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Transcription factor Nrf2 activation by inorganic arsenic in cultured keratinocytes: involvement of hydrogen peroxide

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Abstract

Inorganic arsenic is a well-documented human carcinogen that targets the skin. The induction of oxidative stress, as shown with arsenic, may have a bearing on the carcinogenic mechanism of this metalloid. The transcription factor Nrf2 is a key player in the regulation of genes encoding for many antioxidative response enzymes. Thus, the effect of inorganic arsenic (as sodium arsenite) on Nrf2 expression and localization was studied in HaCaT cells, an immortalized human keratinocyte cell line. We found, for the first time, that arsenic enhanced cellular expression of Nrf2 at the transcriptional and protein levels and activated expression of Nrf2-related genes in these cells. In addition, arsenic exposure caused nuclear accumulation of Nrf2 in association with downstream activation of Nrf2-mediated oxidative response genes. Arsenic simultaneously increased the expression of Keap1, a regulator of Nrf2 activity. The coordinated induction of Keap1 expression and nuclear Nrf2 accumulation induced by arsenic suggests that Keap1 is important to arsenic-induced Nrf2 activation. Furthermore, when cells were pretreated with scavengers of hydrogen peroxide (H_2O_2) such as catalase–polyethylene glycol (PEG-CAT) or Tiron, arsenic-induced nuclear Nrf2 accumulation was suppressed, whereas CuDIPSH, a cell-permeable superoxide dismutase (SOD) mimic compound that produces H_2O_2 from superoxide ($\cdot O_2^-$), enhanced Nrf2 nuclear accumulation. These results indicate that H_2O_2, rather than $\cdot O_2^-$, is the mediator of nuclear Nrf2 accumulation. Additional study showed that arsenic causes increased cellular H_2O_2 production and that H_2O_2 itself has the ability to increase Nrf2 expression at both the transcription and protein levels in HaCaT cells. Taken together, these data clearly show that arsenic increases Nrf2 expression and activity at multiple levels and that H_2O_2 is one of the mediators of this process.

Keywords: Arsenic; Nrf2; Antioxidant response element; Hydrogen peroxide; Keratinocyte; Keap1; Oxidative stress

Introduction

Inorganic arsenic is a well-recognized human carcinogen and exposure is associated with an increased risk for tumors of the skin, bladder, liver, kidney, lung, prostate, and other tissues [1–4]. Inorganic arsenic is also active in rodent models, producing liver tumors after transplacental exposure [5] and skin tumors in combination with UV irradiation or phorbol esters [6,7]. However, the molecular mechanisms of inorganic arsenic carcinogenesis remain poorly understood. Arsenic can stimulate production of reactive oxygen species (ROS) [8] and alters cellular sulfhydryl levels [9], whereas glutathione depletion enhances arsenic cytotoxicity [10]. ROS resulting from an imbalance between antioxidants and oxidants during arsenic metabolism has been implicated as a possible etiologic factor in arsenic toxicity and carcinogenesis [11] and as a general factor in carcinogenic potential of inorganics [12]. The precise cause of arsenic-induced ROS is unclear and its molecular impact has not been well defined.

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The transcription factor NF-E2-related factor 2 (Nrf2), a member of the Cap'n'Collar family of bZIP proteins, is a central mediator in the activation of a variety of genes encoding for antioxidative and phase 2 drug-metabolizing enzymes through antioxidant response elements (AREs) [13,14]. The human ARE is a *cis*-element that contains one perfect and one imperfect AP1 binding element [15]. AREs have been found in the 5′-flanking region of many genes involved with cytoprotection from oxidative stress, such as glutathione *S*-transferase (GST) [16], NAD(P)H:quinone reductases (NQO1) [17], γ-glutamylcysteine synthetase heavy (γ-GCS_h) and light subunits (γ-GCS_L) [18], and heme oxygenase 1 (HO-1) [19] among many others [20–22]. The induction of these enzymes can be viewed as a strategy for cellular protection against the adverse effects of excess ROS production, including, potentially, carcinogenesis. Prestera et al. [23] previously identified arsenic as one of 28 compounds that can activate an incompletely defined GST enhancer element from the 5′ upstream region inserted into a plasmid containing a human growth hormone reporter. Furthermore, overexpression of HO-1, an Nrf2-activated gene [19], is common with arsenic exposure [24]. In this regard, peritoneal macrophages obtained from Nrf2-deficient mice show a greatly diminished induction of HO-1 when compared to wild-type cells after exposure to sodium arsenite [25]. Although these data infer a potential role for Nrf2 in the cellular response to arsenic [23,25], little is specifically known about the effects of this metalloid on the Nrf2-mediated response to oxidative stress.

Several models have been proposed for the activation of the Nrf2-ARE-mediated pathway. The most commonly invoked model for regulation of Nrf2 function is dissociation of Nrf2 from a cytoskeleton-associated protein Keap1 (human/rat homolog also known as KIAA0132/INrf2), which appears to act as a cytoplasmic repressor of Nrf2 [26]. When Nrf2 dissociates from Keap1, it can translocate to the nucleus and there interact with other transcription factors, bind to the ARE, and subsequently stimulate expression of target genes [26,27]. Modification of sulfhydryl groups in Keap1 [28] and phosphorylation of Nrf2 through a protein kinase C-(PKC) based mechanism [29] appear to mediate the dissociation of Nrf2 from Keap1. Recent results demonstrated that the phosphatidylinositol 3-kinase (PI3-kinase) pathway regulates the rearrangement of actin microfilaments and depolymerization of actin facilitates the nuclear translocation of Nrf2 [30,31]. Furthermore, mitogen-activated protein (MAP) kinase pathways activated by extracellular signal-regulated kinase kinase kinase 1 (MEKK1), transforming growth factor-β-activated kinase (TAK1), and apoptosis signal-regulating kinase (ASK1) are reported to be signaling pathways for Nrf2 activation [32]. Interestingly, Nrf2 has been shown to autoregulate its own expression through an ARE-like element located in the proximal region of the Nrf2 promoter, leading to persistent nuclear accumulation of Nrf2 and protracted induction of target genes [33]. An increase of Nrf2 stabilization represents another important posttranscriptional regulation mechanism that can enhance Nrf2 activity [34,35]. Thus, it is likely that multiple mechanisms play a role in Nrf2-induced gene expression in response to oxidative stress.

It is well established that arsenic can induce oxidative stress [8,9] and that Nrf2 is a key player in the cellular oxidative stress response [13,14]. However, a clear interrelationship between Nrf2 and arsenic-mediated oxidative stress has not been established. In the present study, the effects of inorganic arsenic exposure on Nrf2 expression and nuclear translocation, as well as the downstream expression of Nrf2 related genes, were studied. We selected HaCaT cells, a human keratinocyte cell line that models the skin as a target of arsenic carcinogenesis. In this study, we provide direct evidence that arsenic activates the Nrf2 system probably through production of H_2O_2. This indicates the Nrf2 gene as a novel target of inorganic arsenic and provides evidence of a primary molecular response in a potential target cell of this important inorganic carcinogen.

