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Low Levels of Arsenite Activates Nuclear Factor- κ B and Activator Protein-1 in Immortalized Mesencephalic Cells

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Abstract

Degeneration of dopaminergic neurons is one of the major features of Parkinson's disease. Many redox-active metals such as iron and manganese have been implicated in neuronal degeneration characterized by symptoms resembling Parkinson's disease. Even though, arsenic, which is another redox-active metal, has been shown to affect the central monoaminergic systems, but its potential in causing dopaminergic cell degeneration has not been fully known. Hence, the present study was designed to investigate arsenic signaling especially that is mediated by reactive oxygen species and its effect on early transcription factors in dopamine producing mesencephalic cell line 1RB₃AN₂₇. These mesencephalic cells were treated with low concentrations of sodium arsenite (0.1, 0.5, 1, 5, and 10 μ M) and incubated for different periods of time (0–4 h). Arsenite was cytotoxic at 5 and 10 μ M concentrations only after 72-h incubation period. Arsenite, in a dose-dependent manner, induced generation of reactive oxygen species (ROS) and activation of early transcription factors such as nuclear factor- κ B (NF- κ B) and activator protein-1 (AP-1) as shown by electro mobility shift assay. Incubation of antioxidants, either *N*-acetyl-L-cysteine (50 μ M) or α -tocopherol (50 μ M) with 1 μ M arsenite, suppressed ROS generation. Arsenite at 1 μ M concentration was sufficient for maximal activation of NF- κ B and AP-1 activation. Time kinetics studies showed maximal activation of NF- κ B by 1 μ M concentration of arsenite was seen at 120 min and correlated with complete degradation of I κ B α at 60 min. Similarly, maximal activation of AP-1 by 1 μ M concentration of arsenite occurred at 120 min. *N*-acetyl-L-cysteine at 50 μ M concentration inhibited arsenite-induced NF- κ B and AP-1. In addition, arsenite was shown to induce phosphorylation of extracellular signal regulated kinase (ERK) 1/2 at concentrations of 1 μ M and above. These results suggest that arsenite, at low and subcytotoxic concentrations, appears to induce oxidative stress leading to activation of early transcription factors whereas addition of antioxidant inhibited the activation of these factors.

Keywords

Arsenite; Reactive Oxygen Species (ROS); Nuclear Factor-Kappa B (NF- κ B); Activator Protein-1 (AP-1); Extracellular Signal Regulated Kinase (ERK); Electrophoretic Mobility Shift Assay (EMSA)

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INTRODUCTION

Metals are trace elements that are essential for the human body. However, certain metals and their derivatives are toxic and have a wide variety of adverse effects, including carcinogenicity, neurotoxicity, and immunotoxicity [1]. Arsenic (As) has long been considered as a toxic metal. Arsenic is an environmental contaminant found naturally in ground water. Arsenic contamination also results from industrial and agricultural uses [2].

Arsenic may also be a teratogen [3] and affects the development of the central nervous system. Both pentavalent and trivalent arsenic have been shown to cross the placental barrier and selectively accumulate in the fetal neuroepithelium in early gestation [4–6]. Developmental exposure to arsenate or arsenite causes exencephaly and neural tube defects [7–13]. Arsenite, a trivalent arsenic [As(III)] is about 10 times more toxic than arsenate that is a pentavalent arsenic [As(V)] in causing neural tube effects [8]. Some important effects of chronic arsenic exposure on the CNS are changes in the content and release patterns of dopamine and other neurotransmitters [14,15]. The mechanisms of arsenic toxicity are not clearly known.

The selective degeneration of dopaminergic neurons in the substantia nigra is one of the principal features of pathogenesis of human Parkinson's disease [16]. Mechanism of dopaminergic neuronal degeneration is not fully understood in Parkinson's disease, but evidence suggests that oxidative stress is involved [17]. One source of oxidative stress that is unique to dopaminergic neurons is the presence of dopamine (DA) itself, as DA can form ROS and quinines through two different pathways. First, DA is metabolized via monoamine oxidase (MAO) to produce hydrogen peroxide (H_2O_2) and dihydroxyphenylacetic acid [18]. H_2O_2 , if not reduced by cellular antioxidant mechanisms such as GSH (glutathione) and GSH peroxidase, can react with transition metals such as iron to form hydroxyl radical [19]. Second, the catechol ring of DA can undergo oxidation to form DA quinone and ROS such as H_2O_2 and superoxide anion ($O_2^{\bullet -}$) in a reaction that can occur either spontaneously in the presence of transition of metals or enzymatically [20,21].

