Sodium Arsenate Induces Overproduction of Interleukin-1α in Murine Keratinocytes: Role of Mitochondria

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It has recently been demonstrated that arsenic induces overexpression of keratinocyte-derived growth factors, which are likely to have a significant role in arsenic-induced skin hyperkeratoses and cancer. The mechanism(s) involved in this induction are, however, still elusive. The purpose of this study was to investigate the early intracellular events that follow *in vitro* treatment with sodium arsenate in a murine keratinocyte cell line (HEL30), which leads to cytokine overproduction. First, we observed that sodium arsenate induced a concentration-dependent production of interleukin-1α and a significant increase in cell proliferation, that could be suppressed by the addition of a neutralizing antibody against murine interleukin-1α, confirming the ability of arsenic to induce keratinocyte growth-promoting cytokines. Electron microscopic analysis revealed that arsenate induced a dramatic alteration in keratinocyte mitochondria. This effect could be prevented by rotenone pretreatment, which suggests the possible involvement of mitochondria-derived reactive oxygen species. Arsenic induced a concentration- and time-dependent increase in cellular oxidative activity, which was followed by activation of redox-sensitive transcription factors such as nuclear factor-κB and activator protein-1, that are essential for interleukin-1α synthesis. Prior treatment with rotenone or prolonged treatment with ethidium bromide, an inhibitor of mitochondrial DNA and RNA synthesis, to deplete cells of functional mitochondria, completely prevented sodium arsenate-induced interleukin-1α production, this indicates the pivotal role of these organelles in sodium arsenate-induced keratinocyte growth factors. ***Key words: cytokines/hyperkeratosis/reactive oxygen species/skin tumor. J Invest Dermatol 113:760–765, 1999***

The use of arsenic as a therapeutic agent and poison dates back 2400 y. Arsenic is a human carcinogen in skin, lung, urinary bladder, kidney, and liver. Although over the last 50 y general exposure to arsenic has greatly decreased, particularly of sodium arsenate (As) in pesticides, this element is commonly found in drinking water (naturally or as a smelter by-product), in certain foods and in cigarette smoke. In recent years, arsenic contamination of drinking water has become a public health concern. The current US Environmental Protection Agency standard for inorganic arsenic in drinking water is 50 μg per liter. The Environmental Protection Agency risk analysis predicts an increased lifetime skin cancer risk of 3 or 4 per 1000 population from chronic exposure at that concentration (Brown *et al*, 1997).

Because of the affinity of arsenic for SH groups and its binding to keratin (Lindgren *et al*, 1982), skin is a critical organ in arsenic toxicity. A wide variety of skin lesions have been associated with arsenic toxicity, particularly from chronic exposure in drinking water and certain types of occupational exposure. A characteristic finding is verrucous hyperkeratoses of the palms and soles of the feet. Hyperpigmentation or melanosis is also common. Skin cancers induced by arsenic include Bowen's disease, basal cell carcinoma, and squamous cell carcinoma (Maloney, 1996). Arsenic is not a direct genotoxic carcinogen; there is currently no accepted mechanism for its carcinogenic action. It has recently been suggested that arsenic may induce skin tumors and hyperkeratosis by chronic stimulation of keratinocyte-derived growth factors (Germolec *et al*, 1997). Both *in vitro* in primary human keratinocytes and *in vivo* in transgenic TG.AC mice an increase has been shown (Germolec *et al*, 1996) in mRNA transcripts of keratinocyte growth factors, including transforming growth factor-α (TGF-α) and granulocyte/macrophage-colony stimulating factor following treatment with sodium arsenite. Injection of anti-granulocyte/macrophage-colony stimulating factor antibody following application of 12-*O*-tetradecanoylphorbol 13-acetate in these transgenic mice significantly reduced the number of papillomas: this supports the role of keratinocyte-derived growth factors in arsenic-induced skin toxicity (Germolec *et al*, 1997). The mechanism(s) responsible for the induction of cytokines by arsenic exposure is, however, unknown.

It has been known for many years that several sulfhydryl-containing proteins and enzyme systems are altered by exposure to arsenic. In particular, arsenic affects mitochondria enzymes and impairs tissue respiration (Brown *et al*, 1976). Mitochondria accumulate arsenic and it has been suggested that arsenic inhibits energy-linked functions of mitochondria in two ways: competition with phosphate during oxidative phosphorylation and inhibition of energy-linked reduction of nicotinamide adenine dinucleotide (Mitchell *et al*, 1971). We have recently shown that mitochondria play an important part in skin irritant-induced interleukin (IL) -1α production. In particular, we

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Abbreviations: AP-1, activator protein-1; As, sodium arsenate; BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid; EMSA, electrophoretic mobility shift assay; NF-κB, nuclear factor-κB; Rot, rotenone; AU, arbitrary unit of fluorescence.

