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# Cyclooxygenase-2 (COX-2) Mediates Arsenite Inhibition of UVB-Induced Cellular Apoptosis in Mouse Epidermal Cl41 Cells

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## Abstract

Inorganic arsenic is an environmental human carcinogen, and has been shown to act as a co-carcinogen with solar ultraviolet (UV) radiation in mouse skin tumor induction even at low concentrations. However, the precise mechanism of its co-carcinogenic action is largely unknown. Apoptosis plays an essential role as a protective mechanism against neoplastic development in the organism by eliminating genetically damaged cells. Thus, suppression of apoptosis is thought to contribute to carcinogenesis. It is known that cyclooxygenase-2 (COX-2) can promote carcinogenesis by inhibiting cell apoptosis under stress conditions; and our current studies investigated the potential contribution of COX-2 to the inhibitory effect of arsenite in UV-induced cell apoptosis in mouse epidermal Cl41 cells. We found that treatment of cells with low concentration (5 μM) arsenite attenuated cellular apoptosis upon UVB radiation accompanied with a co-inductive effect on COX-2 expression and nuclear factor-κB (NFκB) transactivation. Our results also showed that the COX-2 induction by arsenite and UVB depended on an NFκB pathway because COX-2 co-induction could be attenuated in either p65-deficient or p50-deficient cells. Moreover, UVB-induced cell apoptosis could be dramatically reduced by the introduction of exogenous COX-2 expression, whereas the inhibitory effect of arsenite on UVB-induced cell apoptosis could be impaired in COX-2 knockdown Cl41 cells. Our results indicated that COX-2 mediated the anti-apoptotic effect of arsenite in UVB radiation through an NFκB-dependent pathway. Given the importance of apoptosis evasion during carcinogenesis, we anticipated that COX-2 induction might be at least partially responsible for the co-carcinogenic effect of arsenite on UVB-induced skin carcinogenesis.

### Keywords

Apoptosis; arsenite; COX-2; NF-κB; UVB

## INTRODUCTION

Inorganic arsenic is a well-known environmental toxin that is distributed throughout the earth's crust and is released into the environment during the process of energy generation upon utilization of coal, oil shale, and geothermal sources [1, 2]. Ingestion of arsenic has been reported to increase the risk of various cancers, including liver, bladder, kidney, lung,

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**CONFLICT OF INTEREST**
Declared none.

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as well as skin [3]. A dose-response relationship has been established between the prevalence of skin cancers and the arsenic concentration in drinking water [4, 5]. Ultraviolet radiation (UVR) is a main risk factor for skin carcinogenesis [6], and this threat has increased over recent decades because of the depletion of stratospheric ozone, the principal barrier to the biological effect of UVB (290–320nm) on human skin [7, 8]. Instead of being a complete carcinogen, arsenic could enhance the mutagenicity of other carcinogens such as UVR on the mouse skin [9]. However, the molecular mechanisms underlying the co-carcinogenic effect of arsenic with UVR are not well understood [10].

Cyclooxygenase (COX) exists as two distinct isoforms. COX-1 is a constitutive enzyme, whereas COX-2 is the inducible isoform [11]. COX-2 is undetectable in most mammalian cells, but its expression can be induced rapidly (2–6 h) in different mammal cell lines in response to growth factors, tumor promoters and cytokines [12, 13]. Increasing evidence indicates that COX-2 plays an important role in skin carcinogenesis. For example, mice that are deficient in COX-2 experience reduced tumor formation by 75% in comparison to that in wild type littermates in a multistage mouse skin carcinogenesis model [14]. Oral administration of specific COX-2 inhibitors is also effective in the inhibition of UVB-induced skin tumors by up to 90% [15]. COX-2 induction upon arsenite exposure mediates the anti-apoptotic effect in both mouse Cl41 cells and human BEAS-2B cells [10, 16]. It has been reported that apoptosis induction is a key mechanism for the effectiveness of some cancer chemopreventive agents, and failure of apoptosis is now believed to contribute to the development of human cancer [17], whereas COX-2 overexpression increases rat intestinal epithelial cell adhere to the extracellular matrix and be resistant to apoptosis [18]. Thus, we examined our hypothesis that the synergetic induction of COX-2 by arsenite and UVB is responsible for the anti-apoptotic effect of arsenite against UVB radiation-related cell death in mouse epidermal JB6 Cl41 cells.

