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Sodium arsenite-induced inhibition of eukaryotic translation initiation factor 4E (eIF4E) results in cytotoxicity and cell death

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Abstract

Exposure to arsenic (As) is a risk factor for the development of diabetes, vascular diseases and cancer. Several theories have been proposed to account for the mechanisms potentially responsible for As toxicity and carcinogenesis. Currently, we have investigated whether the eukaryotic translation initiation factor 4E (eIF4E), the mRNA cap binding and rate limiting factor required for translation, is a target for As-induced cytotoxicity and cell death. We have also investigated the potential cellular mechanisms underlying the As-induced de-regulation of expression of eIF4E that are most likely responsible for the cytotoxicity and cell death induced by As. Exposure of four different human cell lines – HCT15 (colorectal adenocarcinoma), PLC/PR/5 (hepatocellular carcinoma), HeLa (cervical adenocarcinoma) and Chang (likely derived from HeLa cells) to sodium arsenite ($NaAsO_2$) for time intervals up to 24 h resulted in a concentration-dependent cytotoxicity and cell death. All the $NaAsO_2$-treated cells exhibited significant inhibition of eIF4E gene (protein). The potential involvement of eIF4E gene expression in the $NaAsO_2$-induced cytotoxicity and cell death was investigated by silencing the cellular expression of the eIF4E gene by employing a small interfering RNA (SiRNA) specifically targeting the eIF4E gene's expression. The SiRNA-mediated silencing of eIF4E gene expression also resulted in significant cytotoxicity and cell death suggesting that the toxicity noticed among the $NaAsO_2$-treated cells was probably due to the chemically induced inhibition of eIF4E gene expression. The potential involvement of inhibition of eIF4E gene expression in the $NaAsO_2$-induced cytotoxicity and cell death was further investigated by employing transgenic cell lines overexpressing the eIF4E gene. Overexpression of the eIF4E gene in the Chinese hamster ovary cell line was protective against the $NaAsO_2$-induced cytotoxicity and cell death. Additional studies conducted to understand the potential mechanisms responsible for $NaAsO_2$-induced inhibition of eIF4E gene expression demonstrated that exposure to $NaAsO_2$ resulted in transcriptional down-regulation of the eIF4E gene only in HCT-15 and HeLa cells, while in the $NaAsO_2$-treated and PLC/PR/5 and Chang cells, the eIF4E mRNA expression level was comparable to those of the corresponding control cells. Cellular levels of ubiquitin and the process of ubiquitination were significantly higher in the $NaAsO_2$-treated cells compared with the control cells. Immunoprecipitation of lysates obtained from the $NaAsO_2$-treated cells and the subsequent western blot analysis of the immunoprecipitated protein(s) using the eIF4E antibody detected the presence of eIF4E protein in the immunoprecipitate suggesting possible ubiquitination of eIF4E protein in the $NaAsO_2$-treated cells. Pre-exposure of the $NaAsO_2$-treated cells to proteasome inhibitors blocked the inhibition of eIF4E gene expression as well as the resulting cytotoxicity and cell death. Furthermore, exposure of cells to $NaAsO_2$ resulted in a significant inhibition of expression of the cell cycle and growth regulating gene, cyclin D1. Whether or not the inhibition of cyclin D1 in the $NaAsO_2$-treated cells is mediated through the inhibition of eIF4E was tested by silencing the expression of eIF4E gene in the cells. Transfection of cells with SiRNA specifically targeting eIF4E gene expression resulted in a significant inhibition of cyclin D1 gene suggesting that

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the observed inhibition of cyclin D1 gene in the $NaAsO_2$-treated cells is most likely mediated through inhibition of eIF4E gene. Taken together, our results indicate that the exposure of cells to $NaAsO_2$ resulted in cytotoxicity and cell death, at least in part, due to the inhibition of eIF4E gene expression leading to diminished cellular levels of critical genes such as cyclin D1. (Mol Cell Biochem **279:** 123–131, 2005)

Key words: arsenic, eukaryotic translation initiation factor 4E (eIF4E), gene expression, small interfering RNA (SiRNA), toxicity, ubiquitination, protection

Introduction

Exposure to arsenic (As) as well as to its compounds and the resulting health effects is a major concern for people living in many different parts of the world. Certain geological formations contain significant quantities of As which can leach into ground water resulting in significant contamination of drinking water in certain geographical areas [1]. In addition, approximately 100,000 tons of As-containing compounds are manufactured annually on a global scale [2]. Arsenic and some of its compounds are used in agricultural applications as insecticides, herbicides, fungicides and algicides. Certain As-containing compounds are used in the control of specific veterinary diseases such as tape worms in sheep and cattle and black head disease in turkeys and chickens [2]. In humans, arsenic is used as an anticarcinogenic agent mainly in the treatment of acute promyelocytic leukemia as well as in treating certain tropical diseases such as the African sleeping sickness and amoebic dysentery [2]. The presence of As in the environment, food and water as well as its extensive use for various applications have resulted in significant human exposure to As.