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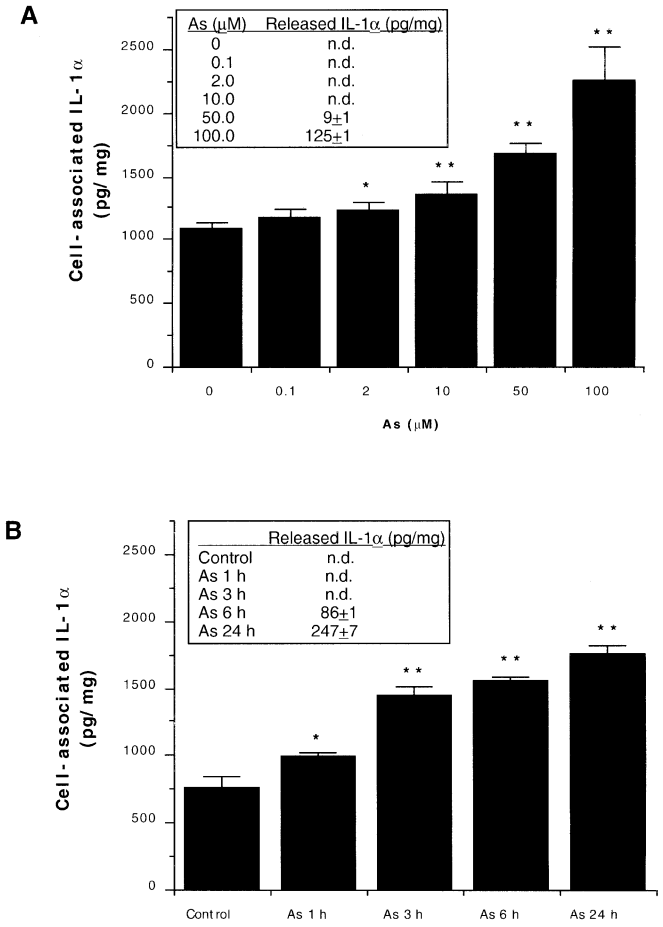

Figure 1. As induces IL-1α production in murine keratinocytes. (*A*) Confluent HEL30 cells treated for 24 h with increasing concentrations of As. (*B*) Confluent HEL30 cells treated for different times with As 100 μM. In the *insets* the release of IL-1α in cultured medium is reported. Results are expressed in pg per mg of protein of the cell extracts. n.d. = not detectable. Each value represents the mean ± SD of three to four determinations. Statistical analysis by Dunnett's test, *$p < 0.05$ and **$p < 0.01$ *versus* control cells (0).

have shown (Corsini *et al*, 1996a) that the skin irritant tributyltin (TBT) induces both *in vivo* and *in vitro* a dose-related production of IL-1α. *In vitro*, IL-1α production by TBT is preceded by the generation of reactive oxygen species (ROS), which activate nuclear factor (NF) -κB (Corsini *et al*, 1996b). Blocking mitochondria metabolism resulted in a dramatic reduction of TBT-induced NF-κB activation and IL-1α production, which indicated that mitochondria act as mediators of TBT effects and gene-regulatory signaling pathways (Corsini *et al*, 1996b). We speculated that disturbance of mitochondria activity by arsenic, may divert electrons from the respiratory chain into the formation of the kinds of ROS known to be involved in the activation of transcription factors and production of cytokines (Ilnicka *et al*, 1993; Lee and Ilnicka, 1993; Baeuerle and Henkel, 1994). Here, we demonstrate that one of the earliest intracellular events following As treatment is the generation of ROS by mitochondria followed by activation of transcription factors, which confirms mitochondria as an important target in As toxicity. We were able to correlate these early events with a later increase in IL-1α and cellular proliferation.

MATERIALS AND METHODS

Chemicals As, ethidium bromide, and rotenone were obtained from Sigma (St Louis, MO); whereas 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate di(acetomethyl ester) (DCFH) and 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA) were obtained from Molecular Probes (Eugene, ON). All reagents were purchased at the highest purity available.

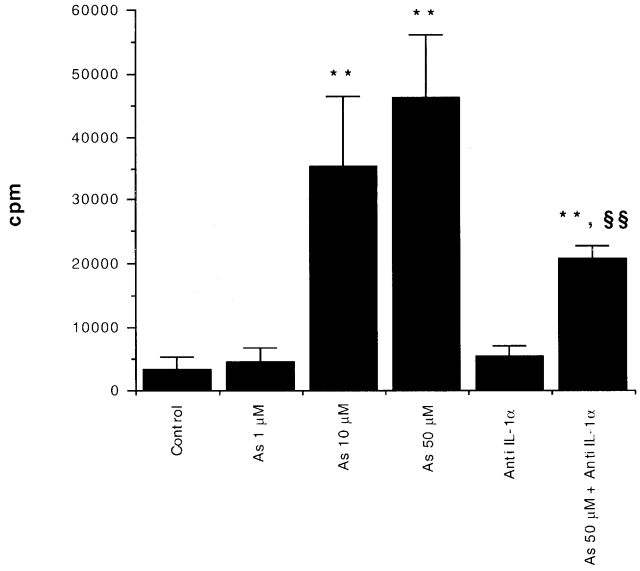

Figure 2. As stimulates keratinocyte proliferation. Keratinocytes were cultured in a 96-well plate. Six replicate wells per condition were used for determination of ^{3}H-thymidine incorporation. Each bar represents the mean ± SD. **$p < 0.01$ *versus* control cultures and §§$p < 0.01$ *versus* As 50 μM.

Cell culture and treatment The C3H mouse-derived keratinocyte cell line HEL30 (kindly supplied by Dr N.E. Fusenig, Cancer Research Center, Heidelberg, Germany) was cultured as previously described (Corsini *et al*, 1994). Cells were maintained in RPMI 1640 medium (Sigma) supplemented with 10% fetal bovine serum (Sigma), penicillin (100 U per ml), and streptomycin (100 μg per ml) (medium) at 37°C in a 5% CO_2 humidified incubator.

Cells were grown to confluence, as judged by optical microscopic observation (≈$5.2 \pm 1.2 \times 10^5$ cells), in a 24-well plate (Corning, NY). Monolayers were washed once with medium and treated with 0.3 ml of medium containing different concentrations of As (0.5–100 μM) dissolved in distilled water or with water alone as the vehicle control. After different times of incubation, the culture medium was recovered and centrifuged for determination of IL-1α release enzyme-linked immunosorbent assay (see below). The monolayers were washed once with phosphate-buffered saline (PBS) and lysed in 0.3 ml of PBS containing Triton X-100 (0.5%), and the intracellular IL-1α was determined.