## MATERIALS AND METHODS

### Cell Culture and Reagents

Mouse epidermal Cl41 cells and their stable transfectants were cultured in Eagle's Minimal Essential Medium (MEM, Calbiochem San Diego, CA) that was supplemented with 5% fetal bovine serum (FBS), 1% penicillin/streptomycin, and 2 mM L-glutamine (Life Technologies, Inc., Rockville, MD) at 37°C in a humidified atmosphere with 5% $CO_2$. Mouse embryonic fibroblasts (MEFs) and the MEFs with either p65 deficiency ($p65^{-/-}$) or p50 deficiency ($p50^{-/-}$) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS, 1% penicillin/streptomycin, and 2 mM L-glutamine at 37°C in a humidified atmosphere with 5% $CO_2$. The cultures were dissociated with trypsin and transferred to new 75-$cm^2$ culture flasks (Fisher) twice a week. FBS was purchased from Nova-Tech, and other cell culture reagents were obtained from Sigma (St. Louis, MO); the luciferase assay substrate was purchased from Promega (Madison, WI); arsenite ($As^{3+}$) was purchased from Aldrich (Milwaukee, WI); and the antibodies against p65, p50 and COX-2 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), whereas antibody specific for β-Actin was obtained from Sigma-Aldrich (St. Louis, MO, USA) or Sungene Biotech (Tianjin, China).

### Stable Transfection

The AP-1-luciferase, NFAT-luciferase, NF-κB-luciferase, and COX-2-luciferase reporter plasmids have been described in studies [19, 20]. The COX-2 expression vector and the COX-2 siRNA vector as well as IKKβ-KM were used in our previous studies [10]. The plasmid DNA isolation was performed according to Qiagen plasmid MIDI kit protocol. Cell transfection was carried out with Lipofectamine 2000 (Invitrogen, Inc.) according to the

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manufacturer's instructions. Briefly, the cells were cultured in a 6-well plate to 75–85% confluence. A mixture of 2 μg plasmid DNA and 6 μL Lipofectamine 2000 reagent was used to transfect each well in the absence of serum. After 4~6 h, the medium was replaced with 5% FBS MEM. Approximately 36–48 h after the beginning of the transfection, the cells were selected with normal culture medium containing 500 μg/mL G418 (Gibco BRL, Rockville, MD). After a 28–45 day selection, the stable transfectants were established and identified using Western blot and/or luciferase assays. All stable transfectants were cultured in G418-free medium for at least two passages before each experiment.

## Western Blot

Cl41 cells and their transfectants ($2\times10^5$) were seeded and cultured in each well of 6-well plates until 70–80% confluence. The cells were exposed to arsenite and/or UVB for the dosage and time indicated, and then extracted with SDS-sample buffer, as described in our previous studies [10]. The cell extracts were applied for Western blotting using specific antibodies as indicated. The protein band, specifically bound to the primary antibody, was detected using an IgG-AP-linked secondary antibody and an ECF Western blotting system (Amersham Biosciences, Piscataway, NJ). Quantification of COX-2 expression was normalized to β-Actin using the Image J program.

## DNA Fragmentation Assay

DNA fragmentation assay was performance as described in our previous study [21]. Briefly, Cl41 cells were cultured in monolayers in 100 mm dishes with 5% FBS MEM. The cells were exposed to arsenite and/or UV radiation in 0.1% FBS MEM after cell density reached 75–80%. All the cells were harvested by centrifugation and lysed with a lysis buffer (5 mM Tris-HCl, pH 8.0, 20 mM EDTA, 0.5% Triton X-100) on ice for 45 min. The fragmented DNA in the supernatant after centrifugation at 14,000 rpm for 30 min at 4°C was extracted twice with phenol/chloroform/isoamyl alcohol (25:24:1, v/v) and once with chloroform before precipitation with ethanol and salt. The DNA pellet was washed once with 70% ethanol and resuspended in TE buffer (pH 8.0) with 100 μg/mL RNase at 37°C for 2 h. The DNA fragments was separated by 1.8% agarose gel electrophoresis and visualized under UV light.

## Flow Cytometry Assay

Cells ($2 \times 10^5$) were seeded into each well of 6-well plates and cultured to 75–80% confluence. After exposure, the cells were harvested and fixed with 3 mL of ice-cold 80% ethanol overnight. The fixed cells were then centrifuged (3000 rpm, 3 min), suspended in lysis buffer (100mM sodium citrate and 0.1% Triton X-100) and incubated for 15 min at room temperature. The cells were incubated with RNase A (10 mg/mL) (Sigma Chemical, St. Louis, MO) for 10 min at room temperature and DNA was stained with propidium iodide (PI, 50 μg/mL) for 1 h at 4 °C. The cell proportions in sub-G1 and S phases were determined using flow cytometry with an Epics XL FACS (Beckman Coulter) and EXPO 32 software [22].

## Cell Viability Assay

Cell viability was determined using MTT-based cell growth assay. Cells were rinsed with PBS, incubated in DMEM-free medium containing 0.5 mg/mL 3-[4,5-dimethylthiozol-2-yl]-2,5-diphenyltetrazolium bromide (also called thiazolyl blue or MTT) for 4 h. The purple crystals yielded were dissolved in isopropanol/0.1N HCl. The cell viability was calculated by measuring the difference between the optical densities of the resulting purple solution at 570 and 690 nm.