Materials and methods

Chemicals

Sodium *m*-arsenite ($NaAsO_2$), copper (II) 3,5-diisopropyl salicylate hydrate (CuDIPSH), 4,5-dihydroxy-1,3-benzenedisulfolic acid (Tiron), and catalase–polyethylene glycol (PEG-CAT) were obtained from Sigma-Aldrich (St. Louis, MO).

Cell culture

The human keratinocyte cell line HaCaT is a spontaneously transformed human epithelial cell line developed by Boukamp et al. [36]. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 U of penicillin/ml, and 100 μg of streptomycin/ml. Cultures were maintained at 37°C in a humidified 5% CO_2 atmosphere.

Preparation of protein extracts

Cells were treated with various chemicals as detailed in respective figure legends. After washing three times with ice-cold phosphate-buffered saline (PBS), whole-cell extracts were obtained by using cell lysis buffer (Cell Signaling Technology, Inc., Beverly, MA) with 0.5% of Protease Inhibitor Cocktail (Sigma-Aldrich). Nuclear and cytosolic fractions were separated using a TransFactor Extraction kit (BD Biosciences Clontech, Palo Alto, CA) according to the manufacturer's recommendation. All the protein fractions were stored at −70°C until use and the protein concentrations were determined by Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA) with bovine serum albumin as a standard.

Western blot analysis

Proteins were separated by Novex 12% Tris–glycine gel (Invitrogen, Carlsbad, CA) and transferred onto nitrocellulose membranes. The blots were probed with polyclonal rabbit anti-Nrf2 antibody (1:2000) (sc-H300, Santa Cruz Biotechnology, Inc., Santa Cruz, CA), followed by incubation with horseradish peroxidase-conjugated secondary antibodies. Antibody incubations were performed in Blocker BLOTTO in TBS (Pierce, Rockford, IL). Immunoreactive proteins were detected by chemiluminescence using ECL reagent (Amersham Pharmacia, Piscataway, NJ) and subsequent autoradiography. Quantitation of the results was performed by Bio-Rad Gel Doc 2000 Systems with Bio-Rad TDS Quantity One software. After the blots were striped using Restore Western Blot Stripping Buffer (Pierce, Rockford, IL), the blots were probed for β-actin (Cell Signaling Technology, Inc.), and the level of β-actin was used to normalize for sample loading.

RNA extraction and RT–PCR

Total RNA were extracted using TRI REAGENT (Molecular Research Center, Inc., OH) and purified by RNeasy Mini Kit (QIAGEN Sciences, Valencia, MA) with digestion by Rnase-Free DNase Set (QIAGEN Sciences). Reverse transcription–polymerase chain reaction (RT–PCR) was conducted using a TITANIUM one-step RT–PCR kit (Clontech) and a GeneAmp PCR system 9700 (Applied Biosystems, Foster City, CA) according to the manufacturer's introduction. Amplification conditions were: 60 min at 50°C and 5 min at 94°C followed by 26–40 cycles for 30 s at 94°C, 30 s at 57°C, 1 min at 68°C. Five hundred nanograms of total RNA were used in each amplification. Primers were designed for Nrf2, Keap1, NQO1, γ-GCS_h, and β-actin using Primer 3 Software and were synthesized by Sigma-Genosys (Woodlands, TX) as follows: Nrf2 (Gene Bank accession number AF323119), AGATTCACAGGCCTTTCTCG and CAGCTCTCCCTACCGTTGAG, product size: 201bp (144–344); Keap1 (Accession number NM_012289) CAGAGGTGGTGGTGTTGCTTAT and AGCTCGTTCATGATGCCAAAG, 244 bp (78–321); γ-GCS_h (accession number NM_001498) TTGATTAAGGCTTTCTTTGGTAGG and TTTCAATAAATCAGGTCCCAGG, 273 bp (2133–2405); NQO1 (Accession number NM_000903) GCCTAGCACAAGTACCACTCTTGGTC and CTGAGGCAGGAGAATTGCTGGAACC, 233 bp (1385–1618); β-actin (Accession number XM_037235) AGAGATGGCCACGGCTGCTT and ATTTGCGGTGGACGATGGAG, 406 bp (748–1193). PCR products were electrophoresed on 2% agarose gels and the gel image captured and quantified with a Bio-Rad Gel Doc 2000 Systems with Bio-Rad TDS Quantity One software. The level of β-actin was used to normalize results.

Immunostaining of Keap1

Cells were grown on glass coverslips in six-well plate for 48 h and treated with arsenic (as sodium arsenite) as indicated in the figure legends. Then the cells were washed with PBS and fixed for 10 min at room temperature in 2% (v/v) formaldehyde/0.1% (v/v) glutaraldehyde. After washing in PBS, cells were permeabilized in 1% (v/v) Triton X-100 in PBS, washed, and incubated with 10% goat serum for 1 h at 25°C. The cells were first treated with polyclonal goat anti-Keap1 (E-20) (1:50, Santa Cruz Biotechnology, Inc.) for 16 h at 4°C and subsequently with Cy5-linked rabbit antigoat IgG (H + L) (1:20, Zymed Laboratories, South San Francisco, CA) for 1 h at room temperature. After washing the cells with PBS, coverslips were mounted on microscope slides with ProLong antifade mounting media (Molecular Probes, Inc., Eugene, OR). A laser scanning confocal microscope (LSM 510 NLO) mounted on Axiovert 100M microscope (Carl Zeiss, Inc., Thornwood, NY) was used to obtain the fluorescence and Differential Interference Contrast (DIC) images. The images were obtained simultaneously using the 633-nm line from the included HeNe laser as the excitation source and the Zeiss Plan-Apo 100X oil N.A. = 1.4 as objective lens. For the fluorescence emission, a 650-nm long-pass filter was used, a pinhole of 0.6 Airy units, corresponding to a *z*-resolution of 0.6 microns. All fluorescence images in the same figure were acquired with the same settings. The software used for acquisition was Zeiss LSM510 version 3.0 for Windows NT, and for analysis, LSM Image Examiner (licensed) version 3.0 for Windows. DIC images were further processed to normalize the background using the FFT high-pass filter in MetaMorph version 4.6r5 (Universal Imaging, Inc., Downington, PA).