Increased levels of iron are found in the brain of Parkinson's disease (PD) patients [22,23]. The substantia nigra (SN), one of the most vulnerable brain regions in PD, has selectively increased levels of iron [23,24]. Iron has been implicated because it can trigger redox reactions that are involved in neurodegeneration [25]. Several epidemiological studies suggest that long-term occupational and dietary metal exposure is associated with the occurrence of PD [26, 27]. It has been reported in the literature about significant associations of PD with manganese and copper, as well as combinations of lead with copper and iron, and iron with copper, for workers with more than 20 years of occupational contact [28]. A combination of manganese, iron, and aluminum might favor the development of PD after 30 years of exposure [29]. The only positive dose–response relationship was found between mercury exposure and PD but not for other metals [30,31]. In contrast to an earlier study [32], a later study reported a moderate association between iron intake from foods and PD and apparent joint effect of iron and manganese [27,33]. Thus, other transition metals in combination with endogenous iron could play a role in the neurodegeneration.

Experimental support for the contribution of oxidative damage to arsenic neurotoxicity is accumulating. Arsenic, in the presence of Fe^{2+} , by Fenton reaction or Haber–Weiss reaction, is known to cause cellular injury through ROS generation [34,35]. Some of the most prominent toxic effects demonstrated in arsenic-exposed cells, such as DNA damage [36,37] and apoptosis [38–40], have been traced to the generation of reactive oxygen species (ROS) during oxidative stress caused by arsenite [41–45]. Chronic exposure to low levels of arsenic in rats results in depletion of glutathione and increases oxidized glutathione and lipid peroxidation in the brain [46–48].

Arsenite was demonstrated to promote or alter gene expression and modify intra- and intercellular communication [49]. Interference with signal transduction pathways is one of several modes of action that has been proposed for arsenic [50,51]. Alterations in intracellular oxidation/reduction (redox) reactions have been shown to activate signal cascades that regulate early response genes. These genes are believed to function in a protective or reparative capacity. Stress-induced activation of these early response genes appears to rely, at least in part, on changes in intracellular redox status. Transcription factors, such as activator protein-1 (AP-1) and nuclear factor-kappa B (NF- κ B), play a vital role in these responses. Both AP-1 and NF- κ B are considered stress response transcription factors, which regulate the expression of a variety of downstream target genes, such as proinflammatory genes that are known to be involved with cellular antioxidant defense mechanisms [52]. NF- κ B is maintained in the cytoplasm of nonstimulated cells through interaction with specific inhibitors, I κ Bs [53]. In response to proinflammatory stimuli, the I κ Bs are rapidly phosphorylated and degraded by ubiquitin-dependent proteolysis, resulting in the release of free NF- κ B dimers, which translocate to the nucleus to induce transcription of target genes [54]. In contrast, the AP-1 heterodimers are constitutively localized within the nucleus and transactivation of AP-1 is achieved through phosphorylation of its activation domain by c-Jun N-terminal kinase (JNK) [55].

Arsenic has been shown previously to affect transcription and expression of proto-oncogenes in mammalian cells in culture. However, many previous studies used only short-term exposures and significantly higher (more toxic) doses of arsenic [50,52,56–58]. There are some evidence showing how long-term exposure with low-dose arsenic affects signal transduction pathways [59]. Arsenic concentrations used in all these studies ranged from 1 to 500 μ M. The concentrations below 10 μ M are considered as low where as concentrations greater than 10 μ M are considered as high concentrations [60]. Many previous studies that were conducted to study the short-term exposure of arsenite used significantly higher doses of arsenite (i.e., >10 μ M) that are very toxic and they inhibited NF- κ B where as low and noncytotoxic concentrations of arsenic (i.e., <10 μ M) activated NF- κ B [60]. In human GM847 fibroblast cell line, short-term exposure (3–24 h) of arsenite induced early transcription factors such as NF- κ B and AP-1 even at very low concentrations such as 0.1 and 0.5 μ M [59]. In this study, we report the exposure of dopaminergic cells 1RB₃AN₂₇ to arsenite for shorter durations ($\leq$ 4 h and at low concentrations, i.e., 0.1–10.0 μ M) alters the cellular redox status and activation of these early transcription factors in a very significant manner. This 1RB₃AN₂₇ cell line, which produces dopamine, also expresses tyrosine hydroxylase, dopamine transporter, neuron-specific enolase, and nestin, was derived from fetal rat mesencephalon by transfection with plasmid vector (pSV₃^{neo}) that carries the large T-antigen gene from SV40 virus [61].

