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As(III) inhibits ultraviolet radiation-induced cyclobutane pyrimidine dimers repair via generation of nitric oxide in human keratinocytes

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Abstract

Inorganic arsenic enhances skin tumor formation when combined with other carcinogens including ultraviolet radiation (UVR). The inhibition of DNA damage repair by arsenic has been hypothesized to contribute to the co-carcinogenic activities of arsenic observed *in vivo*. Cyclobutane pyrimidine dimers (CPDs) are an important mutagenic UVR photoproduct and implicated in the genesis of non-melanoma skin cancer. The current study demonstrates that low concentrations of arsenite (As(III)) inhibit UVR-induced CPDs repair in a human keratinocyte cell line via nitric oxide (NO) and inducible nitric oxide synthase (iNOS). Following As(III) treatment, NO production and iNOS expression are elevated. Little is known about regulation of iNOS by As(III) and further investigations indicated that p38 mitogen-activated protein kinase (p38 MAPK) and NF-κB are required for As(III) induction of iNOS expression. This As(III)-stimulated signaling cascade was involved in inhibition of UVR-induced CPDs repair as disruption of p38 MAPK activity and NF-κB nuclear translocation counteracted the effects of As(III) on CPD repair. Selective inhibition of iNOS ameliorated As(III) inhibition of CPDs repair thereby suggesting that iNOS is a downstream mediator of As(III) activity. These findings provide evidence that an As(III) stimulated signal transduction cascade culminating in elevated iNOS expression and NO generation is an underlying mechanism for inhibition of UVR-induced DNA damage repair by arsenic.

Keywords

arsenite; arsenic; nitric oxide; iNOS; UVR

Introduction

Skin cancer accounts for ~40% of all newly diagnosed cancers each year and nonmelanoma tumors represent >95% of these cases (1,2). Squamous cell carcinoma can progress to metastatic spindle cell carcinoma and at this point is highly refractory to treatment (3,4). Experimental and epidemiological evidence implicates solar UVR as the most important etiological factor in development of skin tumors (2–4). UVA (320–400 nm) and UVB (280–320 nm) are the wavelengths of chief concern for human cancers. UVA exposure results in

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oxidative modifications such as hydroxylation of 2'-deoxyguanosine (8-OHdG), protein-DNA cross-linking, base loss and strand breaks (5). In contrast, DNA absorbs UVB photons leading to formation of cyclobutane pyrimidine dimers (CPD) and 6-4 photoproducts (6-4PP) (6-8). UV photoproducts, primarily CPDs, are major causes of mutations identified in *ras* oncogenes, *p53* and *PTCH* tumor suppressor genes identified in non-melanoma skin cancers (7-10). Because the skin is exposed to many environmental carcinogens, interactions between UVR and other carcinogens in the genesis of skin cancer is receiving increasing attention (11,12).

Although human epidemiological data links inorganic arsenic in drinking water and increased risk of non-melanoma skin cancer [reviewed in (13,14)], arsenic as a sole agent is not an effective skin carcinogen in animal models. However, arsenic potentiates skin cancer in transgenic mice expressing activated H-ras or ornithine decarboxylase in hair follicle keratinocytes and phorbol ester treated mice, indicating that arsenic can amplify pathways leading to skin cancer *in vivo* (15). The most striking observation is that UVR-induced skin carcinogenesis is greatly increased in mice receiving 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 (11,12). The mechanism for the dramatic enhancing effect of arsenic is unknown, but a number of potential contributing mechanisms may account for this observation. Low dose arsenic has growth promoting activity in cultured keratinocytes (16) and recent studies propose that arsenic over-rides UVR-induced growth arrest and inhibits apoptosis, fostering the outgrowth of cells harboring DNA mutations (17). In addition, it has been proposed that inhibition of DNA damage repair is an important mode of action of arsenic as a co-carcinogen *in vivo* and *in vitro* (18-20).