Detection of H_2O_2 generation

Intracellular H_2O_2 generation was monitored with the fluorescent probe 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H_2DCFDA, Molecular Probes, Inc.), which is a chloromethyl derivative of H_2DCFDA and exhibits much better retention in live cells. Cells were grown on coverslips for 48 h and then changed to media lacking both serum and phenol red. Cells were preincubated with 1 μM of CM-H_2DCFDA for 20 min, treated with arsenic for 20 min followed by rinsing with PBS 3 times. Then the cells were viewed immediately with the LSM 510 laser scanning confocal microscope, using the 488-nm laser for exciation, the LP 505 filter for emmision, and a wide-open pinhole (nonconfocal mode).

Extracellular H_2O_2 levels were measured by a CM-H_2DCFDA-based fluorescent assay. Briefly, cells were grown up in six-well plate to confluence, rinsed twice with PBS, and changed to serum- and phenol red-free DMEM (0.5 ml). After a 30-min preincubation, CM-H_2DCFDA (4 μM) was added to the system followed by addition of arsenic. After 20 min of incubation, 100 μl of the condi-

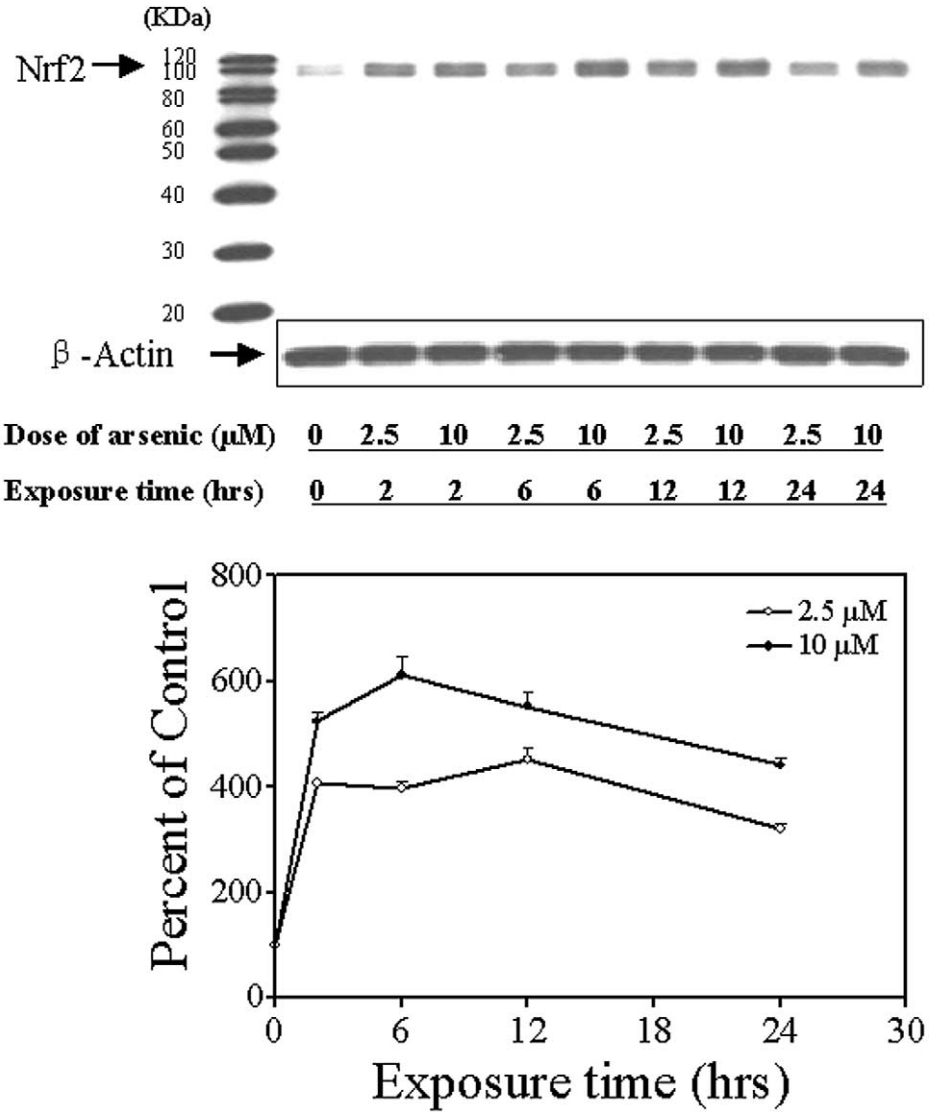

Fig. 1. Arsenic increases nuclear Nrf2 accumulation in HaCaT cells. Cells were grown to confluence and rendered quiescent by incubation in serum-free DMEM. The media was adjusted to contain 2.5 or 10 μM of sodium arsenite at the indicated time point prior to cell harvest. Nuclear protein fractions were then extracted. Each lane was loaded with 15 μg of nuclear proteins. Lower figure is a quantitative analysis of Western blot results.

tioned culture media was moved to a B&W Isoplate 96-well plate (Perkin–Elmer, Boston, MA) to measure the changes of DCF fluorescence using a VICTOR^2 Multilabel Counter (Perkin–Elmer) at excitation and emission wavelengths of 485 and 535 nm, respectively.

Statistical analysis

Data are expressed as mean ± SEM of $n = 3$ to 6 in all cases. A one-way ANOVA followed by Dunnett's multiple comparison test was used to compare the treated groups to the control. A one-way ANOVA followed by a Bonferroni test was used to compare selected data pairs as appropriate. A P value of ≤ 0.05 was considered significant in all cases.

Results

Arsenic increases nuclear Nrf2 accumulation and induces NQO1 and γ-GCS$_h$ expression in HaCaT cells

As shown in Fig. 1, exposure to inorganic arsenic resulted in Nrf2 protein accumulation in the nuclear fraction of HaCaT cells in a time- and dose-dependent fashion that reached a peak at between 6 and 12 h. To evaluate the effects of the nuclear Nrf2 accumulation induced by arsenic on transcriptional activation through the ARE, the expression of the ARE-controlled genes NQO1 and γ-GCS$_h$ was assessed. Both NQO1 and γ-GCS$_h$ expression were induced concomitantly with arsenic induction of Nrf2 (Fig. 2A and B). Importantly, the transcript levels of these two genes