MATERIALS AND METHODS

Chemicals

Sodium arsenite from Fisher Scientific (New Jersey), 2',7'-dichlorofluorescein diacetate (H₂DCF), 3-(4,5-dimethyl-2-thiozoly)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT), *N*-acetyl-L-cysteine (NAC), ($\pm$)- α -tocopherol from Sigma Chemicals Corporation (St. Louis, MO) were purchased. Double stranded oligonucleotides having consensus sequences of NF- κ B and AP-1 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Culture medium RPMI 1640, fetal bovine serum, and horse serum were purchased from Invitrogen Corporation (Grand Island, NY). The rabbit polyclonal antibody namely anti-I κ B α antibody from Santa Cruz Biotechnology (Santa Cruz, CA), the phospho-specific anti-p44/42 antibody (Thr 202/ Tyr 204) from New England Biolabs (Beverly, MA), and monoclonal anti- α -tubulin antibody from Sigma Chemicals (St. Louis, MO) were obtained.

Cell Culture

Mesencephalic cell line namely 1RB₃AN₂₇ was grown in culture medium RPMI 1640 supplemented with 2 mmol/L glutamine, 100 units/mL penicillin, 100 µg/mL streptomycin, 5% fetal bovine serum and 5% horse serum at 37°C and in 5% CO₂ atmosphere. Dr AG Kanthasamy, Department of Biomedical Sciences, Iowa State University, Ames, Iowa, kindly provided the cell line to us.

Cytotoxicity Assay

The cytotoxicity was assayed by the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) dye uptake. Briefly, 1RB₃AN₂₇ cells (5000 cells/well of 96-well plate) were incubated with different concentrations of sodium arsenite with the final volume of 0.1 mL for different periods of time (4, 24, and 72 h) at 37°C. Thereafter, 25 µL of MTT solution (5 mg/mL in PBS) was added to each well. After 2-h incubation at 37°C, 0.1 mL of the extraction buffer (20% SDS, 50% dimethylformamide) was added. After an overnight incubation at 37°C, the absorbance was read at 570 nm using microplate reader VERSAmax (Molecular Devices, Sunnyvale, California), with the extraction buffer as blank. The cell viability in response to the treatment with arsenite was calculated as percentage of cell viability = (O.D. treated/O.D. control) × 100.

Reactive Oxygen Species Generation

Intracellular accumulation of ROS was determined with 2',7'-dichlorofluorescein diacetate (H₂DCF). This nonfluorescent compound accumulates within cells upon deacetylation, H₂DCF then reacts with ROS to form fluorescent dichlorofluorescein (DCF). The 1RB₃AN₂₇ cells (2 × 10⁶/mL) were incubated with different concentrations of arsenite for 2 h at 37°C. In another experiment, antioxidants namely *N*-acetyl-L-cysteine, 50 µM, (NAC), and α -tocopherol (50 µM), each of them was incubated with arsenite for similar duration and the levels of ROS were measured. After treatment, cells were transferred to eppendorf tubes and washed twice in PBS and resuspended in 100 µL of Krebs Ringer-bicarbonate (KRB) buffer. Protein was measured using BioRad protein assay reagent by Bradford method [62]. Appropriate volume of cell suspension equivalent to 100 µg protein was made up to final volume of 100 µL with KRB buffer and incubated with 10 µL H₂DCF (10 µM in DMSO) in microtiter plate for 3 h at 37°C. Cellular fluorescence was monitored on a Fluoroskan Ascent fluorometer (Labsystems, Helsinki, Finland) using an excitation wavelength of 485 nm and emission wavelength of 527 nm.