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### Gene Reporter Assay

A confluent monolayer of AP-1–, NFAF–, NF-κB–, or COX-2 luciferase reporter stable transfectants were trypsinized, and $8\times10^3$ viable cells suspended in 100 μL of medium were added to each well of 96-well plates. After the cell density reached 75–80% confluence, the cells were treated with arsenite and/or UVB for time periods as indicated in the figure legends. The cells were then lysed with 50 μL lysis buffer, and the luciferase activity was measured using Promega Luciferase assay reagent with a luminometer (Wallac 1420 Victor2 multipliable counter system). The results were expressed as AP-1, NFAT or NF-κB activation relative to the medium control (relative AP-1, NFAT or NF-κB activation) or COX-2 induction relative to the medium control (relative COX-2 induction), as described in our earlier studies [23, 24].

### Statistical Analysis

The Student's t test was used to determine the significance of differences in COX-2 induction and AP-1, NFAT or NF-κB activation among various groups as indicated. The differences were considered significant at $P < 0.05$.

## RESULTS

### Exposure of Cl41 Cells to Low Concentration Arsenite Rendered the Cells Resistant to the Apoptosis Due to UVB Radiation

UVB irradiation induces apoptosis and alters differentiation in primary human keratinocytes, HaCaT cells, and human epithelial corneal cells [25–27]. In an earlier study, we demonstrate that arsenite exerts an anti-apoptotic effect on Cl41 cells at low concentrations [10]. Since skin tissue is a major target of both UVB and arsenic, and because arsenite at low concentrations shows a co-carcinogenic effect on mouse skin, mouse epidermal Cl41 cells were employed to test whether low concentrations of arsenite were able to inhibit UVB-induced cell apoptosis. As shown in Fig. (1A), treatment of Cl41 cells with UVB radiation at 1 $KJ/m^2$ led to marked cell death, whereas 5 μM of arsenite alone did not produce an observable effect on cell death. However, the pretreatment of Cl41 cells with 5 μM of arsenite for 30 min showed a dramatic inhibition of UVB-induced cell death under the same experimental conditions (Fig. 1A). These findings were consistent with the results that we obtained from the cell viability assay using MTT (Fig. 1B) and flow cytometry assay with PI staining (Fig. 1C). DNA content analysis using PI staining followed by flow cytometry analysis was used to quantify the cell apoptosis rate upon arsenite and/or UVB radiation. The results showed that UVB radiation led to 73.5% Cl41 cell apoptosis at 48 h after exposure, while co-treatment of arsenite and UVB radiation only resulted in 32.9% cell death, in comparison to the treatment of cells with arsenite alone, which led to 6.8% cell apoptosis (Fig. 1C). Accordingly, assays for DNA fragmentation and clevage caspase 3, two classic indicators of apoptosis [21], were further employed to verify the anti-apoptotic effect of arsenite on UVB-treated Cl41 cells (Figs. 1D and 1E). Kapahi *et al.* reported that arsenite at doses over 10 μM could inhibit IKK/NF-κB activation by binding to Cys-179 in the activation loop of the IKK catalytic subunits and promoted cell apoptosis, whereas it did not show observable inhibition on IKK/NF-κB activation and induction of cell apoptosis at the dose at 6.25 μM [28]. Those results were consistent with our previous findings that high dose of arsenite treatment led to cell apoptosis *via* p50/JNK pathway [29], while low doses of arsenite exposure resulted in cell transformation [30]. Thus, the differential effects of arsenite on NF-κB activation might be due to arsenite doses that were applied. Collectively, our results demonstrated that low concentrations of arsenite protected UVB-exposed Cl41 cells from apoptosis in mouse epidermal Cl41 cells. This was consistent with the findings demonstrated in human keratinocytes [31].

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### Arsenite Had a Co-Inductive Effect with UVB Radiation on COX-2 Expression in Cl41 Cells

COX-2 is an inducible early responsive gene, and plays a role in the mediation of inflammation and carcinogenesis [11]. Although studies demonstrate that exposure of cells to either UVB or arsenite induces COX-2 expression both *in vivo* and *in vitro* [10, 18, 32–35], no study has been conducted to investigate whether or not arsenite has a co-inductive effect on COX-2 expression in combination with UVB radiation. Thus, we examined the COX-2 induction by arsenite and/or UVB in Cl41 cells. Consistent with the previous reports, exposure of Cl41 cells with either UVB or arsenite alone resulted in increases in COX-2 expression (Figs 2A and 2B), whereas arsenite at 5 μM had a co-inductive effect with UVB on COX-2 protein expression (Fig. 2C). Moreover, our results indicated that treatment of Cl41 with arsenite and 1KJ/$m^2$ UVB also resulted in a significant co-induction of COX-2 expression in the COX-2-luciferase reporter assay (Fig. 2D), suggesting that an co-inductive effect of arsenite with UVB on COX-2 expression might occur at transcriptional level.

