p53 Response to Arsenic Exposure in Epithelial Cells: Protein Kinase B/Akt Involvement

Marisol Sandoval,* Moisés Morales,* Rocío Tapia,† Luz del Carmen Alarcón,‡ Montserrat Sordo,§ Patricia Ostrosky-Wegman,§ Arturo Ortega,* and Esther López-Bayghen*,†

*Departamento de Genética y Biología Molecular, †Departamento de Fisiología, Biofísica y Neurociencias, Centro de Investigación y de Estudios Avanzados del IPN, México D.F., México; ‡Laboratorio de Citopatología, Unidad Académica de Ciencias Químico Biológicas, Universidad Autónoma de Guerrero, Chilpancingo, Guerrero, México; and §Departamento de Genética y Toxicología Ambiental, Instituto de Investigaciones Biomédicas, UNAM, México D.F., México

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Inorganic arsenic is a major environmental contaminant associated with an increased risk of human skin cancer. Arsenic modulates cellular signaling pathways that affect diverse processes such as cell proliferation, differentiation, and apoptosis, including genotoxic damage. The p53 protein plays a central role in mediating stress and DNA damage responses, leading to either growth arrest or apoptosis. Several signal transduction pathways activated under a plethora of stressing conditions increase p53 protein levels. To further understand the molecular mechanisms involved in the arsenic mode of action, we explored the effects of this metalloid on the activation of the phosphatidylinositol 3-kinase (PI3K)/Ca2+/diacylglycerol dependent protein kinase/protein kinase B (PKB) signaling cascade and its repercussion in p53 activation in two epithelial cell types: primary normal human keratinocytes cultures (NHK) and the carcinoma-derived C33-A cell line. Although in both cell systems arsenic leads to an increase in p53 and its binding to DNA, the final outcome is different. In NHK, arsenic triggers a sustained activation of the PI3K/PKB/glycogen synthase kinase-3 beta pathway, driving the cell into a cell-differentiated stage in which the proliferation signals are turned down. In sharp contrast, in C33-A cells, arsenic leads to a transient increase in p53 followed by a drastic reduction in its nuclear levels and an increase in cell proliferation. These findings favor the notion that p53-stage and transcriptional abilities are important to understand modifications in the proliferation–differentiation balance, an equilibrium that is severely impaired by arsenic.

Key Words: arsenic; p53 protein; protein kinase B; Akt; human keratinocytes.

Arsenic is a ubiquitous element that has represented a human health concern for centuries. The contamination of water supplies with this metalloid has resulted in a very high incidence of skin lesions and cancer. Human exposure to inorganic arsenic at elevated levels is widespread in several populous regions of the world as demonstrated in a large amount of epidemiologic studies (Chen et al., 1985; Chiou et al., 1995, 2001; Lokuge et al., 2004; Nordstrom, 2002; Sun, 2004). Benign skin lesions related to chronic arsenic exposure act as early warning signals, including hyperpigmentation and hyperkeratosis (reviewed in Tchounwou et al., 2004; Tondel et al., 1999). The molecular mechanisms underlying arsenic carcinogenicity have been explored, noticing that exposure-associated effects are genotoxicity, cell proliferation changes, and altered DNA repair (Dong, 2002). Recently, new animal models of transplacental carcinogenesis and cocarcinogenesis are adding new molecular insights about the specific gene alterations associated with carcinoma development (Liu et al., 2007; Liu et al., 2006a; Liu et al., 2006b). Also in vitro transformation studies demonstrate the importance of altered DNA methylation (Xie et al., 2007). Mode-of-action studies suggest that arsenic might be acting as a cocarcinogen, a promoter or a progressor of carcinogenesis.

The skin epithelium provides a barrier between the internal and external regions of the body. It is constantly subjected to physical and chemical stress, and consequently has high renewal rates. The epidermis consists of a stratified squamous epithelium composed mainly of keratinocytes at variable differentiation stages (Fuchs, 1990a; Zinkel and Fuchs, 1994). In consequence, there is a highly regulated balance between epithelial cell growth and cell differentiation. A number of laboratories have described responses of cultured keratinocytes to inorganic arsenic treatment, including increased growth factors production, (Germolec et al., 1996), changes in differentiation (Jessen et al., 2001), and global (Germolec et al., 1996), effects assessed by DNA microarray (Hamadeh et al., 2002; Rea et al., 2003). In epithelia, damage to DNA results in increased levels of the tumor suppressor p53 (Colman et al., 2000). This protein mediates genotoxic stress responses and has been regarded as a key element in maintaining genomic stability (Appella and Anderson, 2001). At the cellular level, p53 is a central regulator of stress reacting pathways, becoming active in response to...
abnormal proliferation, hypoxia, DNA damage, nucleotide depletion, and disruption of the mitotic spindle. Increased levels of active p53 can have many consequences including cell cycle arrest, apoptosis, senescence, and differentiation that depend on numerous cell intrinsic and extrinsic factors. Upon several cellular stress stimulus, p53 can induce reversible cell cycle arrest and participate in damage repair mechanisms (reviewed in Hofseth et al., 2004; Menendez et al., 2007).