Results of several *in vitro*, *in vivo* and epidemiological studies have demonstrated the toxicity associated with exposure to As and its compounds. Exposure of cultured Syrian hamster embryo cells to As induces morphological changes [3]. Exposure to As results in toxicity of several organs and organ systems in experimental animals such as mice, rats, hamsters, guinea pigs and rabbits [4, 5]. In humans, epidemiological studies have found an association between exposure to As and liver injury, peripheral neuropathy, vascular diseases, type II diabetes, jaundice, hyperkeratosis of skin, etc. [6, 7]. Furthermore, a significant increase in the risk for developing cancers of multiple organs and tissues including skin, lungs, liver and bladder have been seen under conditions of substantial exposure to As [8–10].

Although not clearly understood, several mechanisms potentially responsible for toxicity due to the exposure to As have been proposed. These include chromosomal abnormalities [11, 12], oxidative stress [13], alterations in the DNA repair and methylation patterns [14, 15], enhanced cell proliferation [16], gene amplification [17], alterations in the expression of growth factors [18, 19] and alterations in signal transduction pathways [20–22]. Transcriptional de-regulation of gene expression, mainly through modification of signal transduction pathways resulting in modulation of expression of target genes has been proposed as a major mechanism responsible for As toxicity [23].

In the present study, the role of eukaryotic translation initiation factor 4E (eIF4E) – the rate-limiting translation initiation factor involved in eukaryotic protein synthesis, in As-induced cytotoxicity and cell death has been investigated. Results of the studies described in this communication have demonstrated that eIF4E is a novel cellular target for As-induced cytotoxicity and cell death. The underlying mechanisms responsible for the As-induced down-regulation of eIF4E gene expression and the potential implications pertaining to cytotoxicity and cell death are discussed.

Materials and methods

Cytotoxicity studies

Human cell lines, viz: HeLa (cervical adenocarcinoma, catalogue number CCL-2), HCT15 (colon adenocarcinoma, catalogue number CCL-225), PLC/PR/5 (hepatocellular carcinoma, catalogue number CRL-8024), and Chang (likely derived from HeLa cells, catalogue number CCL-13), were purchased from ATCC (Manassas, VA) and were cultured in medium containing fetal bovine serum and all required supplements as recommended by the supplier. Exponentially growing cells were used to determine the lethal concentration 50 (LC50) for sodium arsenite ($NaAsO_2$). One hundred thousand cells each were plated in the individual wells of a 96-well cell culture plate. The cells were allowed to attach and grow overnight. An aqueous stock solution of $NaAsO_2$ (Sigma Chemical Company, St. Louis, MO) prepared in sterile water was diluted with fresh serum-free medium to get the desired final concentrations. The cells were allowed to grow in the control medium as well as in medium containing increasing concentrations of $NaAsO_2$ for 24 h. The number of surviving cells following the 24 h of exposure to $NaAsO_2$ was determined by the MTT cell proliferation assay (ATCC, Manasas, VA) following the instructions provided by the supplier. The LC50 for $NaAsO_2$ was determined for each of the

cell lines based on their response to increasing concentrations of $NaAsO_2$ resulting in 0–100% cell death.

Effect of $NaAsO_2$ on eIF4E gene expression

Quantitative real-time PCR and western blot analysis were employed to determine the effect of $NaAsO_2$ on the expression level of the eIF4E transcript and protein, respectively. Exponentially growing HeLa, HCT-15, PLC/PR/5 and Chang cells were treated for 24 h with $NaAsO_2$ at the respective LC50 values obtained for each of the cell lines as determined by an independent cytotoxicity experiment. Following the chemical exposure, the cells were washed with PBS. Total RNA, free of contaminating genomic DNA and cellular proteins, was isolated from one-half of the control and $NaAsO_2$-treated cells using RNeasy Mini Kit (Qiagen Inc., Valencia, CA). Cell lysates were prepared from the remaining half of the control and the chemically treated cells using a mammalian protein extraction reagent (Pierce, Rockford, IL). The cell lysates prepared were centrifuged at 10,000 rpm for 10 min at 4 °C and the clear supernatant was collected. Protein concentrations of the lysates were determined spectrophotometrically using Bio-Rad protein assay reagent (Bio-Rad, Hercules, CA). Thirty micrograms of protein from the control and the $NaAsO_2$-treated cells were electrophoresed on a 12% SDS containing denaturing gel and were transferred onto a polyvinylidene membrane for western blot analysis. The eIF4E protein reacting with a mouse anti-human eIF4E antibody (BD Biosciences, Palo Alto, CA) was detected using an enhanced chemiluminiscent system (Amersham Biosciences Corporation, Piscataway, NJ). Subsequently, the blots were stripped of the eIF4E antibody and the abundance of the house-keeping gene – GAPDH, was determined by western blot analysis. Intensity of the eIF4E and GAPDH protein bands reacting with the corresponding antibodies was determined by scanning the western blot images using a densitometer equipped with the ImageQuant software (Amersham Biosciences Corporation, Piscataway, NJ).