The major pathway eliminating DNA base damage and helix distortions is excision repair, subdivided into nucleotide excision repair (NER) and base excision repair (BER) (21). There is substantial evidence that BER and NER are inhibited at low, non-cytotoxic concentrations of carcinogenic metals Ni(II), Co(II), Cd(II) and As(III) (22). With respect to NER, As(III) impaired the incision step and ligation of repair patches after induction of UVR-induced DNA damage, affecting both global genome repair and transcription-coupled repair (23). CPDs are repaired primarily by NER and there is limited evidence indicating that As(III) interferes with repair of CPDs in Chinese hamster ovary cells (24) and TK6 human lymphoblastoid cells (25), although As(III) interference with CPDs repair was not observed in mouse keratinocytes (26). Furthermore, the mechanism by which As(III) inhibits CPDs repair is unclear. Because of evidence that As(III) induced the generation of NO in human keratinocytes (27) and in other cells types (28), we set out in the present study to determine whether As(III)-mediated NO production interferes with repair of UVR-induced CPDs formation in human keratinocytes, and to identify the As(III)-stimulated signaling pathway that leads to production of NO. We find that low concentrations of As(III) inhibit UVR-induced CPDs repair and this activity is dependent upon p38 MAPK-mediated signaling via NF- κ B leading to induction of iNOS expression. These findings suggest that generation of NO by low concentrations of As(III) is an underlying mechanism for inhibition of DNA repair that may contribute to the observed co-carcinogenicity of As(III) and UVR in skin cancer.

Materials and Methods

Cell culture and treatment

HaCat cells are a spontaneously immortalized human keratinocyte line (29) and were maintained in Dulbecco's Modified Eagle's Medium F:12 HAM (DMEM F:12), supplemented with 10% newborn calf serum from Life Technologies/Gibco, four-fold concentration of MEM Amino Acids Solution, 2 mM L-glutamine and antibiotics (penicillin, 100 U/ml and

streptomycin, 50 µg/ml). The cells were cultured at 37 °C in 95% air/5% CO₂ humidified incubators (27,30).

Stock solutions of As(III) (Sigma, St Louis, MI), the NF-κB inhibitor SN50 (Biomol, Plymouth Meeting, PA), the NO donor 3-morpholinosydnoimine (SIN-1) (Sigma, St Louis, MI) and the iNOS inhibitor aminoguanidine (AG) (Sigma, St Louis, MI) were prepared in doubled-distilled water and sterilized by passing through a 0.22 µm syringe filter. Stock solutions of the p38 MAPK inhibitor SB239063 (Sigma, St Louis, MI) and the iNOS inhibitor N-[3(aminomethyl)benzyl]acetamide (1400W) (Cayman Chemical, Ann Arbor, MI) were prepared in sterile DMSO. Working concentrations were prepared by dilution in serum free medium DME: F12 medium containing 0.1% (w/v) bovine serum albumin (BSA).

UVR exposure

Cells were pre-treated with As(III) in serum free medium for 24h at concentrations as indicated in the Figure Legends. Cell culture medium was removed, cells were rinsed three times with PBS, and then cells were covered with a thin layer of PBS and placed on ice in the dark. Cells were maintained on ice during UVR exposure. Cells were exposed to solar-simulated UVR including 50 J/m² UVB using an Oriel 300 W Solar Ultraviolet Simulator (Newport Corporation, Irvine, CA). This solar simulator produces a high intensity UVR beam with 91% UVA (320–400 nm) and 9% UVB (280–320 nm). After UVR exposure, PBS was removed and replaced with serum-free medium. Cells were returned to incubators until collection for further experimental procedures.

Measurement of CPDs by ELISA

DNA was isolated using the QIAamp Blood Kit (QIAGEN Inc., Valencia, CA). The concentration of DNA was measured by a Beckman DU 800 spectrophotometer (Beckman Instruments, Fullerton, CA). Enzyme-linked immunoabsorbent assay (ELISA) was used to determine the quantities of CPDs as described by Mori et al. (31). Briefly, Falcon polyvinylchloride flat-bottom 96-well assay plates (Becton Dickinson Labware, Franklin Lakes, NJ) pre-coated with 0.003% protamine sulfate (Sigma) were incubated with 15 ng purified genomic DNA in PBS at 37°C for 20 hr. TDM-2 (Medical & Biological Laboratories Co. Woburn, MA) was used as the antibody for CPDs detection. Subsequently, after the incubation with biotinylated F(ab')₂ goat anti-mouse IgG fragments and streptavidin-peroxidase (Zymed, San Francisco, CA), the optical density from *o*-phenylene diamine at 492 nm was measured using a Spectra Max 340 (Molecular Devices, Sunnyvale, CA).