Electrophoretic Mobility Shift Assays (EMSA)

Mesencephalic cells were plated in 60-mm petridish and pretreated at 60% confluent state with different concentrations of sodium arsenite for a period of 4 h at 37°C and in 5% CO₂ atmosphere. In some of the experiments, the cells were treated with antioxidant *N*-acetyl-L-cysteine (50 µM) along with 1 µM arsenite for a period of 2 h. And then, nuclear extracts were prepared and activation of NF-κB and AP-1 were analyzed by EMSA as previously described [63].

Briefly, cells were washed with cold PBS and suspended in 100 µL of lysis buffer (10 mM HEPES; pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF, 2 µg/mL leupeptin, 2 µg/mL aprotinin, and 0.5 mg/mL benzamidine). Cells were lysed with 3.1 µL of 10% Nonidet P-40. The nuclear pellet was resuspended in 25 µL of ice-cold extraction buffer [20 mM HEPES (pH 7.9), 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, 2 µg/mL leupeptin, 2 µg/mL aprotinin, and 0.5 mg/mL benzamidine]. The supernatant, i.e., nuclear extract, was either used immediately or stored at -70°C for later use. The protein content was measured by the method of Bradford [62]. EMSAs were performed by incubating

4 μ g protein of the nuclear extract with 16 fmol of 32 P end-labeled, 45-mer double-stranded NF- κ B oligonucleotide from the HIV-1 long terminal repeat, 5'-TTGTTACAAGGGACTTTCCGCTGGGGACTTT-CCAGGGAGGCGTGG-3' (bold indicates NF- κ B binding sites), in the presence of 2–3 μ g of poly(dI-dC) in a binding buffer [25 mM HEPES (pH 7.9), 0.5 mM EDTA, 0.5 mM DTT, 1% Nonidet P-40, 5% glycerol, and 50 mM NaCl] for 15 min at 37°C as described previously. The DNA–protein complex formed was separated from the oligonucleotide on 7.5% native polyacrylamide gel using buffer containing 50 mM Tris, 200 mM glycine (pH 8.5), and 1 mM EDTA and then the gel was dried. Visualization and quantitation of radioactive bands were performed using Personal Molecular Imager FX (BioRad, Hercules, CA) using Quantity One software.

The EMSA for AP-1 was performed as described for NF- κ B using 32 P end-labeled double-stranded oligonucleotides. To assay AP-1, 4 μ g of nuclear extract protein was incubated with 16 fmol of the 32 P-end-labeled AP-1 consensus oligonucleotide 5'-CGCTTGATGACTCAGCCGGAA-3' (bold indicates the AP-1 binding site) for 15 min at 37°C, and then the DNA–protein complexes formed were resolved from free oligonucleotide on 6% native polyacrylamide gels. The radioactive bands were visualized and quantified as indicated above.

Western Blotting for I κ B α

The cytoplasmic extracts from treated and untreated cells were used to examine I κ B α by Western blot method as described previously [64]. After the NF- κ B activation reaction as described above, postnuclear extracts were resolved on 10% SDS-polyacrylamide gels to assay I κ B α . After running the gels, the proteins were electrotransferred onto nitrocellulose filters and probed with rabbit polyclonal antibody against I κ B α and bands were detected by chemiluminescence (ECL, Amersham, Arlington Heights, IL). The bands were quantitated by densitometer scan using LabWorks software version 4.0 (UVP Bioimaging Systems, Upland, CA).

Western Blot Analysis of ERK

ERK activity was checked from total cell lysate by Western blot analysis using phospho-specific anti-p44/42 ERK (Thr²⁰²/Tyr²⁰⁴) antibody as described previously [64]. Briefly, after incubation with various concentrations of arsenite for 120 min at 37°C, 1RB₃AN₂₇ cells were washed with Dulbecco's PBS and then extracted with lysis buffer containing 20 mM HEPES (pH 7.4), 2 mM EDTA, 250 mM NaCl, 0.1% Nonidet P-40, 2 μ g/mL leupeptin, 2 μ g/mL aprotinin, 1 mM PMSF, 0.5 μ g/mL benzamidine, 1 mM DTT, and 1 mM sodium *o*-vanadate. The protein concentration in the supernatant was determined and then resolved with 50 μ g of protein/lane on 10% SDS-PAGE. After the electrophoresis, the proteins were electrotransferred to nitrocellulose filters, probed with the phospho-specific anti-p44/42 ERK (Thr²⁰²/Tyr²⁰⁴) Ab (New England Biolabs, Beverly, MA) raised in rabbits (1/3000 dilution). Then the membrane was incubated with peroxidase-conjugated anti-rabbit IgG (1/3000 dilution), and bands were detected by chemiluminescence (ECL, Amersham).

