increased by 1.16 fold with UVA, 2.15 fold with arsenite and combined treatments resulted in a 4.15 fold increase (Fig. 3B). These results suggest that the increased MAPK signaling observed following combined UVA and arsenite exposure leads to significant changes in downstream HO-1 expression.

Mechanisms of ROS production by arsenite and UVA

Because arsenite and UVR induce ROS production (Fig. 2) and ROS is upstream of p38 and HO-1 induction [30], we investigated mechanisms of ROS production by arsenite and UVR. There are several potential sources for the production of ROS, with NADPH oxidase and mitochondria being prime candidates [31-33,35,36]. Arsenite and UVR have been reported to induce or increase phosphorylation of the subunits of NADPH oxidase [34,35], thereby increasing the overall activity of the enzyme and ROS production [34,35,48-51]. We assessed NADPH oxidase activity following arsenite and UVA exposure and found that both agents increase activity of the enzyme, but with different kinetics (Fig. 4A). UVA exposure resulted in rapid and transient NADPH oxidase activation whereas the response to arsenite was slightly delayed, but more pronounced and persistent. Immunoblotting showed that arsenite, but not UVA, exposure led to upregulation of the p22^{phox} subunit while both treatments increased phosphorylation of the p67^{phox} subunit of NADPH oxidase (Fig. 4B&C). Inhibiting NADPH oxidase activity with diphenylene iodonium (DPI) or apocynin significantly decreased enzyme activity for both arsenite and UVA (data not shown). However, inhibition of NADPH oxidase only affected ROS production in cells treated with arsenite (Fig. 5A). Since NADPH oxidase is not the only potential source of ROS, the mitochondrial contribution to increased ROS was also investigated. Rotenone and Atpenin A5 were concomitantly added to inhibit complexes I and II of the electron transport chain, respectively. This addition did not affect cellular viability during the short exposures employed (data not shown). Mitochondrial inhibition resulted in significant decreases in UVA- induced ROS at all time points. Arsenite-induced ROS was also reduced, but was not significantly affected at the 30 min. time point (Fig. 5B). These results show that both arsenite and UVA induce ROS and are sensitive to mitochondrial inhibition.

Inhibition of NADPH oxidase decreased the arsenite-induced activation of p38 MAP kinase alone as did arsenite in combination with UVA (Fig. 6A). HO-1 induction by arsenite was abolished with the addition of the NADPH oxidase inhibitor apocynin (Fig. 6B). In contrast, UVA induction of HO-1 was not disrupted by apocynin. With combined exposures, the response was partially inhibited by apocynin, likely reflecting inhibition of arsenite-dependent ROS production (Fig. 6B). These results highlight that there are differences in the mechanisms underlying ROS production by arsenite and UVA and may provide some explanation for enhanced responses observed with combined exposures.

Discussion

Skin cancer accounts for approximately 40% of all newly diagnosed cancers each year [52] and nonmelanoma tumors represent greater than 95% of these cases. Experimental and epidemiological evidence implicates solar UVR as the most important etiological factor in development of skin tumors [5,53-55]. Because the skin is exposed to many environmental carcinogens, for example polycyclic aromatic hydrocarbons and various metals, interactions between UVR and other carcinogens in the genesis of skin cancer are receiving increasing attention [5]. Of particular interest are interactions between UVR and arsenic, a known skin carcinogen. UVR-induced skin carcinogenesis is greatly increased in mice that receive arsenic in drinking water with i) nearly 5-fold increase in tumor number per mouse, ii) accelerated time to tumor development, and iii) increase in tumor size and invasiveness [12,13]. The observed decrease in tumor yield with the addition of either vitamin E or an organoselenium compound (*p*-XSC) to food suggests a role for reactive oxygen species (ROS) in the observed cooperation between arsenite and UVR in the development of skin cancer [14].

Reactive oxygen species are implicated in carcinogenesis through epigenetic mechanisms (i.e. modulation of signal transduction pathways and gene expression) and genetic damage. UVR in sunlight, particularly at the UVA waveband, is able to produce a variety of ROS in the skin [4] and increasing evidence indicates that exposure to arsenic results in the generation of ROS,