Measurement of NO in cell culture medium

The measurement of NO release was performed with the inNO-T nitric oxide measurement system using the amino-700 sensor electrode (Innovative Instruments, Inc. Queen Brooks Courts, FL) connected to an amplifier with a digital output (InNO-T; Innovative Instruments). The sensor was calibrated by the conversion of nitrite to NO in acidic solution in the presence of iodide ion from 50 nM to 250 nM. Following calibration, the sensor was inserted into the collected cell culture medium, and the output current was monitored and recorded on a Windows compatible PC system using analysis software provided with the inNO probe.

Analysis of inducible nitric oxide synthase mRNA expression

Real-time reverse transcription (RT)-PCR was used to measure the iNOS mRNA levels. Total cellular RNA was extracted using the RNeasy minikit (QIAGEN, Valencia, CA) according to the manufacturer's protocol, with additional on-column DNase I digestion (RNase-free DNase kit, QIAGEN). The RNA concentration was determined by Nano Drop UV/Vis spectrometer (Nano Drop Technologies, Wilmington, DE).

Primer sequences were designed on the basis of Gene Bank data. The primer sequences of human iNOS were as follows: sense (5'-GAGGGGACTGGGCAGTTCTA-3') and anti-sense (5'-CCTGTGTCCTGACTGGAG-3'). Primers were obtained from Sigma (St. Louis, MO). The expected size of the final polymerase chain reaction (PCR) product was 300 bp.

Two-step RT-PCR was performed as follows. RNA (1 µg) was reverse transcribed using Taqman Reverse Transcription Reagents (Applied Biosystems, Foster City, CA), the final cDNA solution volume was 20 µL. PCR was performed using a HotStarTaq Master Mix kit (QIAGEN, Valencia, CA) in a final reaction volume of 25 µL. Each reaction contained 2 µL of the cDNA synthesis reaction as template. The conical tubes were placed in Perkins Elmer 9600 Thermal Cycler (Waltham, Massachusetts) programmed as follows: i) 95 °C for 5 min; ii) 95 °C for 1 min; iii) 48 °C for 1 min; iv) 72 °C for 1 min; and v) 72 °C for 5 min. Steps ii-iv were repeated for 37 cycles. As a control, GAPDH was also amplified (Integrated DNA Technologies, Inc, Coralville, IA).

The amplified products were analyzed on 1.5% agarose gel containing SYBR safe DNA gel stain (Invitrogen, Eugene, Oregon). Ten microliters of each reaction mixture was mixed with loading buffer, separated by electrophoresis for 30 min at 80 V and visualized by UV-Epi illumination. The approximate size was determined by using 1kb Plus DNA ladder (Invitrogen, Eugene, Oregon).

Western blot analysis of activated p38 MAPK

Immunoblot analysis of activated p38 MAPK was conducted according to Cooper et al (30). Briefly cell lysate (30 µg of protein) was resolved in a 12% SDS -polyacrylamide gel and transferred onto nitrocellulose membranes (Bio-Rad, Hercules, CA), and incubated for 1 hr in TBST (10 mM Tris, pH 8.0, 150 mM NaCl and 0.1% Tween 20) containing 5% BSA at room temperature. Membranes were then incubated overnight at 4 °C with the mouse monoclonal anti-Phospho-p38 MAPK (Thr180/Tyr182) (1:1000, Cell Signaling, Danvers, MA). After washing with TBST, membranes were incubated for 1 hr with horseradish peroxidase-linked secondary antibody (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA). In the p38 siRNA experiment, the membranes were incubated overnight at 4 °C with the mouse monoclonal anti-p38 MAPK (Thr180/Tyr182) (1:1000, Cell Signaling, Danvers, MA) and then incubated with horseradish peroxidase-linked secondary antibody (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA). To control sample loading and protein transfer, the membranes were stripped and re-probed to detect β-actin (monoclonal anti-β-actin, 1:4000, Sigma, St. Louis, MO). 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. Three or more independent samples were analyzed per treatment and time point. Paired t-test was used to determine statistical significance of data obtained from densitometric analysis.