For quantitative real-time PCR analysis to determine the expression levels of eIF4E and GAPDH (house-keeping gene) transcripts, total RNA isolated from the cells were reverse transcribed using the Advantage RT-for-PCR kit (BD Biosciences, Palo Alto, CA). Nucleotide sequences of the primers used to amplify the target and the house-keeping genes were: eIF4E–5′-CTACTAAGAGCGGCTCCACCAC-3′ (F) and 5′-TCGATTGCTTGACGCAGTCTCC-3′ (R); and GAPDH - 5′-GCCTTCTGCACCACCAACTGC-3′ (F) and 5′-GGCAGTGATGGCGTGGACTATG-3′ (R). The PCR amplification, detection of the amplified gene products and their quantitation were performed with the SYBR green PCR core reagent kit (PE Applied Biosystems, Foster City, CA) and the ABI-PRISM 5700 sequence detection system following the instructions provided by the manufacturer. The PCR amplified gene products were analysed by agarose gel electrophoresis to ensure that only the intended product was amplified in each case. The expression level of eIF4E calculated using the formula 2ˆ(−(Ct_target − Ct_GAPDH)), was normalized to the expression level of the house keeping gene – GAPDH.

Construction of transgenic CHO-K1 cell lines and cytotoxicity studies

Transgenic Chinese hamster ovary–K1 (CHO-K1) cells permanently overexpressing the eIF4E gene were generated as follows: the open reading frame of the human eIF4E cDNA (GenBank Accession Number NM_10029) was sub-cloned in frame with the V5 epitope and the His_6 tag of the expression vector, pcDNA3.1D/V5-His-TOPO (Invitrogen Corporation, Carlsbad, CA) following the instructions provided by the manufacturer. Plasmid DNA prepared using the Qiagen maxi preparation kit (Qiagen Inc., Valencia, CA) was used to transfect CHO-K1 cells by the calcium phosphate procedure (BD Biosicences Corporation, Palo Alto, CA). Stable transfectants that were resistant to 200 μg/ml G418 (a concentration that was lethal to the non-transfected cells) were developed. Overexpression of the cDNA-encoded protein was determined by western blot analysis using antibody for the V5 epitope of the fusion protein (BD Biosciences, Palo Alto, CA).

Cytotoxicity of $NaAsO_2$ in the control (transfected with the empty vector) and the eIF4E overexpressing transgenic CHO-K1 cell lines was determined by treating the cells for 24 h with increasing concentrations of the chemical and determining the number of surviving cells using the MTT assay as described earlier.

Silencing of eIF4E gene expression and cytotoxicity determination

The small interfering RNA (SiRNA) technique was employed to silence the endogenous eIF4E expression in HeLa and HCT15 cells. Potential SiRNA target sites present in the eIF4E mRNA (GenBank Accession Number NM_10029) were identified following the recommendations for SiRNA design by Ambion, Inc. (Austin, TX). Several potential SiRNA target sites were identified and screened against the GenBank database by the BLAST program to ensure that the selected target sequences did not exhibit similarity to other known gene sequences available in the database. The potential SiRNA target sequences thus selected were used to generate Silencer™ Expression Cassettes (SEC) using the Silencer™ Express SiRNA Expression Cassettes Kit (Ambion Inc., Austin, TX) following the instructions provided by the manufacturer. Three SECs potentially capable of silencing the expression of the eIF4E gene were tested

in HeLa and HCT15 cells. The cells were transfected with the individual SECs using the lipofectamine 2000 reagent (Invitrogen Corporation, Carlsbad, CA). Forty-eight hours following the transfection, cell lysates were prepared and tested for silencing of the eIF4E gene's expression by western blot analysis using human eIF4E antibody.

For cytotoxicity determination, exponentially growing HeLa cells were plated in individual wells of a 96-well cell culture plate and transfected with eIF4E SiRNA ranging in concentrations from 0–1 nmole. Forty-eight hours following the transfection, cytotoxicity was determined by the MTT assay.

Ubiquitination of eIF4E protein in the arsenic-treated cells

Induction of ubiquitin by $NaAsO_2$

Exponentially growing HeLa cells were treated with $NaAsO_2$ at a final concentration of 200 μM (LC50). At the end of the treatment period, cell lysates were prepared from the control and chemically treated cells and the expression of ubiquitin was determined by Western blot analysis using human ubiquitin antibody (Santa Cruz Biotechnology, Santa Cruz, CA) as described earlier.

Immunoprecipitation experiments

The potential interaction between eIF4E and ubiquitin proteins was studied by conducting immunoprecipitation experiments. Exponentially growing HeLa cells were treated with 200 μM $NaAsO_2$ for 24 h. The cells were washed twice with ice-cold PBS and lysates were prepared. Immunoprecipitation was carried out for 16 h at 4 °C using a polyclonal rabbit anti-human ubiquitin antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and protein A-sepharose beads (Amersham Biosciences Corporation, Piscataway, NJ) following procedures as described elsewhere [24]. The immunoprecipitated proteins conjugated to the protein A-sepharose beads were washed twice with lysis buffer and then boiled with denaturing SDS-PAGE loading buffer. The presence of eIF4E in the supernatant collected was determined by Western blot analysis using human eIF4E antibody as described earlier.