The p53 protein is a transcriptional activator with a transcriptional domain at its NH₂ terminus, a regulatory region and tetramerization domain located at the COOH terminus and a central sequence-specific DNA binding domain (reviewed in Kaustov et al., 2006). Ser15 phosphorylation through the DNA-dependent protein kinase (DNA-PK) is critical for p53 interaction with the transcriptional machinery (Woo et al., 1998). Phosphatidyl inositol 3-kinase (PI3K), Ca²⁺/diacylglycerol dependent protein kinase (PKC), and protein kinase B (PKB/Akt) have also been implicated in p53 activation in response to different stressors and damage inducers Abbas et al., (2004), Lee et al., (2006), Magnelli et al., (1998), Nakamura et al., (2000). Phosphorylated p53 accumulates in the nucleus and transactivates a number of key genes that participate in DNA repair and cell proliferation (Appella and Anderson, 2001).

Our current knowledge of p53 response after arsenic exposure is variable. Depending on the cell type, previous p53 stage, and timing, responses are different (Hamadeh et al., 1999, 2002; Hernandez-Zavala et al., 2005; Salazar et al., 1997). Particularly, the role of p53 as gene activator under these circumstances has been barely explored. Following activation by posttranslational modifications such as phosphorylation and acetylation, p53 protects the cell from further stress by activating the transcription of genes involved in cell cycle checkpoints, DNA repair, cellular senescence, and apoptosis. Serine 392 phosphorylation at in COOH terminal site of p53 activates the protein for sequence-specific DNA binding. Furthermore, there is an interdependence between phosphorylation and dose-dependent responses of p53 (Latten et al., 2001). An apparent tissue-specific p53 phosphorylation response takes place, since the same type and level of damage induce a differential p53 phosphorylation pattern in different cell lineages. Another important posttranslational modification of p53 is acetylation on lysine residues (Liu et al., 1999). This modification activates transcription and stabilizes p53 by preventing murine double minute-2 (Mdm-2)–mediated degradation (Li et al., 2002).

In this contribution, we characterized the DNA binding activity of p53 when epithelial cells were exposed to sodium arsenite in varying times and doses. We found marked differences in p53–DNA binding depending on the dose, epithelial cell type, and stage of p53 expression. Upon arsenite, NHK display a long-term stabilization of nuclear p53, induced and maintained by PI3K, PKC, and PKB/Akt, which renders the cell into growth arrest and expression of differentiation markers such as involucrin. On the other hand, differential kinetics in kinases activation seems to be involved in p53 responses to arsenite in the C33-A cell system, where a mutant p53, is rapidly translocated from nuclei in order to inactivate its function in growth arrest, resulting in an increase in cell proliferation.

MATERIALS AND METHODS

Reagents. BAPTA (1,2-bis(2-aminophenoxy) ethane-N,N,N',N'-tetraacetic acid) was obtained from Tocris-Cookson (St Louis, MO). Calcium chloride (CaCl₂), 2-{1-[3-dimethylamino propyl]-1H-indol-3-yl}-3-[1H-indol-3-yl] maleimide (Bis I) and 12-tetradecanoyl-13-acetate (TPA) were purchased from Sigma (St Louis, MO). LiCl and wortmannin were obtained from Calbiochem (La Jolla, CA) and all other chemicals from Merck (Whitehouse Station, NJ).

Cell culture. Keratinocyte primary cultures were obtained from neonatal human foreskin as described previously (Pirisi et al., 1987) and grown in Keratinocyte Serum Free Medium (Invitrogen, Gaithersburg, MD) containing the appropriate antibiotic mix at 37°C in a 5% CO₂ atmosphere. Culture media was replaced every 2 days. The C33-A cell line (Human Papillomavirus negative cervical carcinoma-derived cell line) was routinely cultured in Dulbecco’s modified Eagle’s medium/F-12 medium, 50:50 (DMEM/F-12, Invitrogen) and HeLa cells growth in DMEM at 37°C in a 5% CO₂ atmosphere. In both cases, media were supplemented with 10% fetal calf serum and antibiotics. Primary cultures from cerebellar Bergmann glia were obtained as reported before (Ortega et al., 1991).

Arsenic treatment. An aqueous sterile stock solution of sodium arsenite (10 mg/ml) was prepared and diluted to obtain the desired final concentrations. Cell cultures were grown to approximately 80–85% confluence and treated with sodium arsenite for the indicated time periods.

Cytotoxicity assessment. Cell viability was measured 24 and 48 h after treatment by the MTT reduction assay (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) (Sigma-Aldrich Co.) performed as described by Mosmann (1983). Cells were seeded on 12-well plates in 500 µl of culture media. These cultures were incubated for 24 h with sodium arsenite at 37°C in a 5% CO₂ atmosphere. After that, the cells were treated with the MTT reagent (5 mg/ml) for approximately 4 h. Isopropanol:HCl solution was added to lyse the cells and solubilize the colored crystals. The optical density of the samples was determined at 630 nm using an enzyme-linked immunosorbsent assay plate reader Opsys MR (Dynex Technologies, Frankfurt, Germany).

Cell proliferation rates determination. For cell proliferation rates, cells were seeded on a 12- (NHK) or a 24-well (C33-A) plate in 500-µl culture media with or without sodium arsenite (1.5µM), incubated at 37°C in 5% CO₂, and analyzed in quadruplicates at 12- to 24-h intervals for 4 days. Two hundred microliters of MTT labeling mixture was added to each well 4 h before each spectrometric measurement.