P38 MAPK siRNA

HaCat cells were transfected with p38 MAP Kinase ShortCut siRNA Mix using TransPass R2 Transfection Reagent (New England Biolabs, Inc., Beverly, MA) according to Cooper et al (30). Briefly, p38 siRNA (25 nM) was added to serum-free medium containing transfection reagents and incubated for 20 min. HaCat cells near confluence were trypsinized, diluted and added to the well of 6-well plate or 6 cm plates containing the siRNA mix and incubated for 1 h before the addition of complete medium. Cells were incubated overnight in the transfection mixture, media were changed and cells incubated for 72 h to allow for maximum target gene inhibition. Cells were then treated and analyzed as described.

Determination of NF- κ B translocation

After the indicated treatments, HaCat cells were fixed in 100% ice-cold ethanol for 5 min and washed with PBS and then were treated with 10% goat serum in PBS for 30 min to block non-specific binding. Primary antibodies against p65 (Santa Cruz Biotechnology, Santa Cruz, CA) were diluted 1:100 in 10% goat serum, and the cells were incubated overnight with the diluted antibodies at 4°C. The secondary, FITC-conjugated anti-mouse antibody (Chemicon, Temecula, CA) (diluted 1:200 in blocking reagent) was applied and samples incubated in a humid chamber at 37°C for 1 hr in the dark. Cover glasses were mounted on micro slides in Vetashield mounting medium containing 2 μ g/ml DAPI (Vector Laboratories, Burlingame, CA) to visualize nuclei. Images were obtained using an Olympus BH2-RFCA fluorescence microscope (Melville, NY) and Omegafire digital camera with MagnaFire 2.1 software (Optronix, Goleta, CA).

Statistics

Statistical analysis of data was carried out using Student's *t*-test. Differences between means were regarded as significant if $p < 0.05$.

Results

As(III) inhibits the repair of UVR-induced CPDs in HaCat cells in a concentration-dependent manner

Since CPDs are the major stable UV photoproduct, which has been identified as a cause of mutation in non-melanoma skin cancers, it is important to determine whether As(III) inhibits CPD repair in human keratinocytes. Prior to analysis of CPD repair in a human keratinocyte line (HaCat), the linear response of CPDs generation following exposure to UVR (with UVB dosages from 25 to 200 J/m²) was established using the ELISA assay (data not shown). A solar simulated UVR exposure with 50 J/m² UVB was selected for the current study based on the assay sensitivity and rate of CPD repair in HaCat cells.

HaCat cells were pre-treated with 2 μ M As(III) for 24 hrs, exposed to UVR and CPDs content were measured at various times. The percentages of CPDs remaining normalized to initial CPDs were shown in Fig 1A. As(III) pre-treatment resulted in a significant increase in CPDs remaining and thus a decrease in CPDs repair rates following UVR exposure. Twenty-four hours after UVR exposure approximately 40% of CPDs were retained in the cells without treatment compared to nearly 60% remaining in As(III) pretreated cells (Fig. 1A). The impact of As(III) was concentration-dependent with significant differences detected at As(III) concentrations as low as 0.5 μ M (Fig. 1B). Twenty-four hours after UVR exposure approximately 55% of CPDs were retained in the cells pretreated with 0.5 μ M As(III) compared to 40% remaining in cells without As(III) treatment. These findings indicate that low concentration of As(III) inhibits UVR-induced CPDs repair and this inhibition is in a concentration-dependent manner in human keratinocytes.