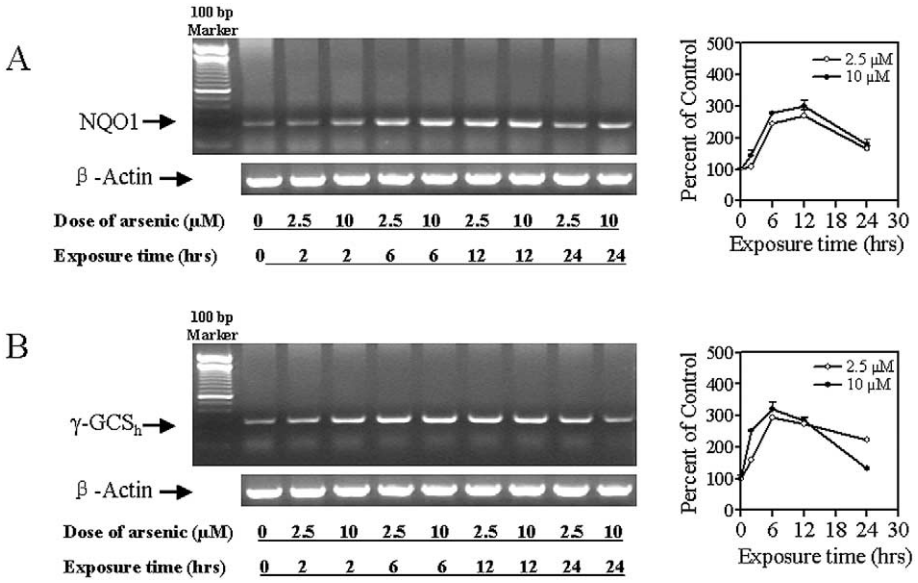

Fig. 2. Arsenic induces NQO1 and γ-GCS_h expression. Cells were treated as in Fig. 1 and total RNA was then extracted. (A) NQO1 mRNA levels. (B) γ-GCS mRNA levels. Figures on right side are quantitative analyses of A and B.

were clearly correlated with nuclear Nrf2 levels (see Fig. 1). It should be noted that although the predicted molecular mass of Nrf2 is 66 kDa, it is known to migrate anomalously in 1D PAGE [37], showing two immunoreactive bands at approximately 96 kDa as in the present study.

Arsenic increases Nrf2 protein and transcript levels

Previous studies have suggested that Nrf2 is sequestered in the cytoplasmic compartment in an inactive form and that oxidants and xenobiotics activate Nrf2, in part, by permitting transport into the nucleus [26,27]. To determine if arsenic regulates Nrf2 activity in this manner, we monitored the time course of Nrf2 levels in subcellular fractions after arsenic exposure. The initial hypothesis was that Nrf2 levels would reveal a time-dependent decay in the cytosolic fraction with a concomitant increase in the nuclear fraction. However, the Nrf2 protein levels in cytosolic extracts increased after 2 h of treatment with arsenic and peaked at 12 to 24 h (Fig. 3A). Nrf2 protein levels in whole-cell homogenates also were found to increase in a similar pattern (Fig. 3B). This arsenic-induced Nrf2 up-regulation was confirmed at the mRNA level by RT–PCR (Fig. 4). Together these data indicate that the elevated levels of Nrf2 protein in arsenic-treated HaCaT cells were, at least in part, due to an increase in Nrf2 gene transcription and that increases occur

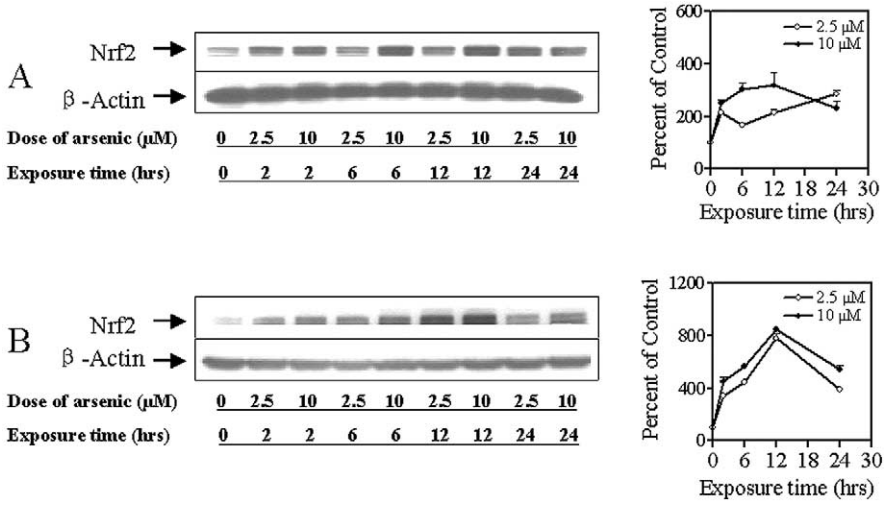

Fig. 3. Arsenic increases cytosolic and total cellular Nrf2 protein levels. Cells were treated as in Fig. 1. (A) Nrf2 levels in the cytosolic fraction. The cytosolic fractions were concentrated using a Microcon concentrator at 14,000*g* at 4°C prior to analysis. Each lane was loaded with 40 μg of proteins. (B) Nrf2 levels in total cellular protein. Each lane was loaded with 30 μg of proteins. Figures on right side are quantitative analyses of A and B.

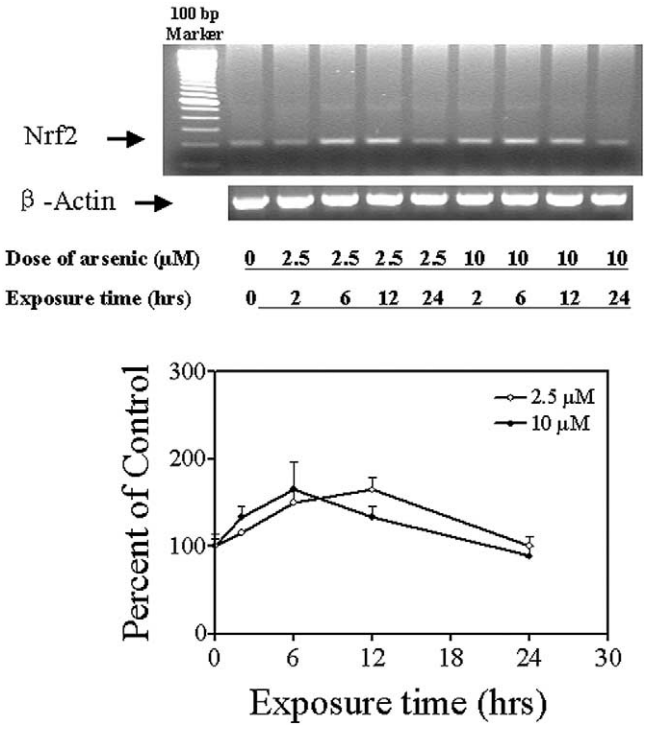

Fig. 4. Arsenic increases Nrf2 transcript levels. Cells were treated as in Fig. 1 and mRNA levels were detected by RT–PCR as described under Materials and methods. Lower figure is quantitative analysis of the results.

in both the cytosolic (Fig. 3) and nuclear (Fig. 1) compartments in a coordinated fashion.