Cytokinesis-block method and nuclear index determination. Keratinoctyes cultures were treated with different sodium arsenite concentrations and incubated with 2 µg/ml cytochalasin B for an additional period of 48 h. After that, the cultures were trypsinized and suspended gently in complete medium. The cells were fixed in methanol:acetic acid solution and centrifuged for 10 min at 50g. Keratinocytes were deposited in slices, air-dried, and stained with eosin and methylene blue. The frequency of cytoplasmic micronuclei was scored under an optic microscope at a magnification of ×100. One thousand binucleated cells were examined for micronuclei presence (per treatment). For cell proliferation, 500 cells were examined and a calculation was done in order to obtain the nuclear index (Fenech et al., 2003a,b).

Protein electrophoresis and immunoblotting. Cells were harvested after arsenic treatment (time and concentrations are indicated in each figure legend) and incubated on ice for 40 min in lysis buffer (50mM Tris HCl; 1mM ethylenediaminetetraacetic acid [EDTA]; 1mM phenylmethylsulfonyl fluoride; 5 µg/ml aprotinin and leupeptin; 10mM NaF; 1mM NaVO₄; and 1mM Na₃VO₄). The lysates were centrifuged for 10 min at 10,000 g and
supernatant fractions were stored at —70°C or used immediately. Standardized amounts of total protein extracts (30–50 μg) were analyzed in sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to polyvinyldene difluoride membranes (Millipore, Bedford, MA). Blots were stained with Poncette S to confirm equal protein loading in all lanes. Membranes were soaked in phosphate-buffered saline (PBS) to remove Poncette S and incubated in PBS containing 5% dried skimmed milk and 0.1% Tween-20 for at least 1 h at room temperature, to block the excess of nonspecific protein-binding sites. After blocking, the membranes were incubated with specific antibodies. The following primary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA): p53 (sc-126), cyclin D1 (sc-717), involucrin (sc-21748), p-Akt1 (sc-7985), p-glycogen synthase kinase-3 beta (sc-11757), and β-actin (sc-32251). Horseradish peroxidase–conjugated anti-mouse or anti-rabbit IgG (Amersham Biosciences, Piscataway, NJ) were used as secondary antibodies, followed by a chemiluminescence detection system ECL + PLUS (Amersham).

Electrophoretic mobility shift assays. Nuclear extracts were prepared as described previously (Lopez-Bayghen et al., 1996). All buffers contained a protease inhibitor cocktail (Complete, Roche Diagnostics Corporation, Indianapolis, IN) to prevent proteolysis. Protein concentration was measured by a Bio-Rad protein assay system. Nuclear extracts (20–30 μg) from keratinocytes and 15 μg for C33-A were incubated on ice with 1 μg poly[dI–dC] as non-specific competitor (GE Healthcare, Piscataway, NJ) to prevent nonspecific binding sites. After blocking, the membranes were incubated with specific antibodies. The following primary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA): p53 (sc-126), cyclin D1 (sc-717), involucrin (sc-21748), p-Akt1 (sc-7985), p-glycogen synthase kinase-3 beta (sc-11757), and β-actin (sc-32251). Horseradish peroxidase–conjugated anti-mouse or anti-rabbit IgG (Amersham Biosciences, Piscataway, NJ) were used as secondary antibodies, followed by a chemiluminescence detection system ECL + PLUS (Amersham).

The reaction mixtures were electrophoresed in a low ionic strength 0.5× TBE buffer in 7% polyacrylamide gels. The gel was dried and exposed to an X-ray film (89mM Tris, 89mM boric acid, 2mM EDTA). For competitive studies, the reaction mixtures were preincubated with different amounts of indicated unlabeled competitor oligonucleotides, before adding labeled DNA. For electro- phoretic super-shift experiments, reactions with the DNA–protein complexes were incubated at 4°C with anti-p53 (sc-126) and anti-sox10 (sc-17342) both from Santa Cruz Biotechnology for 4 h prior to electrophoresis.

Plasmids. The plasmid p53CAT contains the p53 consensus sequence from p21 promoter region (Gohler et al., 2002), under control of the SV40 promoter and chloramphenicol acetyltransferase (CAT) as reporter gene. Vector pXP2-CD1 contains luciferase reporter gene under the control of the human cyclin D1 promoter (Herber et al., 1994) kindly donated by Dr Françoise Thierry (Institute Pasteur, Paris).

Transient transfections and reporter assays. Transient transfections were performed in the C33-A cell line. Cells were grown in 60-mm tissue culture dishes and transfected at 60% of confluence (6 μg of reporter plasmid/dish) by standard calcium mediated method. Protein lysates for CAT assays were obtained as follows: cells were harvested in TEN buffer (40mM Tris–HCl, pH 8.0, 1mM EDTA, and 15mM NaCl), lysed with three freeze–thaw cycles in 25mM Tris–HCl, pH 8.0, and centrifuged at 12,000 × g for 3 min. Standardized amounts of protein lysates were incubated with 0.25 μCi [3H]chloramphenicol (50 μCi/mmol, Amersham Life Science) and 0.8mM acetyl-Co-A at 37°C. Acetylated forms were separated by thin-layer chromatography and quantified using a radioactive image analyzer Typhoon Optical Scanner (Molecular Dynamics, Amersham Biosciences, Buckinghamshire). CAT activities were expressed as relative activities to the untreated control. For luciferase assays, cells were harvested 24-h posttransfection and processed with the Luciferase assay system (Promega). Briefly, protein lysates were obtained in cold PBS by two freeze–thawing cycles and resuspended in 100 μl of reporter lysis buffer. Equal amounts of protein lysates (~70 μg) were incubated with luciferase assay reagent. Light detection was performed in a Fluoroskan Ascent FL 374 (Labsystems, Finland).