As(III) induces NO production and iNOS expression in HaCat cells

We reported previously that exposure of human keratinocytes to As(III) caused the generation of 3-nitrotyrosine, a biomarker of oxidative protein damage induced by excessive NO and superoxide, in a concentration- and time-dependent manner (27). However, there is little information on As(III)-stimulated NO production at low As(III) concentration, and on the source of NO production. Using a highly sensitive electrochemical detection method, the inNO-T nitric oxide measuring systems (32), we demonstrated that treatment of HaCat cells with 2 μ M As(III) for 24 h markedly increased the NO level (Fig. 2A). To determine the source of NO production, As(III) treated HaCat cells were incubated with human iNOS specific

inhibitors, AG and 1400W (33,34). Fig. 2A shows that both AG and 1400W completely blocked the As(III)-induced generation of NO, suggesting that NO production was dependent on iNOS activity. We then performed RT-PCR analysis to further confirm that iNOS expression was indeed significantly elevated after treatment of 2 μ M As(III) (Fig. 2B). These results indicate that As(III) treatment induces NO production through induction of iNOS in HaCat cells.

Involvement of p38 MAPK and NF- κ B in As(III) induction of iNOS

Little is known about the signaling pathways leading to induction of iNOS by As(III), but p38 MAPK and NF- κ B are regulators of iNOS expression in response to other stimuli such as inflammatory stimuli, cytokines, and free radicals (35–39). We reported previously that As(III) stimulates MAPK signaling transduction cascades in HaCat cells (40) and that p38 activation is dependent on generation of reactive oxygen species by As(III) (30). Those findings lead us to speculate that p38 MAPK and NF- κ B may be involved in the iNOS induction by As(III). Our experimental results showed that extended treatment (24h) with 2 μ M As(III) led to a 2.5 fold increase in activated p38 MAPK compared to untreated cells (Fig. 3A).

The transcription factor nuclear factor- κ B (NF- κ B) is known to be a central target for activators or inhibitors of iNOS expression (41). NF- κ B is translocated from the cytoplasm to the nucleus when activated. As shown in Fig. 3B, NF- κ B was translocated from cytoplasmic to nuclear compartments after incubation with 2 μ M of As(III), indicating that NF- κ B was activated by As(III) in HaCat cells. The activation and translocation were observed by fluorescence microscopy and NF- κ B is indicated by green fluorescence. As(III) induced NF- κ B translocation and activation was blocked by both SN50, a peptide inhibitor targeted at nuclear translocation and also the activity of NF- κ B (42), and SB239063, an inhibitor of p38 catalytic activity (Fig. 3B). This result indicates that As(III) induced NF- κ B translocation is dependent upon the activation of p38 MAPK. To determine whether As(III)-mediated activation of p38 and translocation of NF- κ B is functionally involved in the expression of iNOS and NO generation, we investigated the effect of inhibiting of p38 MAPK and NF- κ B on iNOS expression and NO production. Both SB239063 and SN50 blocked As(III)-induced iNOS expression (Fig. 3C) and NO production (Fig. 3D) in HaCat cells. Based on these results, we can conclude that As(III) induces expression of iNOS through the activation of p38 MAPK that leads to the subsequent activation of the transcription factor NF- κ B in HaCat cells.

As(III)-stimulated signaling and inhibition of CPDs repair

Based on the above results, we reasoned that if As(III)-dependent induction of iNOS is involved in inhibition of CPDs repair, then inhibition of the signaling cascade leading to iNOS expression should interfere with the As(III) response. As shown in Figure 4A, treatment of cells with 2 μ M As(III) together with the p38 inhibitor SB239063 abolished the As(III) inhibition of CPDs repair. These results were further confirmed following knock-down of p38 using siRNA. The western blot results shown in Fig. 4B demonstrate that p38 siRNA decreased protein levels and p38 MAPK knockdown by siRNA had similar effect as SB239063 on CPDs repair (Fig. 4C). This is the first demonstration of the involvement of p38 MAPK in As(III) inhibition of UVR-induced CPDs repair.