Proteasome inhibitor studies

Exponentially growing HeLa cells were treated with the proteasome inhibitors – ALLN (25 μM), MG-132 (10 μM) and lactacystin (10 μM), for 24 h. All three proteasome inhibitors used were purchased from Calbiochem (La Jolla, CA). Three hours following the initiation of incubation of cells with the proteasome inhibitors, the cells were treated with $NaAsO_2$ at a final concentration of 200 μM for 21 h. The cell lysates prepared from the control and the $NaAsO_2$-treated cells at the end of the incubation period were analysed for the expression of eIF4E protein by western blot analysis using the human eIF4E antibody.

Statistical analysis of the data

The data were analysed using SAS/STAT software, Version 8.2 of the SAS System for Windows (SAS Institute, Cary, NC). Experiments were performed using a randomized complete block design structure and analysed using mixed model analyses of variance. Post hoc comparisons were made using Fisher's LSD and all differences were considered statistically significant at $p < 0.01$. Western blot and PCR data were normalized to the level of the house-keeping gene GAPDH, while cell survival assays were analysed using the percentage of the control group as the dependent variable. The Pearson product moment correlation coefficients between eIF4E expression and the percentage of surviving cells based on the MTT assay were calculated in cultures treated under identical experimental conditions.

Results

$NaAsO_2$ is toxic and inhibits eIF4E expression in cells

Exposure of human cell lines (HeLa, HCT15, PLC/PR/5 and Chang) to increasing concentrations of $NaAsO_2$ resulted in concentration-dependent cytotoxicity and cell death as evidenced from results of the MTT assay. The LC50 values for $NaAsO_2$, calculated from the dose-response curves of the individual cell lines were as follows: HCT15 – 278.33 $\pm$ 24.05 μM; HeLa – 200.33 $\pm$ 14.33 μM; PLC/PR/5 – 376.66 $\pm$ 24.05 μM and Chang cells – 328.33 $\pm$ 26.53 μM. In addition to the cytotoxicity and cell death noticed among the $NaAsO_2$-treated cells, significant inhibition of eIF4E protein was noticed in all four cell lines tested. The cell lines treated with $NaAsO_2$ at the respective LC50s demonstrated significant inhibition of eIF4E at time intervals of 16- and 24 h and the inhibition was greater at the 24 h exposure period compared to that at the 16 h period (western blot analysis results following the 24 h of exposure to $NaAsO_2$ are presented in Fig. 1A).

The eIF4E transcript level in the control and the $NaAsO_2$-treated cells was analysed to determine whether the $NaAsO_2$-induced inhibition of eIF4E is due to the suppressed transcription of the eIF4E gene. As presented in Table 1, quantitative real-time PCR analysis of the eIF4E transcript showed a significant decrease only in the HCT-15 and HeLa cells in response to $NaAsO_2$ exposure.

Silencing the expression of eIF4E gene results in cytotoxicity and cell death

Transfecting the human cell lines with eIF4E SiRNA resulted in a significant inhibition of eIF4E, and the inhibitory effect was dependent on the concentration of eIF4E SiRNA that was employed in the transfection (Fig. 2A). Expression of

Table 1. Expression of eukaryotic translation initiation factor 4E (eIF4E) mRNA in sodium arsenite treated cells

Cell line	$NaAsO_2$ (LC50)	Gene expression (arbitrary units)
HCT-15	(−)	0.099 ± 0.007
	(+)	0.049 ± 0.007*
HeLa	(−)	0.041 ± 0.008
	(+)	0.029 ± 0.008*
PLC/PR/5	(−)	0.051 ± 0.005
	(+)	0.028 ± 0.003
Chang	(−)	0.018 ± 0.005
	(+)	0.019 ± 0.003

Note. HCT-15, HeLa, PLC/PR/5 and Chang cells were treated with $NaAsO_2$ for 24 h. Expression of eIF4E was determined by real-time PCR as described in the "Materials and methods" section. Data presented are the mean ± S.E. of four independent experiments.
*$p < 0.001$.