including superoxide [17,26,30,44]. In this study we demonstrate that arsenite and UVA in combination generate more ROS than either treatment alone (Fig. 2A-C). Interestingly, we find differences in the mechanisms leading to ROS generation by arsenic or UVA in keratinocytes. NADPH oxidase is an important enzyme for ROS generation by arsenite [35,36,56] and UVR [48-51,57]. We find that both arsenite and UVA increase NADPH oxidase activity (Fig. 4A) and induce phosphorylation of p67^{phox} (Fig. 4B&C); however, only arsenite increased expression of p22^{phox} (Fig. 4B) and caused extended NADPH oxidase activation (Fig. 4A). Mitochondria represent another source of intracellular ROS and selective inhibitors of NADPH oxidase or the mitochondrial respiratory chain revealed different relative contributions of these two mechanisms to arsenite or UVA induced ROS production. Arsenite-induced ROS was sensitive to NADPH oxidase inhibition by apocynin at all time points (Fig. 5A) while UVA-induced ROS displayed greater dependence on mitochondrial contribution throughout the time course examined (Fig. 5B). Recently Valencia and Kochevar reported that NADPH oxidase is the primary contributor to UVA-induced ROS [57]; however their studies detected hydrogen peroxide using the indicator DCFDA. We detected ROS using the indicator DHE (selective for O₂^{•-}) and when we used DCFDA (more H₂O₂ and [•]OH specific) we obtained results comparable to those of Valencia and Kochevar [57, data not shown]. These findings highlight that ROS consist of several unique and distinctive species, including [•]OH, O₂^{•-}, NO, ¹O₂, H₂O₂, and ONOO⁻ [44] and these specific species differ in reactivity, kinetics and sources so studies that focus on specific reactive oxygen species, not “ROS” in general may reveal important distinctions.

Both UVR and arsenite stimulate numerous signal transduction cascades including MAP kinases [23-26,29,58,59]. ROS are signaling molecules and we reported previously that arsenite-induced ROS is upstream of and required for p38 activation [30]. In this study we find that combined arsenite and UVA exposures selectively increase p38 activation compared to either agent alone (Fig. 1). Similarly, expression of a redox sensitive gene (HO-1) was substantially increased by the combined exposure relative to arsenite or UVA alone (Fig. 3). NADPH oxidase is an upstream activator of p38 [31,32]. Both p38 activation (Fig. 1) and HO-1 expression (Fig. 3) were stimulated by arsenite, but not UVA, and were highly sensitive to NADPH oxidase inhibition (Fig. 6 A&B). This suggests that the persistent NADPH oxidase activation by arsenite is an important contributor to arsenite-stimulated ROS production, p38 activation and HO-1 induction.

The observed elevation of ROS and ROS-dependent signaling observed with arsenite and UVA co-exposure may represent a mechanism underlying the synergistic interactions of these agents in producing skin cancer in mouse models [7,10,11]. *In vivo* data shows increased oxidative DNA damage with combined arsenite and UVR exposures [14] which is likely the result of the convergence of stimuli inducing persistently elevated ROS in the skin. The increased ROS also stimulates signaling cascades leading to expression of tumor relevant genes may further contribute to tumor promotion and progression. Since arsenite appears to be a potent co-carcinogen it is imperative that the mechanisms leading to synergistic interactions be thoroughly investigated.

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List of 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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Fig. 1.

Stimulation of MAP kinase signaling by arsenite and UVA. HaCaT cells were treated with 3 μ M arsenite (As) or 10kJ/m² UVA. Dually treated samples were pre-incubated with 3 μ M arsenite (As) for 24 hrs and then exposed to UVA. EGF was used as a positive control for MAP kinase activation. Total protein was collected 2 hrs post exposure and 20 μ g resolved via a 8-12% SDS-PAGE gradient gel and the indicated proteins were analyzed, transferred to nitrocellulose membrane, immunoreacted with the appropriate antibodies and detected by ECL (A) Blots shown are representative of at least 3 independent experiments. Densitometric analysis of immunoblotting was performed (B). Blots were quantified with a Digital Science Image Station on a Kodak 440CF Imager equipped with ID Image Analysis software. Each bar is the mean of at least 3 independent experiments and the error bars indicate $\pm$ S.D. * = significantly different from untreated controls (NT), $p < 0.05$; # = significantly different from singly treated samples, $p < 0.05$.

Fig. 2.

Arsenite and UVA induce cellular ROS. HaCaT cells grown on glass coverslips to 50% confluence were treated with 3 μ M arsenite (As), 10kJ/m² UVA (UVA) or left untreated (NT). Dually treated samples were pre-incubated with 3 μ M arsenite (As) for 24 hrs and then exposed to UVA. A superoxide-specific dye (5 μ M DHE) was added 30 min. prior to fixation. Samples were rinsed with PBS, fixed using 3.7% para-formaldehyde, mounted on glass slide and observed with fluorescence microscopy 4 hours post UV exposure. Lower panels were treated as described above with the addition of the cell permeable SOD mimic MnTMPyP (sod, 5 μ M) 30 min. prior to exposures to demonstrate fluorescence is derived from superoxide (A). ROS quantification was carried out as described in "Experimental Procedures." Each image is

representative of multiple observations, and error bars indicate $\pm$ S.D. (B). ROS generation was monitored over time and quantified as described in “Experimental Procedures” for untreated or dark (◆), arsenite (■), UVA (▲) and dually treated (●) samples (C). All p values were <0.05 versus the untreated control. * = significantly different from untreated controls (NT), $p<0.05$; # = significantly different from singly treated samples, $p<0.05$. DHE, dihydroethidium.