Immunohistochemistry. Cell culture staining with anti-p53 monoclonal and anti-Hi antibodies was performed using NHK grown on ethanol-washed glass coverslips (22 × 22 mm) in the same culture conditions as described above. Cells were fixed by exposure for 10 min to methanol at —20°C, washed twice with PBS, and were immunostained using the biotin–streptavidin–peroxidase DAB (3,3’-diaminobenzidene tetrahydrochloride) (ImmunoDetector horse radish peroxidase/DAB, BIO SB, Inc., Santa Barbara, CA). Control immunolabeling was performed with the same staining procedure but using the visualizing reagents in the absence of the primary antibodies. Slides were treated with 0.3% hydrogen peroxide (H2O2) for 15 min to block endogenous peroxidase. Sections were pressure cooked for 30 min in 10mM citrate buffer, pH 6.0 (ImmunoDNA retriever with citrate, BIO SB, Inc., Santa Barbara, CA) to unmask epitopes. Sections were incubated with primary antibody (1:100 dilution of monoclonal mouse anti-involucrin and 1:100 dilution of monoclonal mouse anti-human p53 (clone DO7, cat # M7001, Dako Cytomation) for 40 min at room temperature and rinsed in Tris-buffered saline (Immuino/DNA Washer, BIO SB, Inc., Santa Barbara, CA). The sections were incubated with biotin-labeled secondary antibody and streptavidin–biotin–peroxidase for 20 min each. DAB (0.05%) was used as the substrate, and positive signal was detected as a brown color under a light microscope. Cells were weakly counterstained with Mayer’s hematoxylin, dehydrated, and mounted in Entellan mounting medium (Merck KgAA, Darmstadt, Germany). The slides were examined under bright field Zeiss Axioscope 40 microscope and the Axiovision software (Carl Zeiss, Inc. Thornwood, NY) at ×400 magnification.

RESULTS

Arsenic Cytotoxicity

Initial experiments were designed to evaluate the effect of sodium arsenite over cell viability of NHK and C33-A cells. Dose–response curves were performed in these cell systems, measuring cell viability by the MTT method. As depicted in panel A of Figure 1, exposure to increasing concentrations of sodium arsenite for 24 h does not induce change in the cell viability and not lead to cell death as compared to untreated control in either of the cultures. In order to prove that 1.5μM is not a toxic dose, we also performed cytotoxicity assays after 24 h incubation with sodium arsenite followed by another 24, 48, or even 72 h of incubation in fresh culture media, results are presented in Figure 1B. No significative changes in cell viability were present under these conditions.

Effects of Sodium Arsenite on p53 Activation

One of the most recognized indicators of DNA damage is p53 activation. The genotoxicity recorded in arsenic-treated keratinocytes may indicate that surveillance by p53 might be activated. NHK cells were exposed to increasing concentrations of sodium arsenite for 24 h, and p53 protein levels were determined via Western blot. The results, depicted in Figure 2, panel A, show that exposure of NHK to sodium arsenite leads to a dose-dependent increase in p53 levels, reinforcing the idea that arsenic induces p53 in our system. Taking into consideration that p53 is capable of inducing the expression

p53

5’-CTAGTACAGAACATGCTAAGCTGCTGGACT-3’

(Gohler et al., 2002)

Sp1

5’-CTAGATTGGATAGCGGGCACGGA-3’

AP-1 (SV40)

5’-CTAGRACTATTAGACTAACTGCTG-3’

MyC

5’-CTAGGGAAAGCACACGTTGCTGCTTCC-3’

(Murre et al., 1991)
of genes containing one or more copies of a p53 DNA–binding element we decided to perform protein–DNA binding assays using a p53 consensus oligonucleotide as a labeled probe and nuclear extracts obtained from NHK treated with sodium arsenite (0.1–1.5 \( \mu \)M). As detected for the p53 protein levels, a specific dose-dependent increase in p53 DNA–binding activity was found (Fig. 2, panel B). Specificity of the p53–DNA interaction was demonstrated by competition assays using the unlabeled p53 oligonucleotide (100-fold molar excess) and the unrelated SP-1 oligonucleotide (100-fold molar excesses). As expected, the unlabeled p53 oligonucleotide efficiently abolishes the formation of p53 complex, while the SP-1 oligonucleotide did not modify the complex. To further demonstrate that p53 is the specific protein that interacts within this region, we performed the immuno-electrophoretic mobility shift assays (immunoEMSA) shown in the right panel of Figure 2B. Addition of anti-p53 antibodies causes a super-shifted complex (SS) that matches with the disappearance of p53 typical complex. In contrast, the preimmune serum did not exert any additional shifting. Taken together these data demonstrate that arsenic exposure of NHK increases nuclear p53 protein, which is capable to interact with its DNA binding element.