Analysis of proteins detected in the cytosol by Western blot (Fig. 5A) from control or arsenic-exposed cells confirmed the marked increase in cytosolic Nrf2 protein after arsenic exposure (10 μM, 6 h). Clear increases were seen in cytosolic proteins of approximately 96 kDa, which corresponds to Nrf2 from nuclear fractions and shows the anomalous migration previously observed [37]. Although other cytosolic bands were observed (at 85, 78, and 41 kDa), these did not correspond to nuclear Nrf2 and were unaltered by arsenic exposure (Fig. 5B). This pattern was typical for all doses and durations of arsenic treatment. Because of the lack of correspondence in size to nuclear Nrf2 and the absence of response to a nuclear Nrf2-inducing agent (i.e. arsenite), the cytosolic bands migrating at 85, 78, and 41 kDa may represent nonspecific binding of the antibody used for Western blot analysis. Only the 96-kDa bands had been used for cytosolic Nrf2 protein quantitation (see Fig. 3).

Arsenic increases Keap1 expression at the transcriptional and translational levels

A well-established mechanism for activation of the Nrf2-ARE pathway is the dissociation of Nrf2 from Keap1 under conditions of oxidative stress [26]. Recent results also show that Keap1 is an effective inhibitor of Nrf2 degradation by the ubiquitin proteasome pathway [38], suggesting it acts as a stabilizer of Nrf2, which may allow for protracted activity. The present results clearly show that arsenic exposure resulted in a marked increase in Keap1 transcript (Fig. 6) and protein (Fig. 7) levels. The peak occurred at the same time point as for the maximal arsenic-induced increases in Nrf2 nuclear accumulation (6 h; Fig. 1) or production (6–12 h; Fig. 3). Thus, arsenic coordinately causes the induction of cellular accumulation of Nrf2 and Keap1.

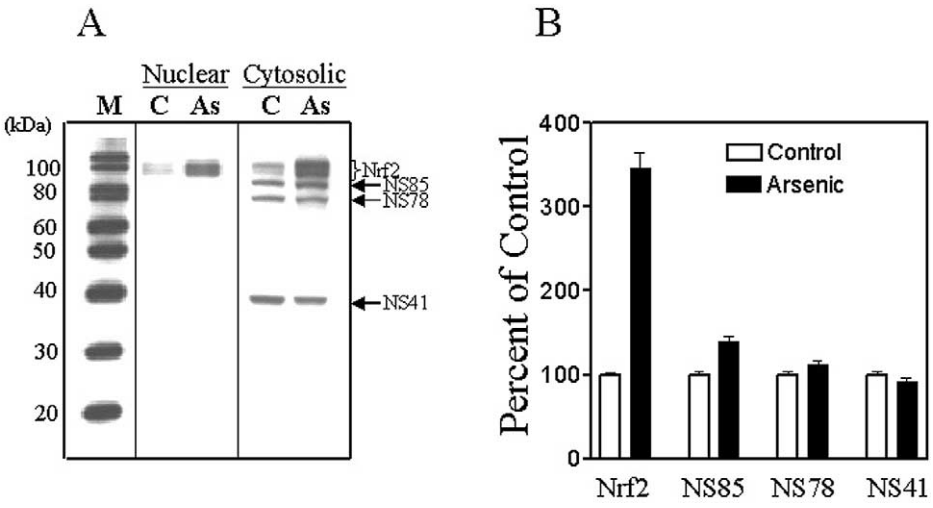

Fig. 5. Specificity of the Nrf2 antibody in Western blot analysis of cytosolic fraction samples. (A) Representative images of Nrf2 Western blot results in nuclear and cytosolic fractions derived from control and arsenic-exposed (10 μM, 6 h) cells. M, molecular weight markers; C, control; As, arsenic-exposed. Only the characteristic double bands at ~96 kDa were observed in nuclear fractions, whereas in the cytosol apparent nonspecific binding also occurred at 85 kDa (termed as NS85), 78 kDa (NS78), and at 41 kDa (NS41). (B) Quantitative analyses of the Western blot results in cytosolic fraction normalized by setting levels in control cytosol to 100%. Arsenic exposure, although clearly increasing Nrf2, did not alter the levels of nonspecific proteins (NS85,-78, and -41). These results are typical of all doses or durations of arsenic exposure.

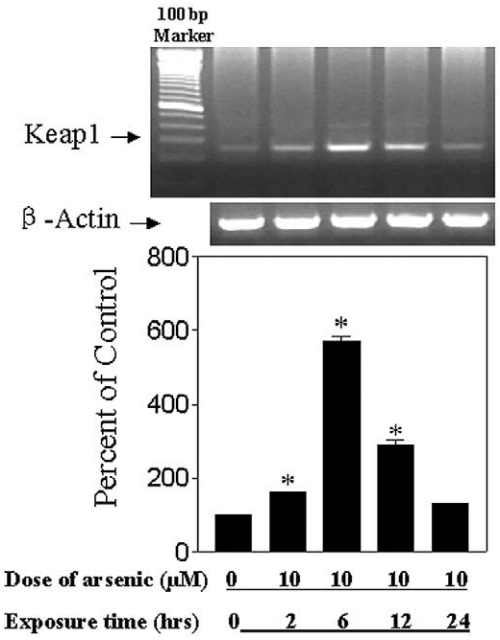

Fig. 6. Arsenic increases Keap1 mRNA levels. Cells were treated as in Fig. 1 and Keap1 mRNA levels were detected by RT–PCR as described under Materials and Methods. Lower figure is quantitative analysis of the results. (*) Significantly different from control (0 time).

Effects of ROS scavengers on the nuclear accumulation of Nrf2 induced by arsenic

To determine if production of ROS mediates the process by which inorganic arsenic increases Nrf2 production and activity, we examined the effects of several ROS scavengers on the Nrf2 levels in the nuclear fraction after arsenic exposure. Common ROS are superoxide ($\cdot O_2^-$) and H_2O_2. $\cdot O_2^-$ is enzymatically converted to H_2O_2 by superoxide dismutase (SOD), which is then converted to H_2O and O_2 by catalase (CAT). As shown in Fig. 8, pretreatment with CuDIPSH (100 μM), a cell-permeable SOD mimic compound which metabolizes the conversion of $\cdot O_2^-$ to H_2O_2, increased nuclear Nrf2 accumulation. In contrast, Tiron (10 mM), a cell-permeable $\cdot O_2^-$ scavenger that reduces the amount of $\cdot O_2^-$ available for conversion to H_2O_2, suppressed the arsenic-induced increase of nuclear Nrf2 levels. PEG-CAT (2000 U/ml), a cell permeable CAT which reduces cellular H_2O_2 levels, markedly reduced the nuclear accumulation of Nrf2. Together these results indicate that H_2O_2 is an important mediator of the process by which arsenic induces nuclear Nrf2 accumulation, whereas $\cdot O_2^-$ appears to play a more limited and indirect role.