### NFκB, but not AP-1 nor NFAT, Was Critical for Co-Induction of COX-2 Due to Arsenite and UVB Exposure

The above results, obtained using the COX-2-luciferase reporter, suggested that co-induction of COX-2 by arsenite and UVB radiation might be regulated at the transcriptional level. Studies show that the COX-2 promoter region contains binding sites for multiple transcription factors, including AP-1, NFκB, and NFAT, and each of these transcription factors is involved in the regulation of COX-2 expression in various experimental systems, depending on cell types and stimulus [18, 36, 37]. To test whether AP-1 was implicated in the regulation of COX-2 co-induction upon exposure to arsenite and UVB radiation, we determined the AP-1-dependent transactivation due to arsenite and/or UVB radiation using Cl41 AP-1-luciferase stable transfectants. The results indicated that arsenite at concentrations of 2.5–10 μM showed neither induction of AP-1 activity, nor any co-inductive effect on AP-1 activity with UVB radiation, eventhough UVB alone induced a high level of AP-1 transactivation (Fig. 3A). Consistent with AP-1 activation, arsenite and/or UVB radiation showed a similar effect on NFAT transactivation in Cl41 NFAT-luciferase reporter assay (Fig. 3B). These results suggested that the transcription factor AP-1 and NFAT might not be involved in the co-inductive effect of arsenite on COX-2 expression with UVB radiation. In contrast with the results observed in AP-1 and NFAT activation, either exposure of cells to arsenite at a dosage range between 2.5–10 μM, and UVB radiation, increased NFκB activity, with similar patterns of their effects on COX-2 induction (Figs. 3C and 2D). Moreover, arsenite showed a significant co-inductive effect with UVB radiation on NFκB transactivation under the same experimental conditions (Fig. 3C). Those results indicated that arsenite alone showed neither any inductive effect on activation of AP-1 and NFAT, nor addictive effect on UVB-induced activation of AP-1 and NFAT, whereas arsenite alone induced NFκB activation by itself and also showed a synergistic effect on NFκB activation with UVB radiation. Thus, we anticipate that NFAT and AP-1 might not be responsible for co-induction of COX-2 expression by arsenite with UVB radiation. It should be noted that we can’t exclude the potential role of AP-1 and NFAT in mediation of COX-2 expression by UVB radiation alone. Our published studies have shown that IKK-α and IKK-β are coordinately required for induction of AP-1 activation through an NF-κB-independent pathway upon apoptotic dose (20 μM) of arsenite exposure [38]. To further elucidate role of NFκB in COX-2 induction by both arsenite and UVB radiation, the stable transfectants of Cl41 cells harboring IKKβ-KM, a dominant mutant of IKKβ were used [10]. As shown in Fig. (3D), combination of arsenite exposure and UVB irradiation led to dramatic increase in COX-2 promoter activity in the control cells expressing mock plasmid, while introduction of IKKβ-KM obviously rendered the blockage in the synergetic effect of arsenite and UVB on COX-2 promoter induction. To further verify the subunit of

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NFκB involved in this process, NFκB p65 knockout MEFs ($p65^{-/-}$) or NFκB p50 knockout MEFs ($p50^{--}$) were employed. As shown in Fig. (3E), knockout of either p65 or p50 dramatically inhibited COX-2 induction by UVB and arsenite (Fig. 3E). It should be noted that there was still marginal COX-2 induction in $p65^{-/-}$ MEFs upon exposure of cells with UVB alone or UVB and arsenite (Fig. 3E). This effect may be mediated by activation of other transcription factors, such as AP-1 and/or NFAT, upon UVB radiation alone. Taken together, these data demonstrated that NFκB, not AP-1 nor NFAT, was responsible for co-induction of COX-2 protein expression due to arsenite and UVB radiation.

### COX-2 Co-Induction Mediated Arsenite Protection of UVB-Exposed Cl41 Cells from Apoptosis

The COX-2 expression is induced in an IKKβ/NFκB-dependent manner after treatment with arsenite in Cl41 cells, and plays an important role in antagonizing apoptosis due to arsenite exposure [10]. Thus, we transfected the COX-2 expression construct into Cl41 cells and determined if COX-2 overexpression provided protection from apoptosis to UVB-exposed Cl41 cells. As shown in Fig. (4A), the COX-2 protein was overexpressed in Cl41 cell transfectants in comparison to that in mock vector-transfectants (Fig. 4A). Overexpression of COX-2 as seen in Cl41-COX2 transfectant showed much less susceptibility to UVB radiation for apoptotic induction in comparison to that in mock vector-transfectant (Fig. 4B), suggesting that COX-2 could provide protection from apoptosis in UVB-irradiated Cl41 cells. To determine whether or not COX-2 co-induction by arsenite and UVB could result in the inhibitory effect of arsenite in UVB-induced cell apoptosis, we used a COX-2 shRNA to knockdown endogenous COX-2 expression in Cl41 cells. We found that introduction of COX-2 shRNA knocked down the serum-induced COX-2 protein expression by 70% (Fig. 4C). Deficiency of COX-2 expression resulted in an increase in UVB-induced cell apoptosis in comparison to the control transfectants (20.7% v. 30.5%, Fig. 4D). Moreover, the reduction of COX-2 expression attenuated the arsenite protective effect on UVB-induced cell apoptosis completely (7.1% v. 25.5%, Fig. 4D). These results demonstrated that co-induction of COX-2 expression upon arsenite and UVB radiation mediated arsenite inhibition of UVB-induced cell apoptosis, and might, at least, be one of the major factors responsible for the arsenite co-carcinogenic effect on mouse skin.