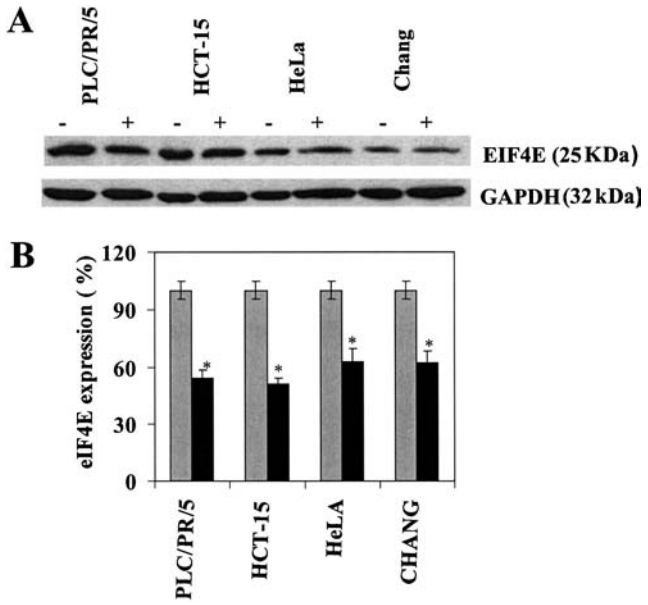

Fig. 1. Expression of eukaryotic translation initiation factor 4E (eIF4E) is inhibited by sodium arsenite. Exponentially growing human cell lines, PLC/PR/5, HCT15, HeLa and Chang, were treated with sodium arsenite for 24 h at the respective LC50 concentrations as determined in a separate experiment. Cell lysates equivalent to 30 μg total protein prepared from the control (−) and the sodium arsenite treated (+) cells were used to determine the expression of eIF4E and the house-keeping gene – GAPDH, by western blot analysis. The experiment was repeated four times and the results of a representative experiment are presented in (A). The western blots were scanned using a densitometer equipped with the ImageQuant software (Amersham Biosciences, Inc.) and the intensities of eIF4E and GAPDH protein bands reacting with the corresponding antibodies were determined. The cellular expression levels of eIF4E protein was normalized to that of GAPDH and the percent inhibitions compared to the corresponding control cell lines were calculated using the data obtained from four independent experiments and are presented (mean ± S.E.) in (B). *Statistically significant compared with the corresponding control cells ($p < 0.01$).

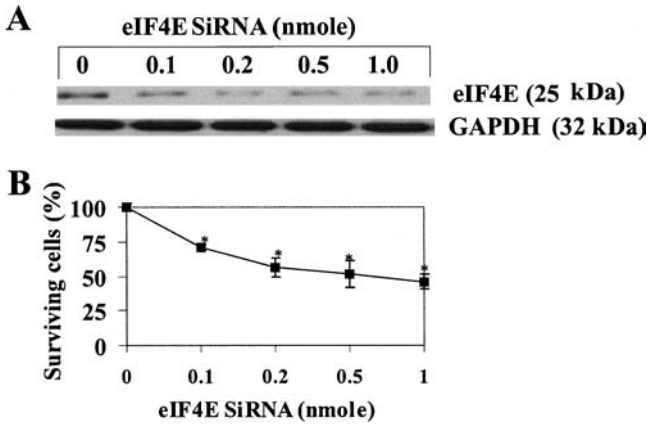

Fig. 2. Transfection of human cell lines with eIF4E-SiRNA resulted in silencing of eIF4E gene expression, cytotoxicity and cell death. HeLa cells were transfected with 0–1 nmole eIF4E-SiRNA. Forty-eight hours following the transfection, cell lysates were prepared from the transfected cells and the expression levels of eIF4E and GAPDH proteins were determined by Western blot analysis using antibodies for the corresponding proteins. In a simultaneously conducted experiment, the cytotoxicity and cell death due to silencing of eIF4E gene expression caused by transfecting the cells with the eIF4E-SiRNA were determined by MTT assay as described in the text. The experiments were repeated 4 times and the results are presented as mean ± S.E. (A) Results of a typical western blot analysis demonstrating the silencing of eIF4E protein. (B) Summary of the results from four independent experiments demonstrating cytotoxicity and cell death following silencing the expression of eIF4E gene by transfecting the cells with the eIF4E SiRNA. The percent of surviving cells following the transfections compared to the control cells was calculated and the results are presented as mean ± S.E. *Statistically significant compared with the corresponding controls ($p < 0.01$).

GAPDH – the house-keeping gene employed in the experiment, was not influenced by eIF4E SiRNA transfection. The specific nature of inhibition of eIF4E gene expression with the eIF4E SiRNA was further evidenced from the lack of inhibition of another translation factor, eukaryotic translation elongation factor 1A1 (eEF1A1), in the transfected cells compared with the control cells (data not presented). As evidenced from results of an MTT assay (Fig. 2B), significant cytotoxicity and cell death was noticed in the cells transfected with the eIF4E SiRNA.