For proliferation studies, 1.5×10^3 cells per well were seeded on to 96-well flat-bottom plates in 100 μl of medium containing different concentrations of As. A neutralizing antibody against murine IL-1α (Genzyme, Cambridge, MA) at 1:200 dilution was added to the medium to assess the role of As-induced IL-1α production in keratinocyte proliferation. After 48 h the cells were pulsed with 1 μCi per well of ^{3}H-thymidine (specific activity 47 Ci per mmol; Amersham, Little Chalfont, U.K.) and incubated for a further 24 h. The cells were then collected on to glass-fiber filters using an automated cell harvester (Dynatech Minimash 2000, PBI, Milan, Italy), and ^{3}H-thymidine incorporation was quantitated by liquid scintillation counting. Results are expressed in counts per minute.

Oxidative activity in living cells For the measurement of oxidative activity (see below) cells were grown to confluence on sterile round glass slips (Knittel Glaser, Germany) in a 24-well plate. The cells were washed once with Hank's balanced salt solution without phenol red, calcium, or magnesium (HBSS), containing 2% of bovine serum albumin and loaded with DCFH 10 μM (stained) or dimethylsulfoxide (unstained) for 1 h at 37°C. Cells were then washed once with HBSS and oxidative activity was assessed as follows. Glass coverslips in quartz cuvettes containing 2 ml of HBSS were treated with different concentrations of As or with water as vehicle control. ROS production was measured as DCFH oxidation by the intensity of the emission of 525 nm fluorescence excited by 503 nm (Perkin Elmer LS 50B, Monza, Italy), at times 0, 5, 15, and 30 min after treatment, both in stained and unstained cells. Results are expressed as the change in fluorescence (in arbitrary units, AU) calculated as follows:

$$AU = [I_{stained} - I_{unstained}]_{tx} - [I_{stained} - I_{unstained}]_{t0}$$

where I represents the intensity of fluorescence.

To assess the modulation of As-induced oxidative activity by Ca^{2+} or

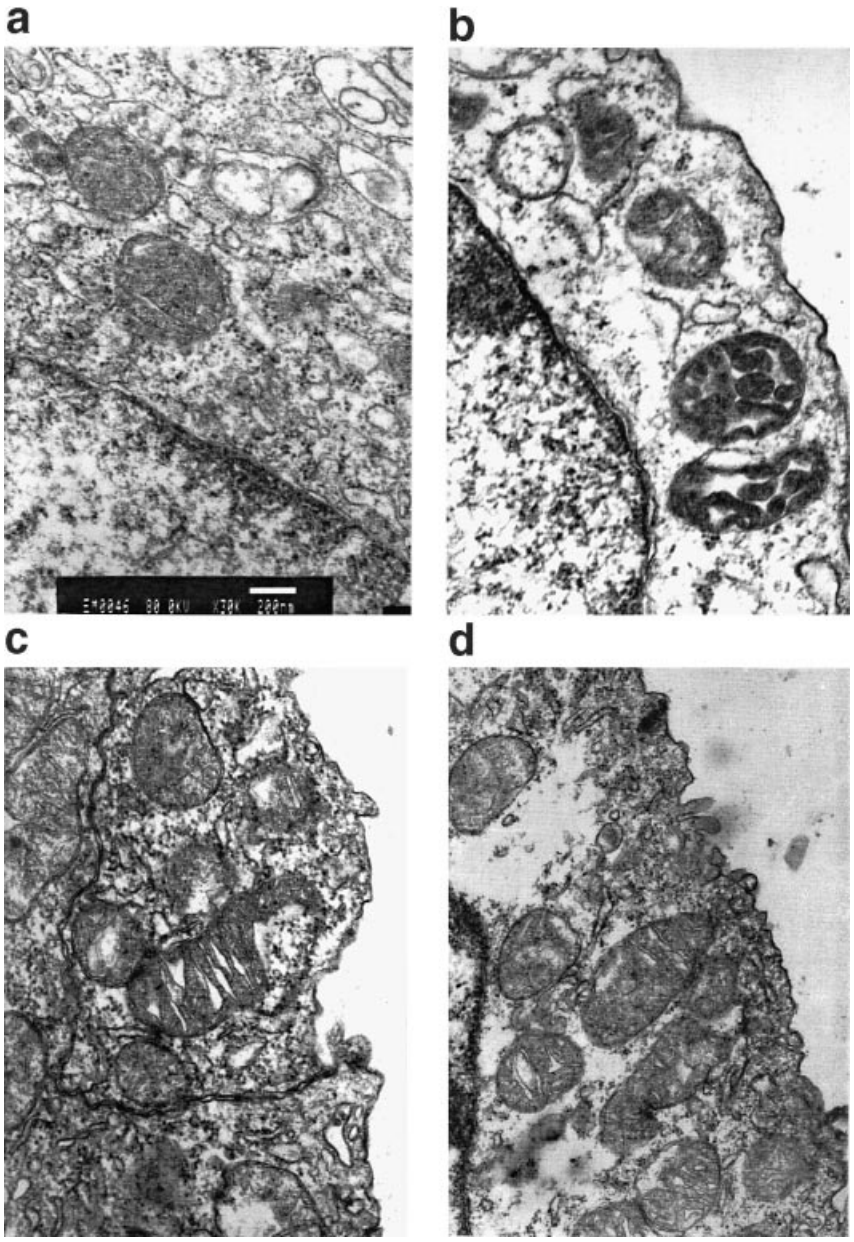

Figure 3. Electron microscopy shows morphologic alterations in HEL30 cells after treatment with As 50 μM, restoration by rotenone treatment. (*a*) Control cells. (*b*) Cells 30 min after treatment with As. (*c*) Cells 1 h and 30 min after treatment with rotenone. (*d*) Cells treated for 1 h with rotenone and As 50 μM added for a further 30 min.