## DISCUSSION

The co-carcinogenic effect of arsenite with UVR on mouse skin is well established [9], but the molecular basis underlying this is largely unknown. Here, we focused on the co-inductive effect of arsenite with UVB radiation on signaling pathways leading to COX-2 induction in mouse epidermal JB6 Cl41 cells. We found that arsenite had a co-inductive effect with UVB radiation on COX-2 expression, which made a significant contribution to arsenite protection from apoptosis on UVB-exposed cells. We anticipated this arsenite protection might be at least one of the key factors responsible for the skin co-carcinogenic effect of arsenite with UVB radiation.

The carcinogenic effect of arsenite by occupational or environmental exposure is convincing [3, 39]. Levels of arsenic in drinking-water in epidemiological studies ranged from 54 ppb (0.72 μM) to 12 ppm (160 μM) [40], and such levels of arsenite exposure are associated with various cancers [40–42]. Although the arsenite doses used for studies in both *in vitro* and *in vivo* vary among different types of cell and animal models, the doses used in the present study (ranging from 2.5–10 μM) are similar to those used in previous studies in cell culture models [43–45]. The inhibition of DNA repair capacity is one of the potential mechanisms for the co-carcinogenic activity of arsenic [46]. Low concentration arsenic (2.5μM) exposure decreases the expression of critical genes involved in nucleotide excision repair of damaged DNA after UV irradiation [47–49]. Ding *et al.,* (2009) demonstrate that arsenite at low

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concentrations inhibits oxidative DNA damage repair by interaction with the PARP-1 zinc finger domain, which leads to the inhibition of PARP-1 activity [50]. Apoptosis typically occurs when cellular genetic damage exceeds the repair capacity and suppression of apoptosis, in the face of significant genetic damage, could facilitate accumulation of aberrant cells and may be a critical step in the pathogenesis of malignancy. Dysregulation of apoptosis may lead to various kinds of pathological conditions, such as cancer development [51]. In this study, we demonstrated that exposure of cells to low arsenite concentrations inhibited UVB-induced cellular apoptosis, revealing that arsenite might have initiated anti-apoptotic signaling and/or gene expression, and provided an anti-apoptotic effect on UVB-exposed cells. This anti-apoptotic effect initiated by arsenite might account for the co-carcinogenesis of arsenite with UV radiation demonstrated in the mouse skin carcinogenesis model.

COX-1 is expressed constitutively, whereas COX-2 is inducible in cells upon treatment with growth factors, tumor promoters, and cytokines [52]. Accumulating evidence suggests that cancer cells which express higher levels of COX-2 may obtain a survival advantage that eventually facilitates tumor development and progression [53, 54]. Over-expression of COX-2 in human skin basal cell carcinoma cells increases antiapoptosis, angiogenesis, and tumorigenesis induced by UVB [55], and NFAT-mediated COX-2 induction is crucial for TNFα-induced cell transformation in Cl41 cells [56]. The promoter region of COX-2 contains a canonical TATA-box and multiple regulatory elements, which can be recognized by transcription factors, such as NFκB, AP-1, and NFAT [22, 57–59]. Arsenite induces COX-2 expression at the transcriptional level by activating the NFAT pathway in human bronchial epithelial BEAS-2B cells, whereas in Cl41 cells, arsenite induces COX-2 through the NFκB-dependent pathway [16, 37]. These published studies from our group suggest that the signaling pathway leading to COX-2 induction depends on the cell types and stimuli. In the present study, we showed that arsenite at low concentration or UVB radiation alone induced COX-2 expression in Cl41 cells, while co-exposure of cells with UVB and low concentration arsenite resulted in a marked COX-2 co-induction at both protein and transcription levels in Cl41 cells. We further found that Cl41 cells transfected with the COX-2 constitutive expression construct were resistant to UVB for apoptotic induction in comparison to that in mock vector-transfected Cl41 cells. Moreover, the knockdown of endogenous COX-2 expression by its specific siRNA in Cl41 cells attenuated the protective effect of low concentration arsenite (5 μM) on cellular apoptosis upon UVB radiation. Collectively, these results demonstrated that low concentrations of arsenite exerted an anti-apoptotic effect by sustaining a relatively high level of COX-2 expression in Cl41 cells exposed to UVB radiation. It should be noted that many mechanistic studies have shown that a number of molecules and gene products are involved in arsenic-induced cell proliferation and growth inhibition in model cell lines including Bcl-xL, Bcl-2, c-myc, Cyclin D1, HO-1 and NADPH oxidase [60–62]. Although our results indicated an important role of NFκB/COX-2 pathway in protection of UV-induced cells from apoptosis, we couldn't exclude other genes involved in such protection.