Transgenic CHO-K1 cell lines overexpressing eIF4E gene are resistant to $NaAsO_2$-induced toxicity

Significant overexpression of the V5-eIF4E fusion protein was noticed in the CHO-K1 cell lines transfected with the recombinant pcDNA3.1D/V5-His TOPO DNA containing the eIF4E cDNA as evidenced from the results of western blot analysis (Fig. 3A). Furthermore, transfection of the cells with the plasmid DNA provided resistance to G418, and this facilitated the selection of stable transfectants overexpressing the

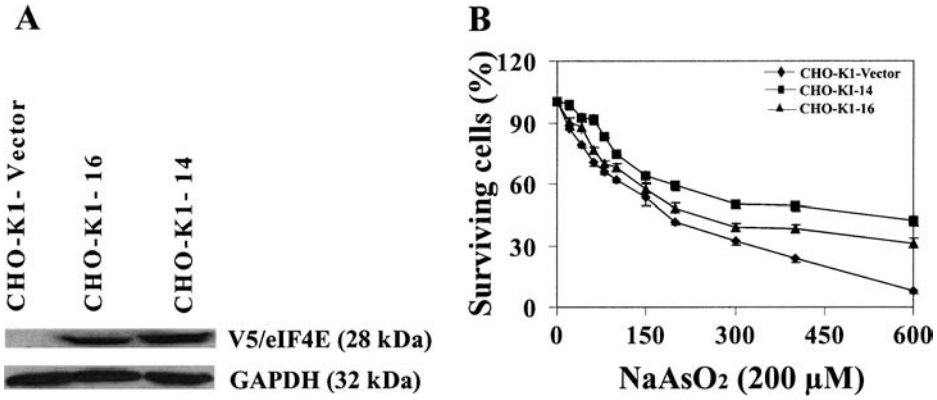

Fig. 3. Overexpression of eIF4E gene in transgenic CHO-K1 cells is protective against $NaAsO_2$-induced cytotoxicity and cell death. Transgenic CHO-K1 cell lines overexpressing eIF4E gene were developed by sub-cloning the open reading frame of eIF4E cDNA in the directional cloning vector, pcDNA3.1 (InVitrogen Corporation, Carlsbad, CA), followed by transfection of the cells with the plasmid DNA and selection of stable transfectants using G418. (A) Lysates prepared from the cells were analysed by western blotting using V5 antibody to determine the expression of the plasmid DNA encoded recombinant eIF4E/V5 fusion protein. The blot was stripped and the abundance of the house-keeping gene – GAPDH, was determined by Western blot analysis. (B) The control and eIF4E overexpressing transgenic CHO-K1 cell lines were treated with increasing concentrations of $NaAsO_2$ for 24 h and the number of surviving cells at the end of the treatment period was determined by MTT assay. The percent of surviving cells among the $NaAsO_2$-treated cells was calculated compared with that of the control and the results are presented as mean ± S.E. [CHO-K1-Vector – CHO-K1 cells transfected with the empty pcDNA3.1 vector; CHO-K1-14 and CHO-K1-16 are two transgenic CHO-K1 cell lines overexpressing eIF4E gene].

eIF4E fusion protein. The transgenic CHO-K1 cells exhibited significant resistance to the cytotoxicity and cell death induced by $NaAsO_2$, demonstrating the protective effect of eIF4E overexpression against $NaAsO_2$-induced cytotoxicity and cell death (Fig. 3B).

Exposure of cells to $NaAsO_2$ resulted in enhanced ubiquitination and proteolysis of eIF4E protein

Results of the studies investigating the potential role of ubiquitination in the inhibition of eIF4E in cells treated with $NaAsO_2$ demonstrated a definite role for the ubiquitination pathway in the $NaAsO_2$-induced inhibition of eIF4E expression. A significant induction of ubiquitin protein was noticed in the cells treated with $NaAsO_2$ compared with the control cells (Fig. 4A). Immunoprecipitation of the $NaAsO_2$-treated cells using an antibody for ubiquitin and further western blot analysis of the immunoprecipitated proteins using eIF4E antibody demonstrated that eIF4E was pulled down by the ubiquitin antibody in the $NaAsO_2$-treated cells (Fig. 4B). The absence of significant amount of eIF4E in the immunoprecipitated proteins of the control cells further supported the observation that exposure of cells to $NaAsO_2$ is required for the interaction between ubiquitin and eIF4E. Furthermore, results of the experiment involving inhibitors of proteasome activity confirmed the role of ubiquitination for the inhibition of eIF4E protein in the $NaAsO_2$-treated cells. The $NaAsO_2$-induced inhibition of eIF4E was reversed by inhibitors of proteasome activity such as lactacystin, MG-132 and ALLN (Fig. 4C).

Cyclin D1 expression is inhibited by $NaAsO_2$ and by silencing of eIF4E gene expression

Results of the Western blot analysis demonstrated that cyclin D1 is a downstream target for both $NaAsO_2$ treatment and eIF4E silencing in cells. Exposure of HeLa cells to $NaAsO_2$ for 24 h as well as silencing the expression of eIF4E gene by transfecting the cells with eIF4E SiRNA resulted in the inhibition of cyclin D1 protein expression (Fig. 5).