Role of arsenic stimulated H_2O_2 production in increased nuclear Nrf2 levels

The nonfluorescent dye CM-H_2DCFDA passively diffuses into cells, where it can then be oxidized by various ROS to a detectable fluorescent form. Fig. 9A shows the fluorescence images of cells treated with CM-H_2DCFDA and then exposed to arsenic. Short-term (20 min) arsenic exposure clearly caused the production of intracellular ROS in HaCaT cells. Pretreatment of the cells with PEG-CAT markedly reduced arsenic-induced ROS production, suggesting most of the fluorescence detected after arsenic exposure originated from H_2O_2. HaCaT cells exposed to arsenic also showed dramatic increases extracellular H_2O_2 levels (Fig. 9B), which likely reflect increased intracellular levels.

To help further define the involvement of the H_2O_2 in the arsenic-induced Nrf2 up-regulation, cells were directly exposed to various concentrations of H_2O_2 and Nrf2 levels were measured. H_2O_2 caused a time- and dose-dependent increase of Nrf2 expression in HaCaT cells (Fig. 10) in a fashion similar to that of arsenic (see Figs. 1, 3, and 5).

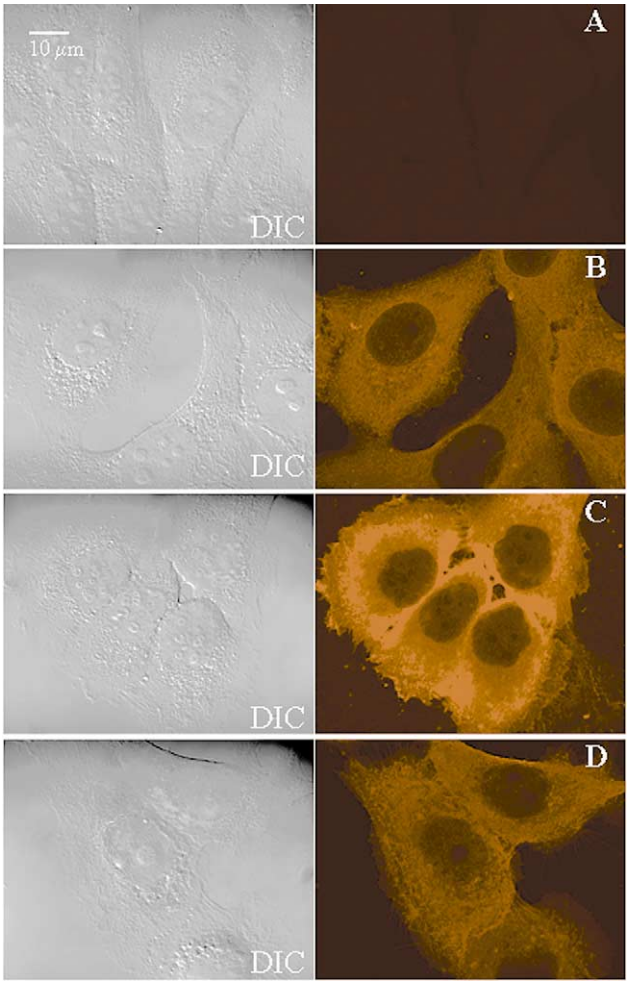

Fig. 7. Arsenic-induced immunoreactive Keap1. Cells were treated as follows: (A) No primary antibody control; (B) control (no arsenic treatment); (C) arsenic (10 μM) for 6 h; (D) arsenic (10 μM) for 24 h. DIC, differential interference contrast.

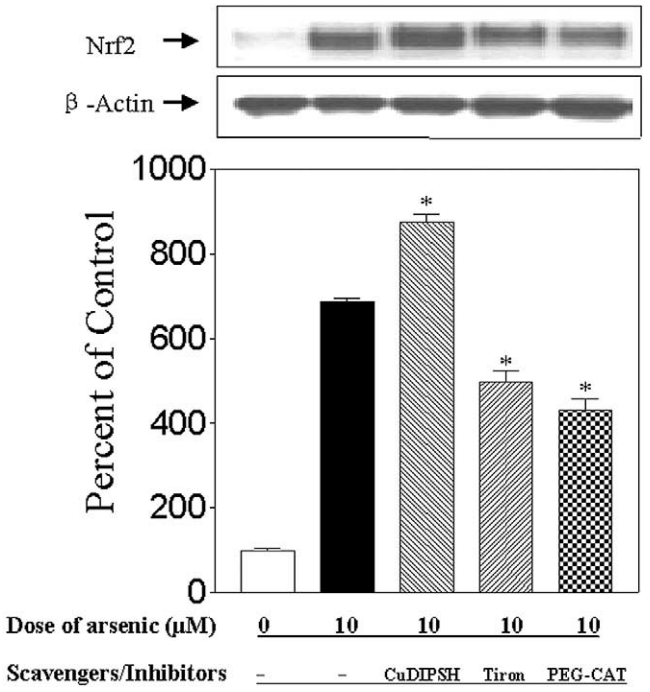

Fig. 8. Effects of agents modifying ROS levels or metabolism on nuclear accumulation of Nrf2 induced by arsenic. Cells were pretreated with CuDIPSH (100 μM), Tiron (10 mM), or PEG-CAT (2000 U/ml) for 30 min prior to arsenic exposure (time = 6 h). (*) Significantly different from arsenic alone.

Discussion

Nrf2 is a ubiquitously expressed transcription factor that occurs in a wide range of tissues and cell types, including keratinocytes [13,39]. Skin is a major target organ for the chronic toxic and carcinogenic effects of inorganic arsenic [1]. Nrf2 has been reported as an important regulator in the skin wound healing process accompanied with excess ROS production [39]. The present work clearly demonstrates the relationship between arsenic exposure and increased cellular and nuclear accumulation of Nrf2, which in turn activates the expression of Nrf2-regulated oxidative stress response genes. Importantly, arsenic activation of Nrf2 was demonstrated in a human skin cell line, implicating this oxidative stress response cascade as an important event in a potential target cell of arsenic carcinogenesis. Previous studies have suggested that Nrf2 is sequestered in the cytoplasm in an inactive form and that oxidative stress activates Nrf2 by permitting translocation into the nucleus, indicating the regulation of Nrf2 activity may be partially or wholly mediated by such posttranscriptional mechanisms [26,40]. However, the present data indicate that the elevated levels of Nrf2 in arsenic-treated HaCaT cells were, at least in part, due to an increase in transcription of the Nrf2. These results are consistent with the findings of Braun et al. [39] which identified the Nrf2 gene as a novel target of keratinocyte growth factor action and showed Nrf2 is similarly regulated at the transcriptional level. However, other work indicates that ROS activation of Nrf2 and the subsequent activation of oxidative stress response gene occurs in the absence of any changes in Nrf2 gene transcript in mouse macrophages [25]. Thus, it would appear that multiple mechanisms may be involved in Nrf2 activation, including transcriptional and posttranscriptional events. Given the remarkable nuclear accumulation of Nrf2 protein and the overproduction of Nrf2 gene transcript seen with arsenic in the present study, it seems likely that arsenic may act at multiple levels to activate this oxidative stress response system.