In our current study, we also found that co-treatment of cells with arsenite and UVB substantially induced NFκB activation in Cl41 cells, whereas it did not show any notable co-inductive effect on AP-1 and NFAT activation. Our previous studies demonstrate that arsenite induces AP-1 activation both in the mouse Cl41 cells and in the skin of AP-1-lucifease transgenic mice, and that the AP-1 induction appears to be mediated by the activation of protein kinase family members [22, 57]. UVB induces COX-2 expression by increasing the activation of AP-1 [34, 36], or NFAT [63], and inhibition of UVB-induced NFAT activation in keratinocytes reduces COX-2 protein expression and increases UV-induced apoptosis [63]. In the current study, although UVB exposure activated both AP-1 and NFAT transactivation, arsenite alone at low concentration showed neither an observable

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effect on transactivation of AP-1 and NFAT, nor a co-inductive effect with UVB on the activation of AP-1 and NFAT. This was not consistent with their effects on COX-2 expression, suggesting that AP-1 and NFAT might not be involved in this COX-2 co-induction by arsenite and UVB treatment. This led us to test the potential contribution of NFκB to COX-2 co-induction by arsenite and UVB. The results indicated that UVB and arsenite at low concentration did result in co-induction of NFκB transactivation in the same Cl41 cells under the same experimental conditions. We further found that knockout of either the NFκB component p65 or p50 resulted in a dramatic reduction of COX-2 expression, demonstrating that NFκB activation was a major pathway responsible for the mediation of COX-2 co-induction due to arsenite and UVB exposure in Cl41 cells. Taken together with the important role of COX-2 in skin carcinogenesis, we anticipated that co-induction of COX-2 by arsenite and UVB radiation, and its role in the inhibition of UVB-induced cell apoptosis, was at least one of mechanisms underlying the reported co-carcinogenic activities of arsenite in UVR-induced skin carcinogenesis. These studies provided additional insights into understanding the molecular mechanisms that underline the co-carcinogenic effect of arsenite with UV radiation.

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## ABBREVIATIONS

| | |
|---|---|
| **AP-1** | activator protein-1 |
| **Bcl-2** | B-cell lymphoma-2 |
| **Bcl-xL** | B-cell lymphoma-extra large |
| **BEAS-2B** | bronchial epithelial cell line |
| **COX** | cyclooxygenase |
| **DMEM** | Dulbecco's Modified Eagle's Medium |
| **FBS** | fetal bovine serum |
| **HO-1** | heme oxygenase 1 |
| **MEFs** | mouse embryonic fibroblasts |
| **MEM** | minimal essential medium |
| **MTT** | 3-[4,5-dimethylthiozol-2-yl]-2,5-diphenyltetrazolium bromide |
| **NADPH** | nicotinamide adenine dinucleotide phosphate |
| **NFAT** | nuclear factor of activated T cells |
| **NFκB** | nuclear factor-κB |
| **UVB** | ultraviolet-B |

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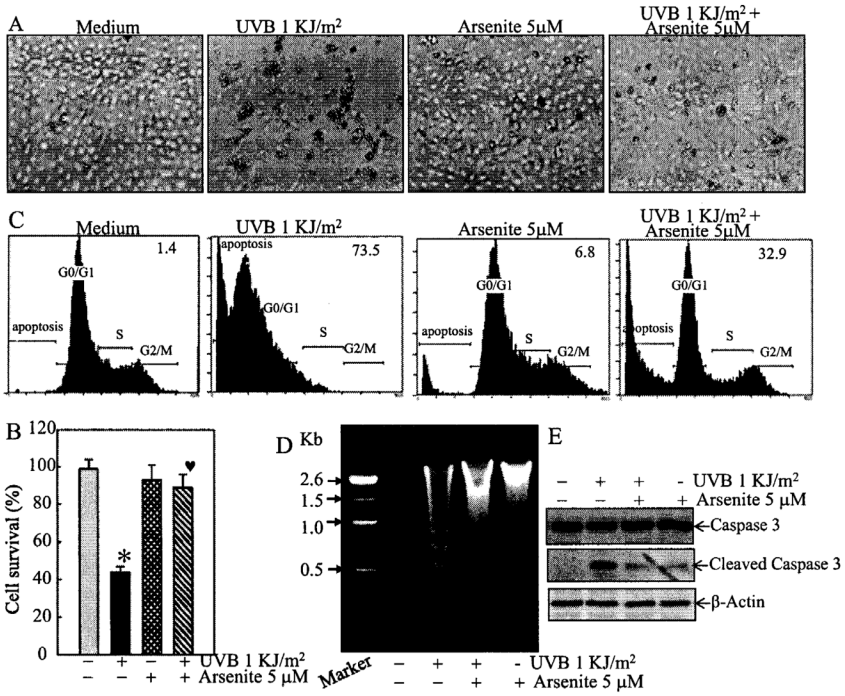