Discussion

The results obtained from the present study indicate that eIF4E plays an important role in arsenic-induced cytotoxicity and cell death. Exposure of four different cell lines to toxic concentrations of $NaAsO_2$ resulted in cytotoxicity and cell death, which were associated with a significant inhibition of the eIF4E gene expression. Transfecting the cells with a SiRNA designed specifically to knock-down the expression of eIF4E and the resulting silencing of eIF4E gene expression also resulted in cytotoxicity and cell death suggesting an important role for the eIF4E gene expression in $NaAsO_2$-induced cytotoxicity and cell death. This argument is further strengthened by the observation that the transgenic CHO-K1 cell lines overexpressing eIF4E were more resistant to the arsenic-induced cytotoxicity and cell death compared to the vector-alone transfected control cells. Taken together, these results indicate that eIF4E is an important cellular target for toxicity and death due to exposure to arsenic.

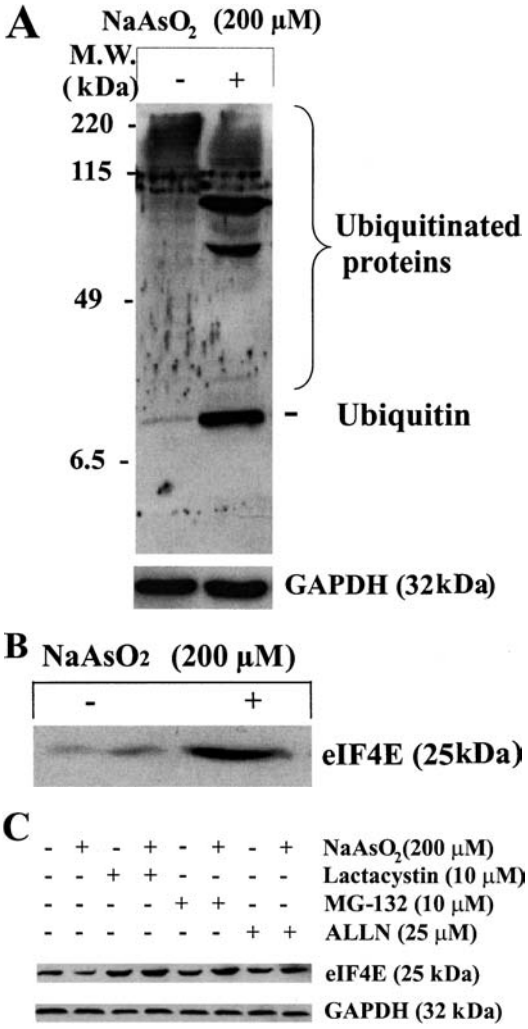

Fig. 4. The sodium arsenite-induced inhibition of eIF4E gene expression is due to enhanced ubiquitination and the resulting proteolysis. (A) Exposure of cells to $NaAsO_2$ resulted in the induction of ubiquitin expression. HeLa cells were treated with 200 μM $NaAsO_2$ for 24 h and cell lysates were prepared from the control (−) and the $NaAsO_2$-treated (+) cells. The cell lysates were used to detect ubiquitin and ubiquitinated proteins by western blot analysis. Subsequently, the blots were stripped to remove the reacted ubiquitin antibody and the expression of the house-keeping gene – GAPDH, was determined. The experiment was repeated 4 times and the results of a representative experiment are presented. (B) Exposure of cells to $NaAsO_2$ results in the interaction between ubiquitin and eIF4E proteins. HeLa cells were treated with 200 μM $NaAsO_2$ for 24 h and the cell lysates were prepared from the control (−) and $NaAsO_2$-treated (+) cells. The cell lysates were subjected to immunoprecipitation using a human antibody for ubiquitin by procedures as described in the Materials and methods section. The immunoprecipitates were electrophoresed and the presence of eIF4E protein in the immunoprecipitate was determined by western blot analysis using a human eIF4E antibody. (C) $NaAsO_2$-induced inhibition of eIF4E gene is reversed by proteasome inhibitors. HeLa cells were treated with $NaAsO_2$ and various inhibitors of proteasome activity (Lactacystin, MG-132, and ALLN) at final concentrations as indicated in the figure label. Lysates prepared from the cells were analysed for the expression of eIF4E and GAPDH proteins by western blot analysis. The experiment was repeated 4 times and the results of a representative experiment are presented.

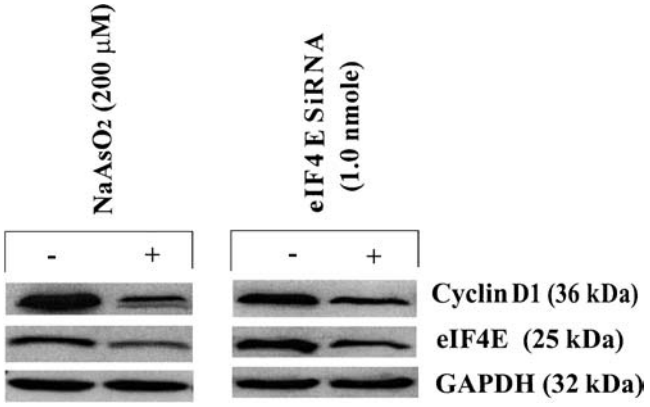

Fig. 5. Exposure of cells to $NaAsO_2$ as well as transfection with eIF4E-SiRNA resulted in a decrease in cyclin D1 expression. Exponentially growing HeLa cells were treated with 200 μM $NaAsO_2$ for 24 h or transfected with the eIF4E-SiRNA as described in the Materials and methods section. Cell lysates were prepared and the expression of eIF4E, cyclin D1 and GAPDH proteins was determined by western blot analysis. The experiment was repeated 4 times and the results of a representative experiment are presented.