Since NHK are nontransformed cells, we wondered if the p53 response was reproduced in a cell line with a mutated form of p53. The point mutations present in the p53 version of C33-A cells result in an amino acid change of Arg-*Cys in codon 273, change that is not involved in the protein–DNA interactions (Scheffner et al., 1991). As shown in panel A of Figure 3, in C33-A cells exposed 24 h to 1.5 \( \mu \)M sodium arsenite, p53–DNA binding is detected albeit to a lower extent than in NHK cells. Specificity of this complex was demonstrated by competition experiments and immunoEMSA. When a dose–response effect was sought, exposing cell for 24 h, a decrease in p53–DNA binding was found (Fig. 3, panel B). This result prompted us to examine in detail the effect of a shorter arsenic exposure (30 min). A dose-dependent increase in p53 DNA–binding activity was found (panel B, Fig. 3).

A quantitative analysis is shown in panel C, where we compare the DNA-binding behavior of p53. As a control of nuclear extracts quality, we tested AP-1 (c-jun/c-fos) transcription factor binding to its cognate sequence, using the same nuclear extracts tested with p53. Note that arsenic effect over the AP-1 DNA–binding activity is different from that of p53. The robust AP-1 complex detected after 24-h exposure to sodium arsenite indicates that the decrease in p53–DNA binding is not the result of protein degradation (Fig. 3, panel D). Arsenic increased significantly the AP-1 DNA–binding activity independently of the exposure time, suggesting a mechanism that regulates the
increase in p53 DNA–binding activity. Additionally, whole cell extracts were analyzed by immunoblotting. Interestingly, when C33-A cells were exposed to sodium arsenite for different time periods using a fixed concentration (1.5 μM), p53 protein levels augmented as early as 15 min, increase that was maintained for up to 24 h (Fig. 3, panel E).

Role of Calcium Influx and PKC in p53 Activation by Sodium Arsenite

Under arsenite exposure, keratinocytes showed an increase in p53 levels which may be associated with cell cycle arrest, but also with changes in gene expression and signaling pathways activation. Ca\(^{2+}\) is an important factor in the regulation of the differentiation process in keratinocytes. It is a requirement for stratification, assembly of desmosomes, and activation of epidermal transglutaminase (Eckert et al., 1997). In this sense, other studies have shown that arsenic increases the intracellular Ca\(^{2+}\) levels in lymphocytes. With this in mind, we evaluated if changes in intracellular Ca\(^{2+}\) levels regulate the p53–DNA complex formation in response to arsenic. We first evaluated the effect of the exposure of NHK cells to increasing CaCl\(_2\) concentrations for 24 h. As expected, an increase in the p53–DNA complex was found (Fig. 4A). When cells were pre-exposed to a 50μM concentration of the Ca\(^{2+}\) chelator BAPTA previous to sodium arsenite, the p53–DNA complex was less evident (Fig. 4B). These results point out that Ca\(^{2+}\) has an important role in the activation of p53.
The differential effect of arsenite in NHK and C33-A cells in terms of p53 DNA-binding activity, might be related to differential signaling pathway activation by arsenite in both cell systems, although 2 mM CaCl₂ increases p53–DNA binding in C33-A cells as in NHK (Fig. 4C). Classical isoforms of PKC are activated in CaCl₂-exposed NHK. So, we asked if PKC induction may directly increase p53–DNA binding in C33-A. As can be observed in panel D of Figure 4, TPA, a PKC activator, mimics CaCl₂ or sodium arsenite responses. Additional confirmation of the link between Ca²⁺ entrance, PKC activation, and p53–DNA binding is provided by the fact that the PKC blocker Bis I diminishes the arsenite-dependent p53–DNA binding.

PKB/Akt Involvement in NHK Response to Sodium Arsenite

Multiple pathways have been proposed for p53 activation in a variety of cell lines. Among them, several kinases activated under elevated Ca²⁺ levels are those of the AGC family, i.e., PKC, cyclic AMP–dependent protein kinase, and PKB/Akt. In fact, arsenite activates PKB/Akt, with an important role for PI3K/Akt/I-kappa-B kinase beta/nuclear factor-kappaB pathway in cyclin D upregulation and arsenite-induced proliferation in mouse epidermal Cl41 cells (Ouyang et al., 2006). Therefore, we investigated if the PKB/Akt signaling pathway is triggered under arsenic exposure in NHK, contributing to p53 activation. To this end, confluent NHK were treated with sodium arsenite (1.5 μM for 30 min) and phosphorylated PKB/
Akt (Ser473) was detected by Western blot. As shown in panel A of Figure 5, sodium arsenite treatment results in the phosphorylation and presumably activation of PKB/Akt. As expected, LiCl, an inositol phosphatase inhibitor, also increases PKB/Akt phosphorylation (Gerling et al., 2004). Although Ser473 phosphorylation has been correlated with PKB/Akt activity, we decided to explore directly its activity over a well-known substrate, GSK3-β (Nicholson and Anderson, 2002). The results are depicted in panel B of Figure 5, sodium arsenite increases Ser9 GSK-3-β phosphorylation after 15 min. As expected, both phosphorylation events are strictly dependent on PI3K. In order to obtain direct confirmation of the involvement of the PI3K/PKB/Akt pathway on the arsenite-promoted p53–DNA binding, EMSA were performed. When cells were treated with the PI3K blocker wortmannin (100nM, 30 min) previous to sodium arsenite, the p53–DNA complex decreased considerably with respect to complex obtained from cells treated with sodium arsenite alone. These results indicate