**Fig. 1. Arsenite at low concentration rendered Cl41 cells resistant to pro-apoptosis effects upon UVB radiation**

Cl41 cells ($2 \times 10^5$) were seeded into each well of 6-well plates. After being cultured at 37°C overnight, the cells were treated with UVB (1KJ/$m^2$), or arsenite (5 μM) for 48 h, or Cl41 cells were pretreated with arsenite (5 μM) for 30 min and then exposed to UVB (1KJ/$m^2$). The cells were incubated for an additional 48 h and cell death was imaged under microscopy (**A**), or monitored with MTT assay (**B**). Each bar indicates the mean ± SD of three replicate assay wells. The symbol (*) indicates that the cell survival rate significantly decreased from the control medium ($p < 0.05$). The symbol (♥) indicates that the cell survival rate significantly increased from UVB radiation alone ($p < 0.05$). Apoptotic cells were analyzed by flow cytometry with PI (propidium iodide) staining (**C**) or by the detection of DNA fragments in agarose gel electrophoresis (**D**) and assay for cleavage of caspase 3 (**E**).

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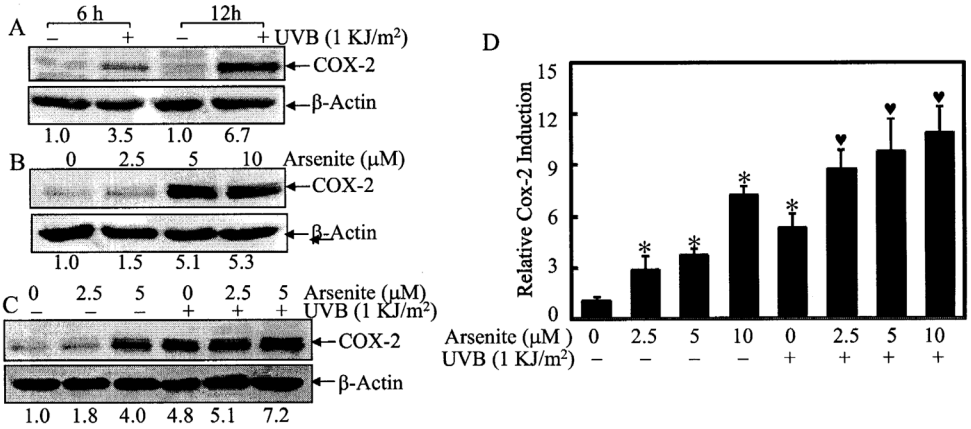


**Fig. 2. Arsenite and/or UVB induced COX-2 expression in Cl41 cells**

**(A–C)** Cl41 cells ($2 \times 10^5$) were seeded into each well of 6-well plates. After being cultured at 37°C overnight, the cells were treated with UVB (1KJ/$m^2$) then cultured for 6 h or 12h (A), or various concentrations of arsenite for 12 h (**B**), or pretreatment of Cl41 cells with arsenite for 30 min then treated with UVB at 1KJ/$m^2$, then cultured for 12 h (**C**). The cells were then extracted with SDS-sample buffer, and cell extracts were subjected to Western blotting with specific antibodies against either COX-2 or β-Actin. Quantification of COX-2 expression was normalized to β-Actin using the Image J program. (**D**) COX-2 luciferase reporter stable transfectant was seeded into 96-well plates. The cells were exposed to arsenite for 6 h, or pretreated with arsenite for 30 min then treated with UVB at 1KJ/$m^2$, then cultured for 6 h. The cells were then extracted with 50 μL lysis buffer for luciferase activity assay, as described in our previous studies. The results are expressed as COX-2 induction relative to the control medium (relative COX-2 induction). Each bar indicates the mean ± SD of three replicate assay wells. The symbol (*) indicates a significant increase from the control medium ($p < 0.05$); the symbol (♥) indicates a significant increase from UVB radiation alone ($p < 0.05$);

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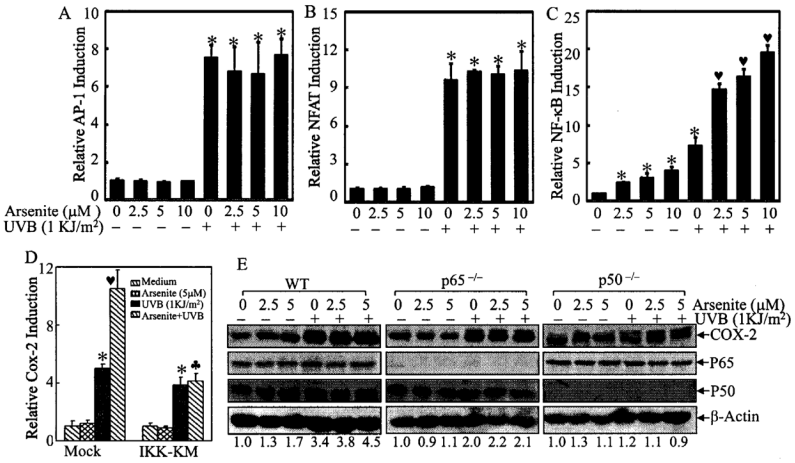