Statistical Analysis

Data were expressed as means $\pm$ SD and two-tailed student's *t* test using Microsoft Excel program was used and accepted levels of significance in all cases were *p* < 0.05.

RESULTS

Arsenite Induced Cell Death and Generation of ROS

In this report, we examined the effect of arsenic on the activation of transcription factors NF- κ B, AP-1, and related kinase. Arsenite, even at the highest concentration used (i.e., 10 μ M) in the study was not cytotoxic when incubated for 4–24 h (data not shown). Only after incubation of 72 h, viability of cells decreased by 25% at 5 μ M concentration of arsenite and by 75% at 10 μ M concentration compared to control, as determined by MTT assay (Figure 1). All our experiments that were conducted to study ROS generation or transcription factors activation, duration of incubation was $\leq$ 4 h during which arsenite even at 10 μ M was noncytotoxic. The reactive oxygen species generation as determined using H₂DCF increased in dose-dependent manner (Figure 2). Addition of either of the antioxidants, NAC at 50 μ M concentration or α -tocopherol at 50 μ M concentration, suppressed the arsenite-induced ROS generation (Figure 3).

Arsenite Induced NF- κ B Activation

The 1RB₃AN₂₇ cells were incubated with different concentrations of arsenite at 37°C for 4 h. They were then examined for NF- κ B activation by electrophoretic mobility shift assay. The results in Figure 4 indicated that arsenite activated NF- κ B in dose-dependent manner. Arsenite concentration of 1 μ M was sufficient to elicit maximal activation of NF- κ B. The 1RB₃AN₂₇ cells were incubated with 1 μ M arsenite for different periods of time (0, 15, 30, 60, 120, 240 min). The EMSA results in Figure 5 indicate NF- κ B activation occurred in time-dependent manner with maximum activation at 120 min. Incubation of cells with antioxidant NAC at 50 μ M concentration for 2 h suppressed the arsenite-induced NF- κ B activation (Figure 6).

Arsenite Activated Degradation of I κ B α

The translocation of NF- κ B to the nucleus is preceded by the phosphorylation and proteolytic degradation of I κ B α in the cytosol. To determine the effects of arsenite on I κ B α degradation, the cytosolic extracts were assayed for I κ B α by Western blot analysis. Results (Figure 7) indicated that arsenic treatment (1 μ M) caused degradation I κ B α by 30 min and with maximum degradation by 60 min. The disappearance and reappearance of I κ B α corresponded with the kinetics of NF- κ B activation.

Arsenite Induced AP-1 Activation

Similar to NF- κ B activation, arsenic also activated another transcription factor, AP-1, in 1RB₃AN₂₇ cells in a dose-dependent manner, with maximum activation at 1 μ M (Figure 8). When examined the time course, AP-1 activation, as induced by 1 μ M concentration of arsenite, reached its maximum in 2 h (Figure 9). But, incubation of antioxidant NAC at 50 μ M concentration for 2 h suppressed the arsenite-induced AP-1 activation (Figure 10).

Arsenic Activated ERK

Effect of arsenic on ERK activity was studied in 1RB₃AN₂₇ cells with different concentrations of arsenic for 2 h and phosphorylated ERK was examined by Western blot. Arsenite of 1 μ M concentration was sufficient to induce maximum phosphorylation (Figure 11).