The observation that p38 MAPK inhibition disrupted As(III) induced activation of NF- κ B (Fig 3B) suggests that NF- κ B is a downstream target of As(III) activated p38 MAPK. As shown in Figure 5, treating cells with 2 μ M As(III) together with the NF- κ B inhibitor SN50, blocked the As(III) inhibition of UVR-induced CPDs repair, which is the first demonstration that NF- κ B is involved in As(III) inhibition of CPDs repair. These data provide important evidence that signaling through p38 MAPK and NF- κ B is an important pathway in the observed As(III) inhibition of UVR-induced CPDs repair.

As(III) induced NO generation as a mechanism for inhibition of CPDs repair

To test whether As(III) generated NO is involved in CPD repair inhibition, cells were pre-incubated with 2 μ M As(III) for 24 hrs with or without the selective iNOS inhibitors AG (300 μ M) and 1400 W (1 μ M). The NO donor 3-morpholinylsydnimine (SIN-1) was used as a positive control and its NO generation capability under our experimental conditions was verified by inNO-T probe measurements (data not shown). The CPDs repairs were measured 4 hrs after UVR exposure. Inclusion of the NO donor SIN-1 decreased UVR-induced CPD repair in the absence of As(III) indicating that elevated NO can inhibit this DNA repair process (Fig. 6). Co-treatment with As(III) and SIN-1 did not further impair CPDs repair. Treating cells with iNOS inhibitors AG and 1400W protected cells from As(III) inhibition of CPDs repair (Fig 6), suggesting that As(III) induced generation of NO through the activation of iNOS, provides a mechanistic basis for the inhibition of UVR-induced CPDs repair.

Discussion

In vivo evidence indicates that inorganic arsenic augments the carcinogenicity of UVR in the induction of mouse skin cancer (11,12), but the molecular mechanisms are not fully understood. In this study we find that As(III) inhibits repair of UVR-induced CPDs through iNOS-mediated NO generation. CPDs represent the most important DNA damage product induced by UVR-exposure and are implicated in mutations identified in *ras* oncogenes, *p53* and *PTCH* tumor suppressor genes identified in non-melanoma skin cancers (7–10). To date, there are very few studies investigating the effect of As(III) on UVR-induced CPDs repair. Danaee et al reported that pre-treating TK6 human lymphoblastoid cells with 1 μ M As(III) inhibited the repair of UVR-induced pyrimidine dimer-related DNA damage (25). In contrast, Wu et al reported that the repair of UVR induced CPDs was not inhibited by pre-incubating mouse keratinocyte cells with 2.5 and 5 μ M As(III) for 24 hrs (26). These conflicting results may be due to the specific experimental conditions used in the study, such as cell type and UVR doses. Our results in the present study provide convincing evidence that As(III) can interfere with CPDs repair even at the environmentally relevant concentration of 0.5 μ M. More importantly, our findings indicate that As(III)-induced NO generation through the activation of iNOS played an important role in the As(III) inhibition of UVR-induced CPDs repair.

NO production in response to arsenic has been reported to be increased, decreased or unchanged, depending on the cell-type, the species of arsenical compound, arsenic dose, as well as the detection methods (28,43). Using a highly sensitive electrochemical detection method, we found that under the conditions of this study that NO production was significantly elevated in HaCat cells following treatment with 2 μ M As(III) for 24 hrs (Fig. 2A). This concurs with previous results from our lab demonstrating generation of 3-nitrotyrosine, a product of peroxynitrite and an indirect indicator of NO generation in HaCat cells (27). Blocking iNOS activity with specific inhibitors not only abolished the increase in NO production (Fig. 2A), but also counteracted As(III) inhibition of UVR-induced CPDs repair (Fig 6), thus linking the As(III)-induced NO generation with As(III) inhibition of CPDs repair. It is not clear at this time how NO would interfere with the CPD repair, but there is ample evidence for NO-mediated inhibition of DNA repair enzymes including Endonuclease III (44), O⁶-Alkylguanine -DNA alkyltransferase (AGT) (45) and a number of DNA repair proteins containing zinc finger motifs. For example, an NO donor caused irreversible damage to the zinc finger-containing DNA repair enzyme formamidopyrimidine -DNA glycolase (46) and the poly (ADP-ribose) polymerase (PARP) family of nuclear enzymes, which play an important role in maintaining genome stability, are also inhibited by NO (47). In view of the fact that environmentally relevant concentration of 0.5 μ M is sufficient to interfere with CPD repair, it seems quite feasible that generation of NO by As(III) represents a potential underlying mechanism for the observed inhibition of DNA repair processes (46–48).