**Fig. 3. NFκB, not AP-1 or NFAT, mediated the COX-2 co-induction due to arsenite and UVB exposure**

**(A–D)**, AP-1 luciferase reporter stable transfectants (**A**), NFAT luciferase reporter stable transfectants (**B**), or NFκB luciferase reporter stable transfectants (**C**), or IKKβ-KM stable transfectants harboring COX-2 promoter luciferase (**D**) were seeded into 96-well plates and cultured in 10% FBS-DMEM at 37°C until the cell density reached 75–80%. The cells were exposed to arsenite for 6 h, or pretreated with arsenite for 30 min, then treated with UVB at 1KJ/m$^2$, then cultured for 6 h, and then extracted with 50μL lysis buffer for luciferase activity assay. The results are expressed as AP-1, NFAT or NFκB activity, or COX-2 promoter activity induction relative to the control medium. Each bar indicates the mean ± SD of three replicate assay wells. The symbol (*) indicates a significant increase from the control medium ($p$< 0.05). The symbol (♥) indicates a significant increase from UVB radiation alone ($p$< 0.05); The symbol (♣) indicates a significant increase from that in transfectants expressing Mock vector ($p$< 0.05) (**E**), mouse embryonic fibroblasts (MEFs), with either p65 deficient (p65$^{-/-}$) or p50 deficient (p50$^{-/-}$), were cultured in 10% FBS DMEM until 75–80% confluence. The cells were exposed to arsenite for 12 h, or pretreated with arsenite for 30 min then treated with UVB (1KJ/m$^2$), then cultured for 12 h, and then extracted with SDS sample buffer. The cell extracts were subjected to Western blotting with specific antibodies against COX-2, P65, P50 or β-Actin. Quantification of COX-2 expression was normalized to β-Actin using the Image J program.

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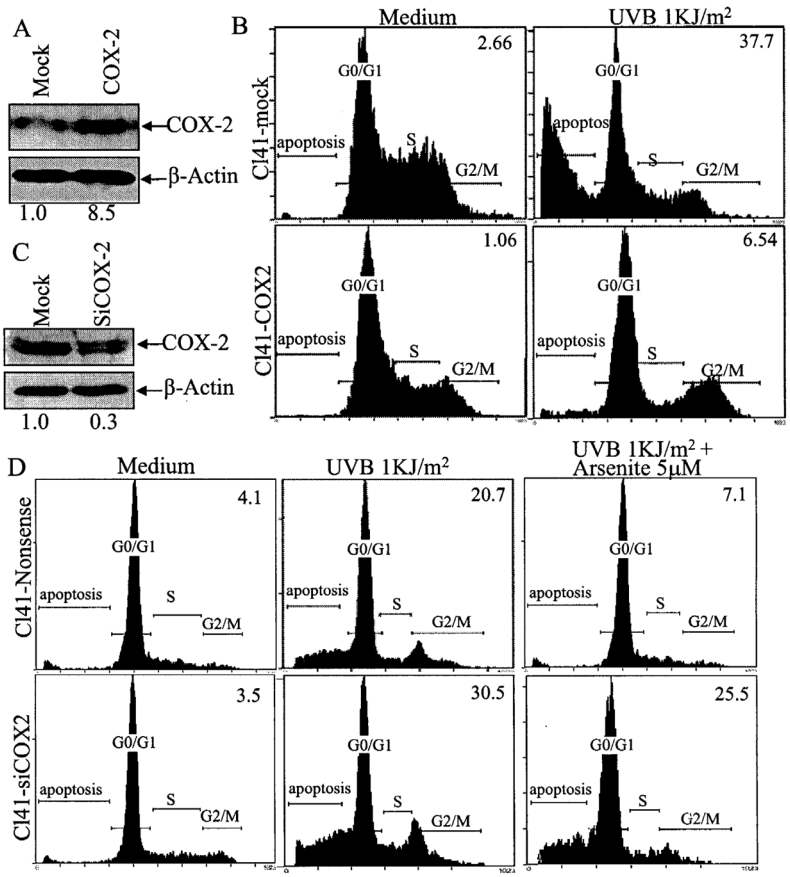


**Fig. 4. COX-2 was the mediator for arsenite protection of UVB-exposed Cl41 cells from apoptosis**

COX-2 constitutive expression (**A**) and knockdown (**C**) were identified with Western blotting as indicated; **B** and **D**, Cl41 cells ($2 \times 10^5$) were seeded into each well of 6-well plates. After being cultured at 37°C overnight, the cells were treated with UVB (1KJ/$m^2$), or pretreated with 5 μM of arsenite for 30 min and then exposed to UVB (1KJ/$m^2$) and cultured for an additional 36h. The cellular apoptosis was analyzed using flow cytometry with PI staining. The experiments were repeated three times.