DISCUSSION

The present study was attempted to investigate the effect of arsenic at low concentrations, i.e., $\leq$ 10 μ M in mesencephalic cell line 1RB₃AN₂₇. Earlier reports on arsenic that were reviewed, considered arsenic below 10 μ M as low concentrations that are nontoxic to most cell types. Whereas, concentrations above 10 μ M are considered as high and are very toxic to majority

of cell types [60]. This cell line produces dopamine (DA) and expresses tyrosine hydroxylase, dopamine transporter, neuron-specific enolase, and nestin [61]. Exposure of cells to durations from 4 to 24 h to arsenite in all the concentrations studied did not show cytotoxicity (data not shown). When cells were exposed to a much longer period, i.e., for 72 h, at concentrations ≤ 1 μM , arsenite was not cytotoxic to this cell line but above this concentration, cell viability was significantly reduced as shown by MTT assay. Thus, the concentrations of arsenite used in the study, i.e., 0.1–10 μM under the experimental conditions used (i.e., ≤ 4 h of incubation period) are nontoxic doses for these dopaminergic cells. Present results have shown that arsenite induced reactive oxygen species (ROS) in a dose-dependent manner in 1RB₃AN₂₇ cells. Arsenic has been demonstrated to induce the formation of ROS in a wide variety of cells, including human vascular smooth muscle cells [65], human–hamster hybrid cells [44], vascular endothelial cells [57], murine keratinocytes [66], acute promyelocytic leukemia cells [67], Chinese hamster ovary cells [68], chronic lymphocytic leukemia cells [69], and peripheral human lymphocytes [70]. Arsenite has been shown to induce oxidative stress in the central nervous system of rats [71]. It was also shown that human fetal brain explants exposed *ex vivo* to arsenite generated ROS and brain cells underwent apoptosis. Addition of antioxidants such as NAC and vitamin E in our study reduced oxidative stress induced by arsenite. In previous studies also, antioxidant vitamins such as C and E had been shown to reverse arsenic toxicity [72]. Similarly coadministration of antioxidants reduced arsenic-induced oxidative stress in brain of rats that were exposed to arsenite in drinking water [73].

Homeostasis of natural oxidation and reduction equilibrium within cells is crucial for maintaining cellular viability. Arsenic compounds are known to imbalance such equilibrium, thereby generating oxidative stress [74]. Arsenic-induced ROS has been demonstrated to cause DNA damage, lipid peroxidation, and protein modification [60]. Trivalent arsenic has been shown to interact with sulfhydryl (SH) group of biomolecules, an important mechanism of toxicity [75]. It was also demonstrated that binding of arsenic to the SH-group of glutathione (GSH) resulted in accumulation of intracellular ROS leading to activation of caspases [41]. Developing brain cells exposed to arsenic was shown to undergo a loss of glutathione and an increase in cellular ROS leading to apoptosis [72]. It was shown that ROS was involved in the cell signaling and affected cytoplasmic and nuclear signal transduction pathways that regulate gene expression [76]. Reports have indicated that ROS can act as signaling messengers to activate transcription factors. Both AP-1 and NF- κ B, which are examples of transcription factors, considered as stress responsive transcription factors that govern the expression of proinflammatory and cytotoxic genes [77].

Our study has clearly shown that arsenite induced activation of transcription factor NF- κ B in mesencephalic cell line in dose-dependent manner and 1 μM was sufficient to induce maximal activation of NF- κ B. Others have also reported similar NF- κ B activation by arsenic at low concentrations (0.1–5.0 μM) in GM487 cells, a human fibroblast cell line [59]. Whereas, high concentrations of arsenic (500 μM) inhibited NF- κ B in human embryonic kidney cells, HEK293, and human bronchial epithelial cells, BEAS 2B [78]. Our results show that arsenic-induced NF- κ B activation was accompanied by characteristic I κ B α degradation in a time-dependent manner in 1RB₃AN₂₇ cells. In As(III) exposed ECV304 cells, a human endothelial cell line, similar phosphorylation and degradation of I κ B α were reported for the activation of NF- κ B [79]. Certain other studies have shown that exposure to arsenic induced basal NF- κ B binding activity but without an increase in I κ B α phosphorylation nor an increase in translocation in lung epithelial cells and alveolar macrophages indicating alternative mechanism [43,80]. We have reported earlier from our laboratory that metals such as lead and manganese (Mn^{2+}) have also induced NF- κ B activation and corresponding degradation of I κ B α in PC12 cells, a dopaminergic cell line [81,64].