mitochondria, we treated cells during the loading period with BAPTA (10 μM), a calcium chelator, or with rotenone (20 μM), an inhibitor of complex-I, for 1 h and then exposed the cells to 10 μM in As or water as vehicle control. Oxidative activity was measured after 30 min

Role of mitochondria in IL-1α production To assess the role of mitochondria in As-induced IL-1α production we used two different strategies. First, confluent cells were treated for 1 h with rotenone (20 μM) and then exposed to As or water for 24 h. In addition, to confirm the role of mitochondria as intracellular signal transducers of As, we disabled the functional mitochondria as previously described (Corsini *et al*, 1996b). Briefly, 10^5 cells per ml were plated in a 24-well plate dish and treated for 5 d with ethidium bromide (200 ng per ml) in medium supplemented with glucose (4.5 mg per ml) and uridine (5 μg per ml), as respiration-deficient cells have been reported to become pyrimidine auxotrophs (King and Attardi, 1989). At this time, cells were confluent; they were treated with As or water for 24 h and intracellular IL-1α was measured. Results are expressed as percentage of control.

Enzyme-linked immunosorbent assay Costar 3690 plates (Costar, Cambridge, MA) were coated overnight at 4°C with 0.1 ml of monoclonal anti-murine IL-1α antibody (Genzyme) diluted to 1 μg per ml in PBS. PBS containing Tween 20 (0.05%) was used to wash the plates. The washed plates were blocked with 0.1 ml of PBS containing bovine albumin (0.5%) and Tween 20 (0.05%) for 30 min at room temperature. Samples (0.1 ml) at different dilutions or recombinant murine IL-1α (Genzyme) were added for 1 h at room temperature. After three washes, 0.1 ml of rabbit anti-murine IL-1α antibody (Genzyme, diluted 1:800) was added to each well for 1 h and then 0.1 ml of goat anti-rabbit IgG conjugated with alkaline phosphatase (Sigma, diluted 1:2000) for 1 h at room temperature. p-Nitrophenylphosphate (Sigma) was used as the substrate, and the absorbance was measured at 405 nm. The limit of sensitivity for this assay was 15 pg per ml. Preliminary studies showed that As did not interfere with the enzyme-linked immunosorbent assay.

Preparation of nuclear extracts and electrophoretic mobility shift assay Nuclear extracts were prepared essentially as described by Schreiber *et al* (1989). Confluent cells in 60 mm Petri dishes were washed once with cold PBS and 2 ml of a hypotonic lysis buffer was added to each dish (buffer A: 10 mM HEPES, pH 7.8, 10 mM KCl, 1 mM dithiothreitol, 0.1 mM ethylenediamine tetraacetic acid, 0.1 mM ethyleneglycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid, 0.5 mM phenylmethylsulfonyl fluoride). Cells were incubated on ice for 15 min, then 125 μl of a 10% Nonidet p-40 (Sigma) solution was added, and cells were scraped, mixed for 15 s and then centrifuged for 30 s at 11,000 × *g*. The pelleted nuclei were washed once with 400 μl of buffer A plus 25 μl of 10% NP-40, centrifuged and then suspended in 50 μl of buffer C (50 mM HEPES pH 7.8, 400 mM NaCl, 1 mM ethylenediamine tetraacetic acid, 1 mM, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride), mixed for 20 min, and centrifuged for 5 min at 11,000 × *g*. The supernatant

containing nuclear proteins was harvested and its protein concentration was determined, and it was then stored at –80°C until used in electrophoretic mobility shift assay. Electrophoretic mobility shift assay was performed as follows. Binding reaction mixtures (20 μl) containing 5 μg protein of nuclear extract, 0.5 μg poly(dI–dC). poly(dI–dC) (Sigma), 10,000 cpm ^{32}P-labeled probe in binding buffer (10 mM HEPES pH 7.9, 50 mM NaCl, 1 mM ethylenediamine tetraacetic acid, 1 mM dithiothreitol, 10% (vol/vol) glycerol, 1% Ficoll, and 0.2 μg per ml albumin) were incubated for 30 min at room temperature before separation in a 7% acrylamide gel in 1 × Tris borate ethylenediamine tetraacetic acid followed by autoradiography. A double-stranded oligonucleotide containing the binding site for NF-κB (5′-GTCTCGCAATTCCCCTCTCTCAG-3′) or for activator protein (AP) −1 (5′-TGATGAGTCAGCCG-3′) was labeled with α-^{32}P-dATP (Amersham) using T4 polynucleotide kinase (Amersham).

Electron microscopy HEL30 cells were treated for 1 h with rotenone (20 μM) and then As (50 μM) or water were added. After 30 min the cells were fixed in 3.3% glutaraldehyde in 25 μM phosphate buffer for 1 h at 4°C, then treated with 1% osmium tetroxide in phosphate buffer for 1 h at 4°C and dehydrated and embedded in Araldite epoxy resin.

Statistical analysis All experiments were performed at least three times; representative results are shown. Results are expressed as mean ± SD. Statistical significance was determined by Student's t test or Dunnett's multiple comparison test, as indicated, after ANOVA.