Fig. 3.

Arsenite and UVA cooperate in HO-1 upregulation. HaCaT cells were incubated with 3 μ M arsenite (As), 10kJ/m² UVA (UVA) or left untreated (NT). Dually treated samples were pre-incubated with 3 μ M arsenite (As) for 24 hrs and then exposed to UVA. Total protein was collected 4 hrs post exposure and 60 μ g resolved via a 12% SDS-PAGE gel. HO-1 was analyzed using anti-HO-1 antibodies and detected by ECL. Equal protein loading was confirmed by reprobing the original membrane with β -Tubulin (A). Blots shown are representative of at least 3 independent experiments. Densitometric analysis of immunoblotting was performed (B). Each bar is the mean of at least 3 independent experiments and the error bars indicate $\pm$ S.D. * = significantly different from untreated controls (NT), $p < 0.05$; # = significantly different from singly treated samples, $p < 0.05$.

Fig. 4. Arsenite and UVA activate NADPH oxidase. HaCaT cells were incubated with 3 μ M arsenite (■) or 10kJ/m² UVA (▲) for the indicated times. NADPH oxidase activity was assessed over time as described in “Experimental Procedures” (A). Total protein was collected at the indicated times and 20 μ g resolved via a 8-12% SDS-PAGE gradient gel and the indicated proteins were analyzed, transferred to nitrocellulose membrane, immunoreacted with the appropriate antibodies and detected by ECL. Panel (B) shows results following arsenite treatment and UVA results are shown in (C). Equal protein loading was confirmed by reprobing original membranes with β -tubulin. Each point and blot is representative of at least 3 independent experiments and error bars indicate $\pm$ S.D. All p values were <0.05 versus the untreated control. * = significantly different from untreated controls.

Fig. 5. Contribution of NADPH oxidase and the mitochondria to arsenite- and UVA- induced ROS. HaCaT cells were treated with 3 μ M arsenite (■), 10kJ/m² UVA (▲). DHE was added 30 min. prior to fixation. Samples were rinsed with PBS, fixed using 3.7% para-formaldehyde, mounted on glass slide and observed with fluorescence microscopy. ROS quantification was carried out as described in “Experimental Procedures.” Samples were incubated with apocynin (10 μ M) (arsenite + inhibitor (□) or UVA + inhibitor (Δ) 30 min prior to any other treatment for inhibition of NADPH oxidase (A) and rotenone and atpenin A5 (arsenite + inhibitors (□) or UVA + inhibitors (Δ) were added for mitochondrial inhibition (B). Each point is representative of multiple observations, and error bars indicate $\pm$ S.D. (A) # = significantly different from uninhibited treatment matched samples, $p < 0.05$. (B) * = significantly different from untreated control; # = significantly different from arsenite or UVA treatment. Comparable results were obtained when employing DPI to inhibit NADPH oxidase. Apocy. = apocynin; DPI = diphenyliodonium

Fig. 6. NADPH oxidase activity is required for arsenite-induced signaling and HO-1 upregulation. HaCaT cells were treated with 3 μ M arsenite (As) or 10kJ/m² UVA (UV). Dually treated samples were incubated with 3 μ M arsenite for 24 hrs and then exposed to UVA (As+UVA). Samples were incubated with apocynin (10 μ M) 30 min prior to any other treatment for inhibition of NADPH oxidase. Total protein was collected 4 hrs post exposure and 20 μ g resolved via 8-12% SDS-PAGE gradient gel and the indicated proteins were analyzed, transferred to nitrocellulose membrane, immunoreacted with the appropriate antibodies and detected by ECL. The effect of NADPH oxidase inhibition on activation of p38 (A). HO-1 upregulation with and without NADPH oxidase inhibition (B). Blots shown are representative of at least 3 independent experiments. Equal protein loading was confirmed by reprobing original membranes with total p38 or β -tubulin as indicated on the figures. Blots were quantified with a Digital Science Image Station on a Kodak 440CF Imager equipped with ID Image Analysis software. Each bar is the mean of at least 3 independent experiments and the error bars indicate $\pm$ S.D. * = significantly different from untreated control (NT); # = significantly different from treatment without inhibitor p<0.05. Comparable results were

obtained when employing DPI to inhibit NADPH oxidase. Apocy. = apocynin; DPI = diphenyliodinium