Many groups have studied mechanism of NF- κ B activation. NF- κ B is a heterodimer composed of p50 and p65 and is inactive in cytoplasm through association with one of many inhibitors, of which I κ B α is the most abundant one [82]. Signal-induced degradation involves phosphorylation of two serine residues, S32 and S36. This phosphorylation leads to polyubiquitination of two specific lysines on I κ B α (K21 and K22) by SCF- β -TrCP complex and its degradation by the 26S proteasome [83]. The phosphorylation is accomplished by a specific I κ B kinase (IKK). This IKK family is further activated by several upstream kinases such as MAPK kinases, Akt, NIK, NAK, and PKC [60]. We have previously reported that toxic metals such as lead and manganese activate NF- κ B through mitogen activated protein kinase (MAPK/ERK) activation in PC12 cells [81,64]. Since many reports suggest that MAP kinase activation is required [84], whether arsenite induced MAP kinase activity was also examined in this cell type. The kinetics of arsenite-induced MAPK activity and NF- κ B activation indicate that MAP kinase may have contributed to NF- κ B activation. It was shown that PD98059, an inhibitor of ERK activation, inhibited the activation of NF- κ B in JB6 cells suggesting upstream activation of MAP kinase (ERK) essential for NF- κ B activation [85].

Our results showed that, as with NF- κ B, arsenite affects AP-1 activation and DNA binding in a time- and dose-dependent manner. We have shown in our earlier studies in dopaminergic cell line namely PC12, metals such as lead and manganese also activated AP-1 along with NF- κ B [64,81,86]. Many groups have studied mechanism of AP-1 activation. Activator Protein-1 (AP-1), is another group of transcription factors is composed of hetero- or homodimer subunits of proteins from fos, jun, jun dimerization partner (JDP), and activating transcription factor (ATF) families. AP-1 is activated by a wide variety of stimuli, including growth factors, inflammatory cytokines, UV radiation, and oxidative stress [87]. Arsenic is known to induce activation of AP-1 in a variety of cell types including epithelial cells, fibroblasts, type II cells, and alveolar macrophages [88]. Similar arsenic-induced AP-1 activation was also reported in GM487 fibroblast cell [59], in rat lung epithelial cells [43], in HeLa cells [89]. AP-1 activation in mouse epidermal JB6 cells, inhibiting PKC activation blocked both arsenic-induced AP-1 activity and phosphorylation of the three major classes of MAP kinases such as ERKs (extracellular receptor kinases), c-Jun-N-terminal kinase (JNK), and p38 kinases, indicating that PKC is required for MAP kinase activation [90]. Certain other studies indicated that arsenic differentially activated ERKs, p38, and JNKs to mediate opposing effects (i.e., cell proliferation and apoptosis), which were dependent of time, dose, and oxidative form of arsenic and the type of target cell [60]. The activation of JNKs and p38 could induce cell growth arrest and apoptosis [91]. In rat cerebellar neurons, it was shown that arsenite, at 10 μ M concentration, induced selective activation of p38 and JNK3 [92].

Collectively, these results indicate that arsenite, at low and subcytotoxic concentrations (i.e., 0.1–10 μ M), with shorter exposure periods ($\leq$ 4 h), can induce the generation of reactive oxygen species causing oxidative stress in this dopaminergic cell line. This in turn leads to activation of early transcription factors such as NF- κ B and AP-1. Induction of ERK phosphorylation by arsenite suggests that upstream activation of MAPK may be involved in the activation of transcription factor NF- κ B. Future studies elucidating the role of Ca²⁺, PKC, other MAP kinases, caspases in this signaling cascade and apoptotic pathway will further help in better understanding of arsenic-mediated neurotoxic effects.

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FIGURE 1.

Effect of arsenite on viability of 1RB₃AN₂₇. Viability was measured by the reduction of the tetrazolium salt MTT, which was assessed after 72-h incubation with arsenite at different concentrations. Results are expressed as the percentage of cell death and are the means $\pm$ SD of three experiments in triplicate. Untreated cells were assumed to be vital (100% viability).

* $p < 0.05$ versus control.

FIGURE 2.

Arsenite-induced generation of reactive oxygen species (ROS) in 1RB₃AN₂₇ cells was measured by the oxidation of 2',7'-dichlorofluorescein (H₂DCF) to the fluorescent 2',7'-dichlorofluorescein (DCF), and it was assessed after 2-h incubation with sodium arsenite at different concentrations. Results are expressed as DCF fluorescence units and are the means $\pm$ SD of three experiments in triplicate. * $p < 0.05$ versus control.