RESULTS

As induces IL-1α production and increases keratinocyte proliferation A murine keratinocyte cell line (HEL30) was examined for its response to As. **Figure 1** shows that As induced a concentration- (**Fig 1*A***) and time- (**Fig 1*B***) related increase in cell-associated IL-1α. Intracellular IL-1α increased at concentrations as low as 2 μM and reached a plateau following 3 h of treatment. The release of IL-1α was detectable only at higher concentrations of As (> 10 μM; **Fig 1*A***, *inset*) and it was preceded by an increase in intracellular IL-1α (**Fig 1*B***). As 100 μM induced a slight reduction (15%) in cell viability as assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-reduction, whereas no cytotoxicity was observed at the other concentrations tested (data not shown).

IL-1α seems to be the most important cytokine in the epidermis capable of initiating the inflammatory hyperplastic process (Gniadecki, 1998). To confirm that the induction of IL-1α by As is associated with keratinocyte proliferation, the effect of As on cell proliferation was assessed my measuring ^{3}H-thymidine incorporation. As, as shown in **Fig 2**, induces a dose-related increase in keratinocyte proliferation, which could be suppressed by an anti-murine IL-1α antibody. This confirms the ability of arsenic to induce keratinocyte growth-promoting cytokines, which are likely to play a significant part in arsenic-induced skin hyperkeratoses and cancer.

Role of mitochondria in As-induced intracellular IL-1α production It has been known for some years that arsenic affects mitochondria enzymes and functions (Brown *et al*, 1976). Indeed, treatment of HEL30 cells with As 50 μM resulted in rapid (30 min) and dramatic morphologic changes in mitochondria: the organelles completely lose their internal organization (**Fig 3*b***), confirming mitochondria as an important intracellular target of As. These alterations can be prevented by a prior treatment with rotenone (**Fig 3*d***), an inhibitor of the entry of electrons from complex I to ubiquinone; this suggests the diversion of electrons from the respiratory chain as a possible cause of mitochondria alterations.

Disturbance of the proton gradient can also result in the formation of ROS, which are known to be involved in the activation of transcription factors and production of cytokines (Ilnicka *et al*, 1993; Baeuerle and Henkel, 1994). The ability of As to increase cellular oxidative activity was measured by a specific fluorimetric assay. A dose-related increase in cellular oxidative activity was detected 15 min after treatment with As; only As at 50 μM induced a slight increase 5 min after treatment (**Fig 4**). The increase in cellular oxidative activity induced by As can be completely abrogated by rotenone (**Table I**), indicating mitochondria as the intracellular source of ROS induced by As. A role for calcium ions can be ruled out, as BAPTA, a calcium chelator, did not affect As-induced ROS production (**Table I**) or IL-1α production (data not shown). We then assessed the ability of As to activate transcription factors. Both NF-kB and AP-1 were activated by As 30 min after treatment (**Fig 5**), and this preceded IL-1α production.

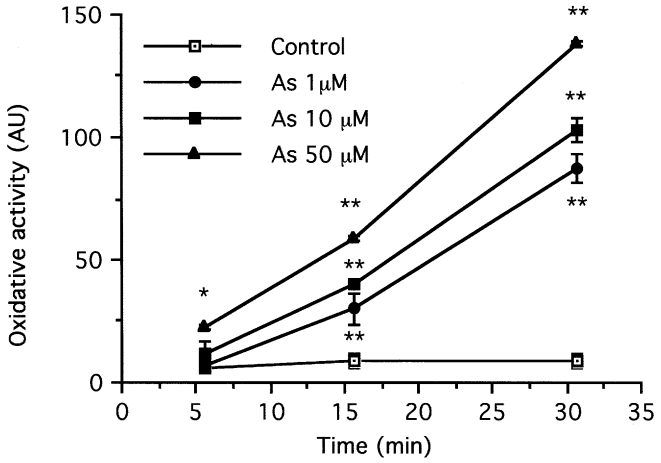

Figure 4. As increases oxidative activity in living cells as measured by DCFH oxidation. Confluent HEL30 cells were loaded for 1 h with 10 μM DCFH and then As (1–50 μM) or water (as vehicle control) was added for up to 30 min. Each value (AU) represents the mean ± SD of three independent samples. Statistical analysis by Dunnett's test, *$p < 0.05$ and **$p < 0.01$ *versus* control cells.

Table I. Rotenone, but not BAPTA, suppresses As-induced cellular oxidative activity

	Cellular oxidative activity (AU)[a]	
	Control	As 10 μM
Experiment 1		
No rotenone	32 ± 8	117 ± 28*
+ Rotenone	32 + 10	42 ± 7**
Experiment 2		
No BAPTA	16 ± 4	89 ± 5*
+ BAPTA	6 ± 3	72 ± 17*

[a]Confluent cells were treated, in two independent experiments, for 1 h with rotenone (20 μM) or with BAPTA (10 μM), and As (10 μM) or water (as vehicle control) was then added. The cellular oxidative activity was measured 30 min after treatment. Each value represents the mean ± SD of three determinations. Statistical analysis was performed by Student's t test. *$p < 0.01$ *versus* relevant control and **$p < 0.01$ *versus* cells treated with As alone.