The known universal property of most of phase 2 enzyme inducers is their reactivity with sulfhydryl groups, which has been proposed as responsible for the initial sensing of inducers [41]. The actin-bound protein Keap1 contains 624 amino acids with 25 cysteine residues, at least 4 of which are known to possess highly reactive sulfhydryl groups [28]. Accordingly, Keap1 has been proposed as a cellular sensor for protection against oxidative and electrophilic stress, possibly through reaction of critical sulfhydryls [28]. One mechanism for Nrf2-induced transcriptional regulation of oxidative stress response enzymes is through the disruption of the cytoplasmic complex between Keap1 and Nrf2, allowing free Nrf2 to migrate to the nucleus where it heterodimerically combines with other transcription factors and activates the ARE [26,42,43]. Dinkova-Kostova et al. [28] recently provided direct evidence that sulfhydryl groups of Keap1 are critical sensors regulating induction of phase 2 enzymes that can protect against reactive carcinogens and/or oxidants. It is generally recognized that a major aspect of the toxicity of arsenic is directly related to its ability to bind with sulfhydryl groups, especially vicinal thiols within proteins, resulting in altered function. This includes arsenic-induced inhibition of enzymatic activity [44] as well as structural alterations of proteins [45]. Indeed, the organometallic phenylarsine oxide

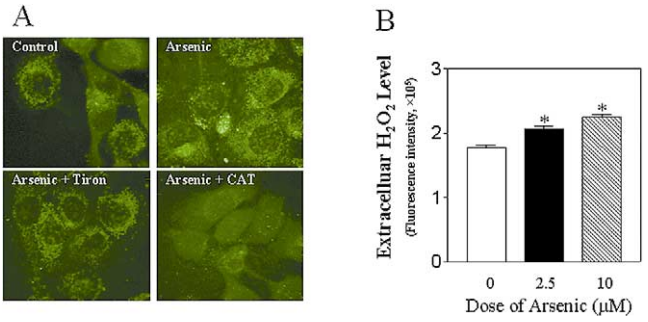

Fig. 9. Arsenic stimulated H_2O_2 production in HaCaT cells. (A) Representative images of fluorescent signals of intracellular H_2O_2 formation. Control: cells treated with CM-H_2DCFDA (1 μM) for 40 min; Arsenic: cells pretreated with CM-H_2DCFDA for 20 min and then treated with arsenic (10 μM) for 20 min; Arsenic + Tiron: cells pretreated with Trion (10 mM) for 20 min prior to addition of CM-H_2DCFDA and arsenic addition; Arsenic + CAT: cells pretreated with PEG-CAT (2000 U/ml) for 20 min prior to CM-H_2DCFDA and arsenic addition. (B) Extracellular H_2O_2 production after arsenic exposure. Cells were treated as described under Materials and methods and fluorescence of the extracellular media was determined. (*) Significantly different from control.

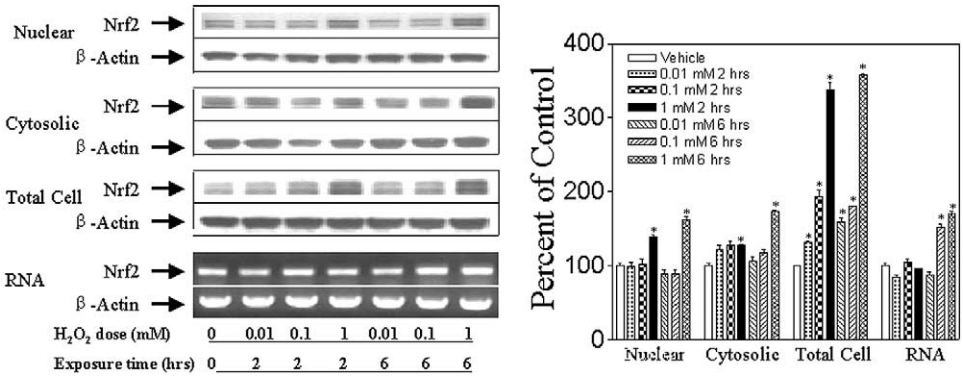

Fig. 10. Direct effect of H_2O_2 on expression of Nrf2. HaCaT cells were treated with H_2O_2 at doses and times indicated. Groups include the following: Nuclear, Nrf2 protein levels in nuclear extracts; Cytosolic, Nrf2 protein levels in cytosolic fractions; Total cell, Nrf2 protein levels in total cell homogenate; RNA, Nrf2 mRNA levels. Panels on left show results of Western blot or RT–PCR as appropriate. Plot on right is the results of quantitative analysis. (*) Significantly different from appropriate control.

which is highly reactive with protein sulfhydryls, has been shown to be a potent competitor of [^{3}H]dexamethasone mesylate (an irreversible modifier of thiols) binding to Keap1 [28]. Thus, it appears that the modification of sulfhydryl groups within Keap1 by arsenic may be one mechanism that the metalloid facilitates nuclear Nrf2 accumulation, although additional research will be require to confirm this contention.

Recent results suggest that free Nrf2 is targeted for ubiquitination- and proteasome-dependent degradation [34,40]. Increased protein stability is a posttranscriptional mechanism that enhances Nrf2-mediated induction of the oxidative stress response [35,40]. Sekhar et al. [38] reported that the association of Nrf2 with Keap1 actually stabilizes Nrf2. Thus, maintaining Nrf2 in a bound form, which appears resistant to ubiquitin–proteasome degradation [38], could provide a critical pool of Nrf2 that is available for immediate Nrf2/ARE-dependent gene transcription when cells are exposed to oxidative stress. However, Itoh et al. [46] recently reported that Keap1 not only regulates cytoplasmic-nuclear Nrf2 shuttling but also enhances the degradation of Nrf2, indicating Keap1 potentially exerts a negative control over the Nrf2-mediated response. In the present study, arsenic exposure resulted in a marked increase in Keap1 at both transcriptional and protein levels. The maximal increases in Keap1 after arsenic exposure occurred in the same time frame as maximal Nrf2 production or nuclear accumulation and preceded Nrf2 activation of NQO1 and γ-GCS_h. The coordinate increase of Keap1 and nuclear Nrf2 accumulation, together with subsequent oxidative stress enzyme induction, suggest that Keap1 takes part in the regulation of arsenic-induced nuclear Nrf2 accumulation. This regulatory function of Keap1 appears to play a major role in the protection of cells from oxidative stress after arsenic exposure. The present results also suggests Keap1 may itself be one target gene for activation by Nrf2. It is also possible that arsenic directly activates Keap1 expression through an undefined mechanism. Regardless, our results support the function of Keap1 as a central regulatory molecule for Nrf2 activity in response to oxidative stress. In addition, because arsenic can influence multiple signal transduction events [46], whether arsenic can affect Nrf2-Keap1 association by other pathways such as PKC-mediated phosphorylation of Nrf2 and PI3-kinase-regulated depolymerization of actin clearly deserve further investigation.