FIGURE 3.

Effect of antioxidants on arsenite-induced generation of reactive oxygen species (ROS) in 1RB₃AN₂₇ cells. ROS was measured by the oxidation of 2',7'-dichlorofluorescein (H₂DCF) to the fluorescent 2',7'-dichlorofluorescein (DCF), and it was assessed after 2-h incubation with sodium arsenite (1 μ M) and each of antioxidants [*N*-acetyl-L-cysteine (50 μ M), α -tocopherol (50 μ M)]. Results are expressed as DCF fluorescence units and are the means $\pm$ SD of three experiments in triplicate. * p < 0.05 versus control.

FIGURE 4.

Dose-dependent activation of NF- κ B by arsenite in 1RB₃AN₂₇ cells. The cells were incubated with different concentrations of arsenite (0–10 μ M) for 4 h and then nuclear extracts were prepared and activation through DNA binding was determined by electrophoretic mobility shift assay. Similar results were obtained in three independent experiments.

FIGURE 5.

Time-dependent activation of NF-κB by arsenite in 1RB₃AN₂₇ cells. Cells were exposed to arsenite (1 μM) for different durations (0–240 min) as indicated and then activation was determined in the nuclear extracts by electrophoretic mobility shift assay. Similar results were obtained in three independent experiments.

FIGURE 6.

Effect of antioxidant *N*-acetyl-L-cysteine on arsenite-induced activation of NF-κB in 1RB₃AN₂₇ cells. Cells were incubated with arsenite (1 μM) and antioxidant *N*-acetyl-L-cysteine (50 μM) for 2 h and activation of NF-κB was determined in the nuclear extracts by electrophoretic mobility shift assay FP = free probe (probe only); C = control (unstimulated); As = arsenite (1 μM); As + NAC = arsenite (1 μM) plus *N*-acetyl-L-cysteine (50 μM). Similar results were obtained in three independent experiments.

FIGURE 7.

Arsenite-induced IκBα degradation in the cytosolic fraction. Cells were incubated with arsenite (1 μM) for different time periods (0–240 min) and IκBα was detected in the cytosolic fraction by Western blot using polyclonal antibody against IκBα as described in Materials and Methods section. Degradation of IκBα is started at 30 min and most of IκBα degraded at 60 min and reappeared at 120 min. Similar results were obtained in three independent experiments. The same Western blot membrane was reprobed with monoclonal anti-α tubulin antibody to check proper loading of samples.

FIGURE 8.

Dose-dependent activation of AP-1 DNA binding by arsenite in 1RB₃AN²⁷ cells. The cells were incubated with different concentrations of arsenite (0–10 μM) for 240 min and then nuclear extracts were prepared and activation was determined by electrophoretic mobility shift assay. Similar results were obtained in three independent experiments.

FIGURE 9.

Time-dependent activation of AP-1 by arsenite in 1RB₃AN₂₇ cells. Cells were exposed to arsenite (1 μ M) for different durations (0–240 min) as indicated and then activation was determined in the nuclear extracts by electrophoretic mobility shift assay. Similar results were obtained in three independent experiments.

FIGURE 10.

Effect of antioxidant *N*-acetyl-L-cysteine on arsenite-induced activation of AP-1 in 1RB₃AN₂₇ cells. The cells were incubated with arsenite (1 μ M) and antioxidant *N*-acetyl-L-cysteine (50 μ M) for 2 h and activation of AP-1 was determined in the nuclear extracts by electrophoretic mobility shift assay. FP = free probe (probe only); C = control (unstimulated); As = arsenite (1 μ M); As + NAC = arsenite (1 μ M) plus *N*-acetyl-L-cysteine (50 μ M). Similar results were obtained in three independent experiments.

FIGURE 11.

Effect of arsenite on phosphorylation of ERK in 1RB₃AN₂₇ cells. The cells were exposed to various concentrations of arsenite (0.1–1.0 μM) and total cell lysates were assayed for ERK activation by Western blotting using antibody specific to phospho-specific p44/42 ERK. Activation of ERK was observed following arsenite exposure at different concentration compared to untreated control. Similar results were obtained in three independent experiments. The same Western blot membrane was reprobbed with monoclonal anti-α tubulin antibody to check proper loading of samples.