To confirm the pivotal role of mitochondria in As-induced IL-1α production, we again used rotenone as well as cells depleted of functional mitochondria by ethidium bromide treatment (Corsini *et al*, 1996b). Confluent keratinocytes were treated with a noncytotoxic concentration of rotenone (20 μM). Rotenone completely abrogated the intracellular increase of IL-1α induced by 10 μM As (**Fig 6*a***). Furthermore, in cells depleted of functional mitochondria the addition of 10 or 50 μM As resulted in no induction of IL-1α, whereas in the absence of ethidium bromide As-induced a dose-related increase in intracellular IL-1α (**Fig 6*b***). This confirms the essential role of mitochondria as mediators of As effects and gene-regulatory signaling pathways.

DISCUSSION

This study has demonstrated the pivotal role of mitochondria as mediators of As-induced IL-1α production and gene regulatory signaling pathways. The concentrations of As used in this study were in the range reached in drinking water (0.01–3 mg per liter, equivalent to 1.5–90 μM). The natural concentration of total arsenic in drinking water varies. In unpolluted areas of the world

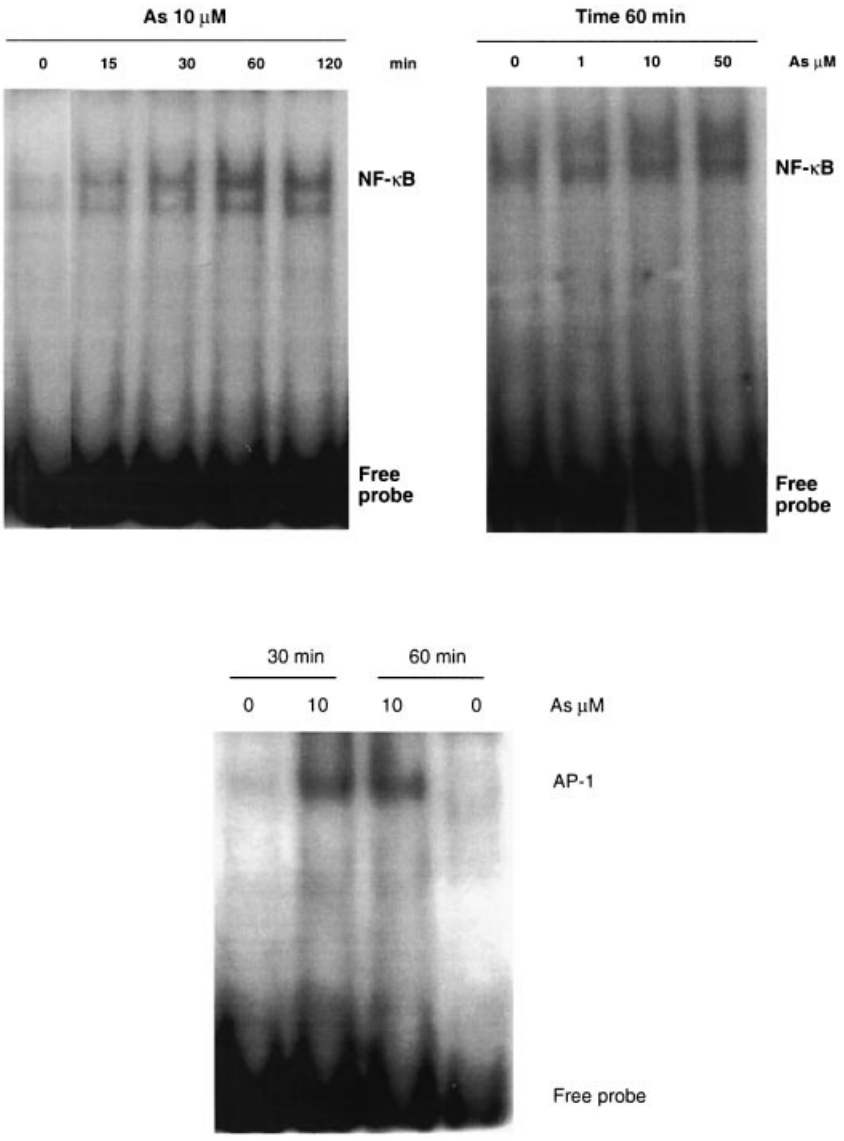

Figure 5. As induces NF-κB and AP-1 activation. Confluent HEL30 cells were treated with As (0–50 μM) for different times. Equal amounts (5 μg) of nuclear extracts were analyzed by EMSA, with ^{32}P-labeled DNA probes detecting, respectively, the binding of NF-κB and AP-1.

it is generally below 0.01 mg per liter, but high levels of arsenic, > 3 mg per liter, have been found in water from polluted areas or with arsenic-rich bedrock (WHO, 1981). The oxidation state of arsenic in the water involved is generally not known. It is likely, however, that trivalent arsenic (arsenite, As(III)) and pentavalent arsenic (arsenate, As(V)) would have similar effects, as absorbed arsenate is to a large extent reduced in the blood to arsenite (Yamauchi and Yamamura, 1979; Vahter and Envall, 1983).

We showed that As induced a dose- and time-related IL-1α production in murine keratinocytes. Statistically significant increases in intracellular IL-1α could be detected at concentrations as low as 2 μM, whereas concentrations > 10 μM induced cellular release of IL-1α. It is well documented that dysregulation of the IL-1 system may play a part in the initiation of cutaneous inflammatory hyperplastic diseases, such as psoriasis, inflammatory allergic diseases, chronic wounds, etc. (Gniadecki, 1998). Interestingly, transgenic mice that express high levels of IL-1α or the IL-1 receptor develop an exaggerated inflammatory and hyperproliferative response (Groves *et al*, 1995, 1996). IL-1α may also induce the production of other keratinocyte growth factors such as transforming growth factor-α (Lee *et al*, 1991), granulocyte/macrophage-colony stimulating factor, and IL-6. Furthermore, IL-1 may induce fibroblasts to produce factors, such as keratinocyte growth factor and hepatocyte growth factor that support keratinocyte growth (Maas-Szabowski, 1996). Thus, the ability of As to induce IL-1α production in keratinocytes may initiate *in vivo* the pathologic process that eventually results in hyperkeratosis and cancer. In our *in vitro* system, we were able to demonstrate the ability of As to increase cell proliferation as well as its modulation by a neutralizing antibody against IL-1α, which again supports the role of this cytokine in the pathologic proliferation of keratinocytes.