FIG. 4. Role of PKC activation in regulating p53 binding to DNA. (A) Nuclear extracts from NHK, control, or exposed to CaCl₂ (for 24 h, concentrations are indicated) were obtained and used for DNA-binding assays with the 32P-labeled p53 oligonucleotide. (B) Nuclear extracts were obtained from NHK treated with sodium arsenite (1.5µM), and the calcium blocker, BAPTA (50µM, added at least 45 min before sodium arsenite). (C, D) For nuclear extracts preparation, C33-A cells treated with sodium arsenite (1.5µM), CaCl₂ (2mM), TPA (50 and 100nM) for 30 min or the PKC inhibitor bisindolemaleimide (1µM) added 30 min before treatment. Extracts were tested with the p53 probe.
that PI3K/PKB/Akt pathway activation is participating in the regulation of p53 protein DNA binding in NHK. Also notice, in Figure 5C, that a similar increase in p53 binding to its consensus sequence is present when the cells are exposed to the phosphatase inhibitor LiCl (stabilizing Akt phosphorylation). Similarly, pretreatment with 1L-6-hydroxymethylchiro-inositol 2-(R)-2-O-methyl-3-O-octadecylcarbonate, an AKT1 blocker, avoids p53 complex activation (Fig. 5D).

Comparative analysis of the arsenite effect in PKB/Akt phosphorylation was performed in the C33-A cell line. Interestingly, a time and dose-dependent increase in PKB/Akt phosphorylation was found and maintained for up to 12 h (Fig. 6, upper panels). Moreover, when we performed EMSA a clear blockage of the formation of p53–DNA complex induced by arsenic in the short term was achieved with the previous application of the PKB/Akt inhibitor, AKT I applied at a 25nM concentration (Fig. 5D).

As a measurement of the pathway behavior in the long term, we decided to test the activity of p53 as a transcription factor in a functional assay. Using a reporter gene system, where a p53 DNA–binding site was cloned to direct the SV40 early promoter activity (see map, Fig. 6D) we evaluated the arsenite effect. The promoter activity was reduced after the continuous exposure to the metalloid. This result is in line with those shown in Figure 3; in the long-term, arsenic treatment is apparently linked to the removal of the mutated p53 from nuclei, lowering the p53–DNA complex formation and obviously its transactivating capacity. Interestingly, the PKB/Akt pathway blockers avoid this effect and the inositol phosphatase mimics it.

**Sodium Arsenite Induces NHK Differentiation**

As p53 seems to be activated for a long period of time under arsenite presence, NHK are expected to be more restricted to the cell growth control exerted by p53. In line with this, we observed that arsenite treatment caused changes in cell morphology that resemble those obtained when cells are induced to differentiate in vitro by adding 2mM CaCl2. Comparative images shown in panel A of Figure 7 reflect a rather similar morphology when cells are differentiated in vitro by adding 2mM CaCl2. Comparative images shown in panel A of Figure 7 reflect a rather similar morphology when cells are differentiated in vitro by adding 2mM CaCl2.
cells were exposed to increasing concentrations of sodium arsenite (Fig. 7, panels B and C). Accordingly, we expected an arsenite-treated NHK augmentation in cyclin D1 levels, assessing these levels as a measure of cell cycle progression and cell proliferation. Indeed this is the case, as shown in Figure 7D, cyclin D1 increases in cells exposed to sodium arsenite, note an inverse concentration-dependent effect. With these results, we next examined cell proliferation effect using nuclear index as a tool to evaluate the changes exerted by sodium arsenite on NHK proliferation rates (Fig. 7E). In fact, we were able to establish a correlation between nuclear index assays and cyclin D1 levels (Figs. 7D and 7E). Accordingly to the cell proliferation rates (Fig. 7F), the cells seem to be withdrawn from cell cycle progression. These data may indicate that different responses can be elicited depending on the arsenite concentration to which NHK are exposed, low concentrations lead to a discrete proliferation, whereas higher concentrations (more than 0.5 μM) seem to gradually stop cell growth, most possibly through p53 arrest.

**Sodium Arsenite Induces Cell Proliferation in C33-A Cells**

As the response to sodium arsenite is clearly different when we compare NHK with C33-A cells, we expected a different behavior in the mutated p53 cell line in terms of cell proliferation. The results are shown in Figure 8, serum-starved C33-A are capable to proliferate under arsenite treatment (panel A). Moreover, a cyclin D1–dependent promoter is activated by arsenite in a dose-dependent manner, indicating that the cell cycle is not stopped by the metalloid (Fig. 8B). Even more, c-myc is also activated by sodium arsenite and may be the connecting link to activate cyclin D1 and cell cycle progression (Fig. 8C). A model with our current understanding of data presented here is shown in Figure 9.