Ubiquitination, a post-translational process, has long been considered to be an adaptive response whereby cells eliminate damaged or misfolded proteins. Proteins involved in cell cycle, apoptosis, stress response, transcription and signalling have all been found to undergo ubiquitination and subsequent proteasomal degradation [25]. As noticed in the present study, accumulation of high molecular weight, ubiquitin-conjugated proteins has been reported previously in cells exposed to arsenic [26, 27]. The present study results indicate that the arsenic-induced inhibition of eIF4E was mainly a post-transcriptional event and was mediated by the ubiquitin-proteasome pathway. Such an assumption is supported by the findings that: (1) eIF4E protein expression was down-regulated in all four cell lines treated with $NaAsO_2$ (Fig. 1), while the eIF4E transcript was down-regulated only in two of the four cell lines treated with $NaAsO_2$ (Table 1), (2) the expression of ubiquitin gene and the process of ubiquitination were significantly higher in arsenic treated cells compared with the corresponding controls (Fig. 4A), (3) the results of the immunoprecipitation/western blot analysis experiments suggested an enhanced interaction between eIF4E and ubiquitin proteins leading to the ubiquitination of eIF4E protein in the arsenic-treated cells compared to the control cells (Fig. 4B), and (4) inhibitors of proteasome activity such as lactacystin, ALLN and MG-132 reversed the arsenic-induced inhibition of eIF4E protein and the resulting cytotoxicity and cell death (Fig. 4C).

How does the arsenic-induced down-regulation of eIF4E gene expression result in cytotoxicity and cell death? Eukaryotic translation initiation factor 4E, which binds with the m^7GpppN mRNA cap structure, is the central molecule in translation initiation. It is the least abundant and therefore

the rate-limiting factor regulating translation in eukaryotic cells [28]. The importance of eIF4E in cell growth and division is documented by results of studies conducted with various cell culture model systems. For example, in the budding yeast (*cdc 33*), mutations in the eIF4E limit protein synthesis [29] and cause a G1 arrest phenotype [30], while in Drosophila cell cycle progression is affected [31]. In mammalian cells, modulation of eIF4E gene expression results in alterations in DNA synthesis [32, 33]. Overexpression of antisense oligonucleotides to eIF4E is lethal in HeLa cells [32] suggesting that eIF4E gene is essential for cell survival. It has also been reported that the translation of mRNAs possessing a complex 5′-untranslated region (UTR) is highly dependent on the cellular eIF4E expression level. Those mRNAs possessing a highly complex 5′-UTR and therefore are translationally more regulated by eIF4E include cyclin D1 [34], c-myc [35], ornithine decarboxylase [36] and Bcr-Ab1 [37]. Results of the present study demonstrate that synthesis of cyclin D1 – a protein essentially required for growth and cell cycle control, was down-regulated in cells by arsenic-induced inhibition of eIF4E as well as by the eIF4E-SiRNA mediated silencing of eIF4E gene expression. Thus, it appears that the exposure of cells to arsenic resulted in a diminished cellular level of eIF4E gene expression which in turn compromised the ability of the cells to synthesize and maintain proper levels of essential proteins, such as cyclin D1, resulting in cytotoxicity and cell death.

Another potential mechanism involved in the arsenic-induced cytotoxicity and cell death due to the down-regulation of eIF4E gene may be related to the role of eIF4E in apoptosis response and the survival of cells. It has been previously reported that eIF4E in addition to its role in protein synthesis at the mRNA translation stage also contributes to the regulation of cell cycle progression and the response of cells to apoptosis induced by various agents [38]. Under conditions of apoptosis induced by various agents in cells, the eIF4E binding protein 1 (eIF4E BP1) undergoes dephosphorylation to facilitate its binding with eIF4E so as to result in the inhibition of eIF4E activity [39–41]. Furthermore, overexpression of eIF4E gene expression results in the inhibition of apoptosis [42–44]. Since exposure to arsenic is known to induce apoptosis in cells [45–47] it is quite logical to assume that the inhibition of eIF4E in the $NaAsO_2$-treated cells might have contributed to enhanced apoptosis leading to cell death.

Similar to the results presented in this communication, we have recently noticed a significant inhibition of eIF4E gene expression in cells exposed *in vitro* to cadmium – another important metal that is known to cause toxicity and carcinogenesis in human [48]. In summary, eIF4E appears to be an important target for the cytotoxicity and cell death induced by metals that are known to be toxic and carcinogenic in human. Inhibition of eIF4E gene expression appears to be, at least in part, responsible for the cytotoxicity and cell death due to exposure to metals such as arsenic and cadmium.

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