As to the mechanism of toxicity, inorganic arsenic has been shown to cause impaired tissue respiration *in vivo*. It inhibits enzyme activity by reaction with the sulfhydryl groups of proteins. In particular, suppression of nicotinamide adenine dinucleotide-linked substrates (pyruvate, glutamate, and α-ketoglutarate) appears to play a crucial part in the toxicity of As. Pentavalent and trivalent forms of arsenic exert similar effects in the inhibition of mitochondrial respiration and uncoupling of mitochondrial oxidative phosphorylation. The mechanism of this inhibition is not clear: one possibility is that arsenate is reduced by the mitochondria to As(III) and that inhibition occurs through the formation of a complex with the lipoic acid cofactor that is necessary for oxidation of the substrate (WHO, 1981). Preliminary data (not shown) indicate that this could be the case in our system, where lipoic acid can indeed inhibit in a dose-related manner As(V)-induced IL-1α production in HEL30 cells.

The uncoupling of mitochondrial oxidative phosphorylation induced by As could divert electrons into the formation of ROS. Indeed, we showed that As induces a dose- and time-related increase in cellular oxidative activity. Early investigations with isolated mitochondria showed that ubiquinone is the major source of ROS derived from the mitochondrial chain (Cadenas *et al*, 1977). Inhibition of

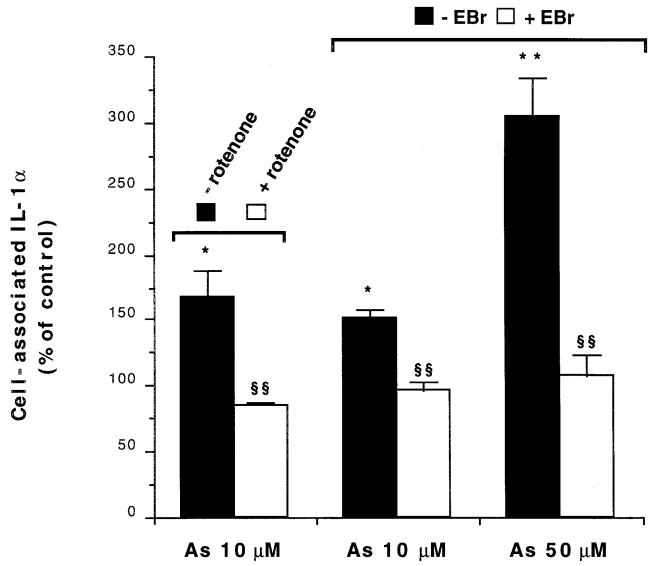

Figure 6. Mitochondria are essential for As-induced cell-associated IL-1α. Effect of 20 μM rotenone on As-induced cell-associated IL-1α 24 h after treatment with As 10 μM is shown in the first group of columns. Effect of mitochondrial depletion obtained by 5 d treatment with ethidium bromide (200 ng per ml) of cell-associated IL-1α 24 h after treatment with As (10–50 μM) is shown in the last two groups of columns. Results are expressed as percentage of control. Each value represents the mean ± SD of three to four independent samples. Statistical analysis by Dunnett's test, *$p < 0.05$ and **$p < 0.01$ *versus* control cells, §$p < 0.05$ and §§$p < 0.01$ *versus* As-treated cells in the absence of ethidium bromide (– Ebr) or rotenone (– rotenone).

electron entry to the ubiquinone pool of the respiratory chain by the complex I inhibitor rotenone indeed resulted in the abrogation of As-induced oxidative activity. We speculate that As increases free radical production at the ubiquinone site of the respiratory chain and that mitochondria are the main source of ROS. In recent years a correlation between Ca^{2+} and oxidative stress has been discovered. Coetzee *et al* (1989) have proposed a link between Ca^{2+} and superoxide formation in ischemia/reperfusion injury. High cytoplasmic Ca^{2+} levels can cause an increased mitochondrial Ca^{2+} uptake and disruption of mitochondrial Ca^{2+} homeostasis, which results in increased ROS formation (Chacon and Acosta, 1991) due to stimulation of electron flux along the electron transport chain. We can, however, rule out that such a disruption initiates As-induced oxidative stress, as BAPTA, a calcium chelator, was ineffective in modulating As-induced cellular oxidative activity.

ROS induced by As act then as a "fast-acting third messenger molecules" in inducing the activation of redox-sensitive transcription factors such as NF-κB (Schreck *et al*, 1991) and AP-1 (Ilnicka *et al*, 1993), both of which are important for the inducible expression of IL-1α (Muegge and Durum, 1990; Fenton, 1992). We indeed demonstrated that As activated both NF-κB and AP-1 and this preceded IL-1α production.

Taken together, these findings indicate that mitochondria act as mediators of As-induced gene regulatory signaling pathways, IL-1α production and cell proliferation. We were able to link the effect of arsenic on mitochondria with the later induction of IL-1α and of cell proliferation, thus helping our understanding of As-induced skin toxicity.