**DISCUSSION**

Previous studies have shown that arsenic is cytotoxic to a variety of cell lines and primary cultures, for example fibroblasts (Lee et al., 2004; Trouba et al., 1999), lymphocytes (Sordo et al., 2001), hepatocytes (Petrick et al., 2000), NHK (Bae et al., 2001), and epidermal carcinoma cells (Huang and Lee, 1996). However, variability between different systems has also been reported (Mei et al., 2003). When we evaluated the sensibility of NHK and C33-A to arsenic, no clear cytotoxicity was detected since cell viability assessed by the MTT method was not severely affected even after 72 h (Fig. 1).
FIG. 7. p53/involucrin association under differentiation like conditions elicited for sodium arsenite. (A) Morphological changes shown in microscopically taken images from arsenic-treated cells (24 h, concentrations are indicated) or those treated with the differentiation inducer CaCl₂ (2mM). (B) Involucrin levels were analyzed via Western blot in treated NHK (24 h). (C) Expanded cells from the primary culture were seeded in eight wells glass slides, treated as indicated and the immunostaining performed with anti-involucrin antibody after 24 h treatment. (D) Cyclin D1 protein levels were analyzed by Western blot in treated NHK, with indicated concentrations and for 24 h. HeLa cells are used as a positive control for cyclin D1 protein. Values represent the mean of three separate experiments ± SE. Plotted data were obtained by densitometric measurement and are shown as % of levels recorded by the nontreated control, normalizing by actin content. (E) NHK exposed to the indicated concentrations of sodium arsenite and cytochalasin B (to block cytokinesis, 2 mg/ml) during 48 h were fixed, and cell proliferation was evaluated by nuclear index. Binucleated cells counting was performed, assessing the frequency of micronucleated cells. (F) Cell proliferation rates were done by the MTT method. Data are expressed as % of untreated control at time zero, values plotted are the mean ± SE.
Arsenic has profound genotoxic effects (Gradecka et al., 2001). Many studies have shown that exposure to arsenic increases the frequency of chromosome aberrations and micronuclei in humans and experimental animals (Gebel, 2001; Waclavicek et al., 2001). When we evaluated the arsenic clastogenic activity over NHK, we found the presence of micronuclei in the cytoplasm, a common feature indicative of genotoxic damage (data not shown). A number of stimuli, including DNA damage, reactive oxygen species, nucleotide depletion, and other genotoxic stressors induce p53 activation (Appella and Anderson, 2001; Colman et al., 2000). Arsenic exposure results in the activation of p53 in different cultured cell systems (Hernandez-Zavala et al., 2005; Salazar et al., 1997; Yih and Lee, 2000). Protection against cancer by p53 is due mainly to its activity as a transcription factor. In fact, p53 accumulates in the nucleus and transactivates a number of key genes that regulate the DNA repair processes and cell proliferation. The function of p53 in transactivation of target genes deals with the dilemma of inducing cell growth arrest or apoptosis pathways. Among the induced p53 genes, p21waf is a gene critically involved in senescence (Noda et al., 1994) and identified as an universal inhibitor of cyclin dependent kinases, which block the cell cycle at G1/S transition and inhibits cell growth (Harper et al., 1993). Interestingly, p21waf promoter harbors two p53 DNA-binding sites. In this sense, we evaluated the effects of arsenic on p53 protein levels and its transactivator capabilities and how these effects are related to parameters such as cell growth in two systems of epithelial cells, a primary culture of NHK and a well-characterized cell line that exhibits p53 mutations, the C33-A cell line. In the latter, mutations map to codons 245, 248, 249, and 273 (Scheffner et al., 1991). Under a p53 mutant, exposure to ultraviolet (UV) and arsenite may lead to enhanced metalloid effects. For example, it is well characterized that a higher p53 expression is present in keratinocytes adjacent to small cell carcinoma focuses exposed to UV (Lee et al., 2004; Rossman et al., 2004). Sun-exposed human skin contains thousands of clones of p53-mutant keratinocytes (Jonason et al., 1996) and the p53-mutant keratinocytes clones grew only during chronic UVB exposure (Zhang et al., 2001). Moreover, in endemic zones of arsenic contamination, both factors are usually concurrent. It is quite possible that arsenic exerts a differential effect in a cell with a previously mutated p53 protein.

![Graph A](image1.png)

**FIG. 8.** Sodium arsenite induces cell proliferation in C33-A. (A) C33-A cells were seeded in 12-well culture plates in 2% serum media (starving conditions), treated with sodium arsenite for indicated times, and cell proliferation rates were evaluated by the MTT method. Data are expressed as % of untreated control at time zero; values plotted are mean ± SE. (B) Transcriptional activity of cyclin D1 promoter was determined after transient transfection assays in C33-A. The pXP2-CD1 construct was transfected (6 μg of plasmidic DNA) in 80% confluent C33-A, which were treated or not with the indicate concentrations of sodium arsenite, along 48 h in media with 0.5% serum and harvested 24-h posttreatment. Relative luciferase units were obtained from at least three independent experiments, data plotted are mean values ± SE (*p < 0.05; vs. untreated, by ANOVA). (C) c-myc DNA binding increases in C33-A exposed to sodium arsenite. Cells were harvested 24-h posttreatment and processed to obtain nuclear extracts, which were normalized by protein content and incubated with [32P]-labeled c-myc probe. FREE, no nuclear extract added.
Therefore, mutated p53 could act as a negative additional risk factor.

Reports concerning the arsenic effects on p53 protein levels are conflicting. For example, it has been reported that a 14-day exposure to environmental relevant arsenic concentrations results in p53 diminished levels in the human keratinocytes (Hamadeh et al., 1999, 2002). In contrast, in most of cell types, including other epithelial cell systems, arsenic exposure causes p53 and p21waf augmentation (Hernandez-Zavala et al., 2005; Salazar et al., 1997; Yih and Lee, 2000). Therefore, we decided to investigate the mechanisms by which arsenic regulates p53 through different approaches. When we evaluated the p53 DNA-binding activity we observed that arsenic increases p53 bound to DNA, due to an increase in its protein levels (immunodetected) (EMSA, Fig. 2). In line with this interpretation, we were able to find p53 nuclear accumulation.