Prolonged production of elevated levels of ROS can adversely affect a variety of cellular functions and can damage critical biomolecules, including DNA or protein [47]. It is postulated that elevation of ROS will lead to early events in carcinogenesis and may be a general mechanism of action for various inorganic carcinogens [12]. In addition, elevated levels of ROS, including H_2O_2, can mediate aberrant intracellular signaling, including signaling involved in growth and malignant transformation [48]. H_2O_2 is uncharged, freely diffusible within and between cells, and, compared with other ROS, is quite stable. The primary origin of cellular H_2O_2 is superoxide ($O_2^{\cdot-}$), which is rapidly dismutated to H_2O_2 by the SOD. Removal of H_2O_2 is regulated by two important enzymes, CAT and glutathione peroxidase [49]. Based on the characteristics of stability and diffusibility and together with tight regulation of its production and removal, it is suspected that H_2O_2 may act as a second messenger [50]. In this regard, of particular interest in the present study are the results showing differential effects of $\cdot O_2^-$ and H_2O_2 scavengers on arsenic-induced nuclear Nrf2 accumulation. Pretreatment of HaCaT cells with agents that reduced cellular H_2O_2 levels (PEG-CAT and Tiron) partially suppressed nuclear Nrf2 accumulation. On the other hand, the specific SOD mimic CuDIPSH, which enhances production of H_2O_2 from $\cdot O_2^-$, increased Nrf2 induction by arsenic. Thus, treatments that enhance or suppress cellular H_2O_2 have a concordant effect on arsenic-mediated alterations in Nrf2. Indeed, arsenic exposure at levels enhancing Nrf2 activity rapidly resulted in increased production of H_2O_2. As little as 20 min of arsenic exposure

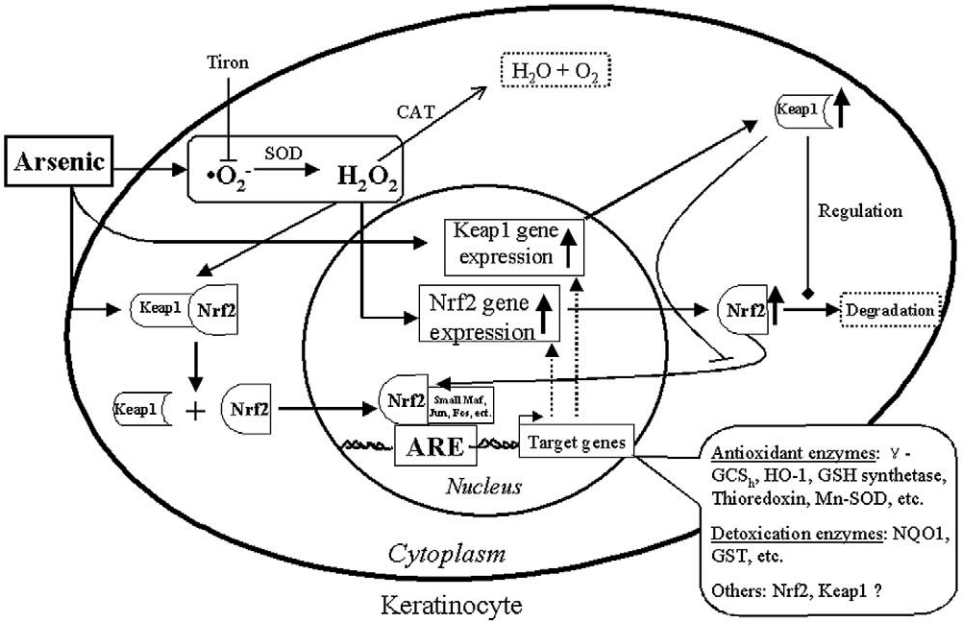

Fig. 11. A putative mechanism for nuclear Nrf2 accumulation caused by arsenic. Based on accumulated findings, it appears arsenic can dissociate Nrf2 from Keap1 in cytosol. This appears to be caused both directly by arsenic and through production of excess ROS, especially H_2O_2. Nrf2 can then translocate to the nucleus. It is known Nrf2 requires some other leucine zipper proteins, such as small Maf proteins, Jun or c-Fos proteins, for final activity at the ARE. Together with these interaction partners, Nrf2 binds to ARE and increases the expression of target genes. In addition, arsenic, possibly through arsenic-derived H_2O_2, up-regulates Nrf2 transcription which then amplifies the signaling at the ARE. It also appears that arsenic increases Keap1 production which takes part in regulation of Nrf2 by sequestering and stabilizing effects on Nrf2. These components appear to work together to allow ARE-related gene expression after arsenic exposure.

resulted in dramatic increases in both intracellular and extracellular H_2O_2 in HaCaT cells, and pretreatment with PEG-CAT or Tiron inhibited the arsenic-induced stimulation of H_2O_2 production. The current study also provides the first evidence that direct exposure of cells to H_2O_2 can cause a time- and dose-dependent nuclear Nrf2 accumulation in a fashion similar to that of arsenic. These findings are consistent with the results of Li et al. [51] showing H_2O_2 induces ARE-regulated chloramphenicolacetyl transferase and indicate H_2O_2 may be a normal mediator of the Nrf2-based oxidative response. Together, the data of the present study strongly implicate H_2O_2, rather than $\cdot O_2^-$, as the ultimate ROS mediator of arsenic-induced nuclear Nrf2 accumulation. This does not, however, eliminate $\cdot O_2^-$ or other ROS as indirect participants in the arsenic-mediated Nrf2 response as precursors of H_2O_2. It is also noteworthy that, although exogenous CAT essentially abolishes arsenic-induced increase in intracellular H_2O_2, there is still a significant increase of nuclear Nrf2 accumulation induced by arsenic, fortifying the concept that arsenic acts through multiple mechanisms to enhance the nuclear accumulation of Nrf2 (Fig. 11).

In summary, nuclear Nrf2 accumulation was induced by arsenic exposure in cultured keratinocytes and this appears to be regulated at multiple levels by a coordinated process partly mediated by H_2O_2. These findings provide evidence of a primary molecular response in a potential target cell of arsenic carcinogenesis and, as such, may help further our understanding of the mechanism by which arsenic is active as a carcinogen.

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