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REFERENCES

Baeuerle PA, Henkel T: Function and activation of NF-κB in the immune system. *Annu Rev Immunol* 12:141–179, 1994

Brown MM, Rhyne BC, Goyer RA, Fowler BA: Intracellular effects of chronic arsenic administration on renal proximal tubule cells. *J Toxicol Environ Health* 1:505–514, 1976

Brown KG, Guo HR, Kuo TL, Greene HL: Skin cancer and inorganic arsenic: uncertainty-status of risk. *Risk Anal* 17:37–42, 1997

Cadenas E, Boveris A, Ragan CI, Stoppani AO: Production of superoxide radicals and hydrogen peroxide by NADH-ubiquinone reductase and ubiquinol-cytochrome reductase from beef-heart mitochondria. *Arch Biochem Biophys* 180:248–257, 1977

Chacon E, Acosta D: Mitochondrial regulation of superoxide by Ca2+: an altered mechanism for the cardiotoxicity of doxorubicin. *Toxic Appl Pharmacol* 107:117–128, 1991

Coetzee WA, McLoughlin JL, Opie LH: The damaging effects of superoxide oxygen radicals in guinea pig ventricular myocytes are mediated by calcium. *J Mol Cell Cardiol* 21:S41, 1989

Corsini E, Marinovich M, Marabini L, Chiesara E, Galli CL:. (1994). Interleukin-1 production after treatment with non-ionic surfactants in a murine keratinocyte cell line. *Toxicol Vitro* 8:361–369, 1994

Corsini E, Bruccoleri A, Marinovich M, Galli CL: Endogenous interleukin-1α is associated with skin irritation induced by tributyltin. *Toxicol Appl Pharmacol* 138:268–274, 1996a

Corsini E, Schubert C, Marinovich M, Galli CL: Role of mitochondria in tributyltin-induced interleukin-1α production in murine keratinocytes. *J Invest Dermatol* 107:720–725, 1996b

Fenton MJ: Review: transcriptional and post-transcriptional regulation of interleukin 1 gene expression. *Int J Immunopharmacol* 14:401–411, 1992

Germolec DR, Yoshida T, Gaido K, *et al*: Arsenic induces overexpression of growth factors in human keratinocytes. *Toxicol Appl Pharmacol* 141:308–318, 1996

Germolec DR, Spalding J, Boorman GA, *et al*: Arsenic can mediate skin neoplasia by chronic stimulation of keratinocyte-derived growth factors. *Mut Res* 386:209–218, 1997

Gniadecki R: Regulation of keratinocyte proliferation. *Gen Pharmacol* 30:619–622, 1998

Groves RW, Mizutani H, Kieffer JD, Kupper TS: Inflammatory skin disease in transgenic mice that express high levels of interleukin-1α in basal epidermis. *Proc Natl Acad, Sci USA* 92:11874–11878, 1995

Groves RW, Rauschmayer T, Nakamura K, Sarkar S, Williams IR, Kupper TS: Inflammatory and hyperproliferative skin disease in mice that express elevated level of IL-1 receptor on epidermal keratinocytes. Evidence that IL-1 inducible secondary cytokines produced by keratinocytes in vivo can cause skin disease. *J Clin Invest* 98:336–344, 1996

Ilnicka M, Lee SW, Eugui EM: Antioxidants inhibit activation of transcription factors and cytokine gene transcription in monocytes. *Ann NY Acad Sci* 696:396–398, 1993

King MP, Attardi G: Human cells lacking mtDNA: repopulation with exogenous mitochondria by complementation. *Science* 246:500–503, 1989

Lee SW, Ilnicka M: Hydrogen peroxide primes promonocytic U937 cells to produce IL-1β. *Ann NY Acad Sci* 696:399–401, 1993

Lee SW, Morhenn VB, Ilnicka M, Eugui EM, Allison AC: Aurocrine stimulation of interleukin-1 alpha and transforming growth factor alpha production in human keratinocytes and its antagonism by glucocorticoids. *J Invest Dermatol* 97:106–110, 1991

Lindgren A, Vahter M, Dencker L: Autoradiographic studies on the distribution of arsenic in mice and hamsters administered ^{74}As-arsenite or -arsenate. *Acta Pharmacol Toxicol* 51:253–265, 1982

Maas-Szabowski N, Fusening NE: IL-1-induced growth factor expression in postmitotic and resting fibroblasts. *J Invest Dermatol* 107:849–855, 1996

Maloney ME: Arsenic in dermatology. *Dermatol Surg* 22:301–304, 1996

Mitchell RA, Change BF, Huang CH, DeMaster EG: Inhibition of mitochondrial energy-linked functions by arsenate. *Biochemistry* 10:2049–2054, 1971

Muegge K, Durum SK: Cytokines and transcription factors. *Cytokine* 2:1–8, 1990

Schreck R, Rieber P, Baeuerle PA: Reactive oxygen intermediates as apparently widely used messengers in the activation of the NF-κB transcription factor and HIV-1. *EMBO J* 10:2247–2250, 1991

Schreiber EP, Matthias MM, Schaffner W: Rapid detection of octameric binding proteins with 'mini-extract' prepared from a small number of cells. *Nucleic Acids Res* 17:6419, 1989

Vahter M, Envall J: In vivo reduction of arsenate in mice and rabbits. *Environ Res* 32:14–24, 1983

World Health Organization: Environmental Health Criteria 18—Arsenic. International Programme on Chemical Safety, Geneva, 1981

Yamauchi H, Yamamura Y: Dynamic change of inorganic arsenic and methylarsenic compounds in human urine after oral intake as arsenic trioxide. *Ind Health* 17:79–83, 1979