Several reports show that arsenic modulates a number of signaling transduction pathways that regulate cell growth, differentiation, and apoptosis (Dong, 2002; Simeonova and Luster, 2000). On the other hand, Ca²⁺ influx is induced by sodium arsenite (Goytia-Acevedo et al., 2003). Using DNA-binding assays, we show here that Ca²⁺ levels are important for the appearance of the p53–DNA complex. The pivotal role of Ca²⁺ in epidermal differentiation is reflected by a fourfold increase in extracellular Ca²⁺ from the basal layer to the stratum corneum (Tobin, 2006). Moreover, addition of Ca²⁺ to keratinocytes in vitro arrests the growth of proliferating cells, while induces the expression of differentiation markers like K1/ K2, filaggrin, involucrin, and loricrin (Bikle et al., 2001; Deucher et al., 2002). In accordance to this, inhibition of the rise in intracellular Ca²⁺ levels by BAPTA, confirms the importance of Ca²⁺ in p53–DNA induction by arsenite. It seems that as part of the differentiation program, cells activate p53, which in turn activates the transcription of other genes that participate in this program, at least in cultured human keratinocytes. This scenario may occur also in normal epithelia. In this context, immunocytochemical analysis show increased levels of p53 in foreskin epithelia, located in spinous stratum, coincident with involucrin expression (data not shown).

A recent study showed that arsenite induces the activation of PKB/Akt and its downstream effector, eNOS, in cultured human keratinocytes (Souza et al., 2001). We observed that arsenic was able to activate the Ca²⁺/PI3K/PKB/GSK-3β/p53 pathway. We noticed that arsenic-induced phosphorylation of a typical PKB/Akt target, GSK-3β, known to play diverse roles in cellular processes as differentiation, proliferation, and transformation (Nicholson and Anderson, 2002). Activation of PKB/Akt and GSK-3β by arsenic is dependent on PI3K in NHK (Fig. 5). Interestingly, when we inhibited the PI3K–PKB/ Akt pathway, the p53–DNA complex was severely affected. Withdrawn from cell cycle is an essential step for the cell for the differentiation process (Fuchs, 1990b). In this sense, we wonder if arsenic exposure could induce NHK morphological changes. When cells treated with sodium arsenite cell morphology remained as Ca²⁺ treated control (Fig. 7). Accordingly, sodium arsenite increased the differentiation marker involucrin while diminished the proliferation marker cyclin D1 (Fig. 7). In line with this, it has been shown that arsenite can induce differentiation in other cells systems different responses over the same cells system depending on the concentrations used (Dong, 2002).

Mutations that inactivate or alter p53 are present in different types of human cancer including colon, breast, lung, brain, and esophageal (Nigro et al., 1989). When cells are under stress conditions, p53 activation plays a definite role in cell survival. While in several epithelial cell lines it is possible to notice a clear change in cellular localization (from cytoplasm to nuclei) when p53 is activated, in C33-A cells the nuclear p53 levels remain constant. In terms of transcriptional regulation, activation of a p53-responsive promoter (p53CON reporter gene) increased significantly using Neocarzinostatin in wild type p53 cell lines but not in C33-A (Banuelos et al., 2003). We found clear differences when sodium arsenite effects were analyzed in C33-A. It is tempting to speculate that in the short
term, p53 is activated in response to arsenic-dependent DNA damage, most likely through Ser15 phosphorylation via DNA-PK resulting in the documented dose-dependent augmentation of the p53–DNA complex (Fig. 3). In the long term, the decrease of the p53–DNA complex might be the result of a MDM-2–induced p53 nuclear export and degradation. This interpretation is favored by the fact that the mdm-2 gene is transcriptionally activated by p53 (Moll and Petrenko, 2003). This model suggests that arsenic-dependent p53 activation drives the transcription of its target genes including p21, cyclin D1, PTEN, and mdm-2 among others (Rocha et al., 2003; Stambolic et al., 2001).

A different outcome resulted in C33-A cells. When the PKB/Akt signaling was activated, as indicated by the increase in phosphorylation of PKB/Akt and GSK3-β, p53 behaves differently. In the short term, activation is detected, but under a long-term exposure, activity decreases considerably. LiCl, normally used to promote phosphorylated PKB/Akt stabilization, caused the same effect, this is, a clear decrease in nuclear p53 available for DNA binding and consequently for transcriptional control. This might indicate a more accelerated rate of p53 nuclear export and degradation, rendering it inactive for activate cell growth controllers such as p21waf. In line with this, the activity of a reporter construct based in p53 binding and controlling SV40 promoter, presented a diminished activity under arsenite exposure. In terms of cell ability to growth, this can be translated in the activation of molecules involved in response to mitogens as cyclin D1 or the c-myc factor, as well as the ability to keep a proliferating cell even under starving conditions, as demonstrated for C33-A (Fig. 8).

In summary, our findings clearly suggest that arsenic toxicity is severely augmented in p53-mutant epithelial cells. Work currently in progress in our lab is aimed at establishing the early signaling events triggered by arsenic in epidermal cells.

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