The p38 MAP kinases are serine/threonine protein kinases that play important roles in cellular responses to external stress signals (49,50). We reported previously that ROS generation by As(III) was an important upstream signal for p38 activation (40). In this study we find that As(III) activation of p38 MAPK leads to activation of NF- κ B (Fig 3A, 3B) and p38 MAPK and NF- κ B are involved in the regulation of iNOS expression in response to As(III) (Fig 3C). Furthermore, p38 MAPK kinase inhibitors, p38 MAPK siRNA, and an NF- κ B inhibitor have a protective effect on As(III) inhibition of UVR-induced CPDs repair (Fig 4A, 4C,5) providing further evidence for the role of this signaling cascade in the response. There are reports that both p38 MAPK and NF- κ B regulate iNOS gene expression after LPS stimulation (41,51), but this is the first demonstration of a relationship between As(III), p38 MAPK and NF- κ B and iNOS expression.

Chronic elevation of NO above normal physiological levels may play a role in initiation, promotion, and progression of some arsenic-related human cancers (28,52). Inhibition of DNA repair processes is one potential mechanism by which As(III) contributes to carcinogenesis. Further investigations into the signaling cascades and molecular processes required for inhibition of DNA damage repair by As(III) should lead to a greater understanding of the carcinogenic activities of arsenic.

In summary, the results from the present study demonstrate that treating HaCat cells with low concentration As(III) activates p38 MAPK, which subsequently directly or indirectly activates the transcription factor NF- κ B. The activation of the transcription factor NF- κ B induced the expression of iNOS. The product of iNOS, NO, is involved in the inhibition of UVR-induced CPDs repair. The process revealed in this study is likely an underlying mechanism for As(III) inhibition of DNA repair that may contribute to the observed co-carcinogenicity of arsenic and UVR in skin cancer.

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Abbreviation

As(III)	arsenite
UVR	ultraviolet radiation
CPDs	cyclobutane pyrimidine dimers
NO	nitric oxide
iNOS	inducible nitric oxide synthase
p38 MAPK	p38 mitogen-activated protein kinase
NF-κB	nuclear factor-kappaB

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A

B

Figure 1.

As(III) inhibits UV-induced CPDs repair in a concentration-dependent manner. (A) HaCaT cells were pretreated with or without 2 μ M As(III) for 24 hrs, rinsed once with PBS and covered with PBS. Cells were then exposed to solar UVR on ice. After UVR exposure, PBS was removed and cells were placed in tissue culture medium. Cells were returned to the tissue culture incubator for the indicated times. CPDs were measured using an ELISA assay. (B) HaCaT cells were pretreated with 0.0, 0.5, 2 and 5 μ M As(III) for 24 hours and exposed to UVR as described in (A). Values are expressed as a percentage of the total CPDs detected immediately after UVR exposure. The results represent the mean and standard deviation of three independent experiments. * p<0.05 comparing UVR+As to UVR alone.

A **B** **Figure 2.**

As(III) induces NO generation and iNOS expression in HaCat cells. (A) Effect of As(III) on NO generation. HaCat cells were pre-incubated 2 μ M As (III) with or without the iNOS inhibitors AG (300 μ M) or 1400W (1 μ M) for 24 hrs. The concentrations of the iNOS inhibitors were chosen based on their reported IC₅₀ values. The culture medium was collected 24 hrs after As(III) treatment and the NO levels were measured using electrochemical detection as described in Materials and Methods. The results are expressed as NO concentration [nM]/protein (μ g). Results represent the mean and standard deviation of three independent experiments. * $p < 0.05$ compared to untreated control. # $p < 0.05$ compared to cells treated with 2 μ M As(III). (B) Effect of As(III) on iNOS expression. HaCat cells were treated without or

with 2 μ M As(III) for 24 hrs and RT-PCR analysis was performed as described in Materials and Method. Equal loading of RNA was confirmed by measurement of GAPDH mRNA levels. The lower panel presents the mean and standard deviation of results obtained from three independent experiments. * $p < 0.05$ compared to untreated negative control.

C

D

Figure 3.

As(III) activates iNOS expression through the activation of p38 MAPK and NF- κ B. (A) HaCat cells were treated with 2 μ M As(III) for 24 hrs. The active, phosphorylated form of p38 MAPK was detected by western blot. Blots were reprobed with β -actin to confirm equal protein levels in each sample. Representative results are shown in the left panel. The right panel presents the results obtained from three independent experiments. * $p < 0.05$ compared to untreated control. (B) HaCat cells were treated with 2 μ M As(III) in the absence or presence of the p38 MAPK inhibitor SB239063 (10 μ M) or the NF- κ B translocation inhibitor SN50 (40 μ g/ml) for 24 hrs. The localization of NF- κ B is shown by green fluorescence; nuclei were stained with DAPI (blue). (C) iNOS expression in HaCat cells that were treated without or with 2 μ M As(III) and

with or without 10 μ M SB239063 or 40 μ g/ml SN50. RNA was collected and RT-PCR analysis performed. Equal loading of RNA was confirmed by measurement of GAPDH mRNA levels. Representative results are shown in the left panel. The right panel represents the mean and standard deviation of results obtained from three independent experiments. * $p < 0.05$ compared to untreated negative control. (D) NO production in HaCat cells that were treated without or with 2 μ M As(III) and with or without 10 μ M SB239063 or 40 μ g/ml SN50. The conditions were the same as in Fig. 2A. * $p < 0.05$ compared to untreated control. # $p < 0.05$ compared to cells treated with 2 μ M As(III).

A **B**

C

Figure 4.

As(III) inhibition of CPDs repair requires p38 MAPK. (A) HaCat cells were pre-incubated with 2 μM As (III) with or without the P38 MAPK inhibitor SB239063 (10 μM) for 24 hrs. Cells were exposed to solar UVR and CPDs measured as described in the legend of Figure 1. (B) HaCat cells were treated with p38 MAPK siRNA as described in Materials and Methods. The protein levels of p38 MAPK were detected by western blot analysis and equal protein loading was confirmed by detection of β-actin in each sample. (C) HaCat cells treated with or without p38 MAPK siRNA were pre-incubated with 2 μM As (III) for 24 hrs. Cells were exposed to solar UVR and CPDs were measured. The results shown represent the mean and standard deviation of at least three independent experiments. * $p < 0.05$ compared to UVR exposed cells treated with 2 μM As(III).

Figure 5.

Involvement of NF- κ B in As(III) inhibition of UVR-induced CPDs repair. HaCat cells were pre-incubated with 2 μ M As (III) with or without the NF- κ B translocation inhibitor SN50 (40 μ g/ml) for 24 hrs. Cells were exposed to solar UVR and CPDs measured as described in the legend to Figure 1. The results shown represent the mean and standard deviation of at least three independent experiments. * $p < 0.05$ compared to UVR exposed cells treated with 2 μ M As(III).

Figure 6.

Effect of selective iNOS inhibitors on UVR-induced CPDs repair. HaCat cells were pre-incubated with 2 μM As (III) with or without the iNOS inhibitors 300 μM AG and 1 μM 1400W for 24 hrs. The NO donor SIN-1 was used as a positive control for the impact of NO on CPDs repair. Cells were exposed to solar UVR and CPDs measured as described in the legend to Figure 1. The results shown represent the mean and standard deviation of at least three independent experiments. * p<0.05 compared cells exposed to UVR alone. # p<0.05 compared to cells exposed to UVR after pre-incubating with 2 μM As(III).