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Involvement of Calcium-Dependent Protein Kinase C in Arsenite-Induced Genotoxicity in Chinese Hamster Ovary Cells

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Abstract Arsenic is the first metal to be identified as a human carcinogen. Arsenite, one inorganic form of arsenic, has been found to induce sister chromatid exchange, chromosome aberrations, and gene amplification in a variety of in vitro systems. In this study of arsenite-induced genotoxicity represented as micronuclei production in Chinese hamster ovary cells (CHO-K1), we found that the calcium channel blocker, verapamil, can potentiate arsenite-induced micronuclei. And after arsenite treatment, the elevation of intracellular calcium was observed. When extracellular calcium was depleted during arsenite treatment, the arsenite-induced micronuclei formation was significantly suppressed. These data indicated that a calcium ion plays an essential role in arsenite-induced genotoxicity. Further, it was found that the cotreatment of arsenite and a calcium ionophore, A23187, can increase the micronuclei induction. In contrast, pretreatment of the intracellular calcium chelator, quin 2, significantly inhibited micronuclei production of arsenite administration. In addition, we measured the activity of calcium- and phospholipid-dependent protein kinase C (PKC) and found that arsenite can activate PKC activity in a dose-dependent manner. Subsequently, some PKC activators and inhibitors were applied to investigate the involvement of PKC on arsenite-induced micronuclei formation. It was found that H7, a PKC inhibitor, can depress but TPA, a PKC activator, can enhance arsenite-induced micronuclei significantly. These data indicated that arsenite exposure perturbs intracellular calcium homeostasis and activates PKC activity. As a result, the activation of PKC activity may play an important role in arsenite-induced genotoxicity. J. Cell. Biochem. 64:423–433.

Key words: arsenite; protein kinase C; calcium; genotoxicity; carcinogen

Arsenic is a well-documented human carcinogen [Ishinishi et al., 1986]. Like some metals, arsenic can form inorganic and organic compounds. From epidemiological investigations, it is known that long-term arsenic exposure results in promotion of carcinogenesis especially in lung and skin via inhalation and ingestion, respectively [IARC, 1980]. A review of the epidemiological studies suggested that ingestion of arsenic may also cause fatal internal cancers, including bladder cancer [Bates et al., 1992]. There is some evidence that nutritional and lifestyle factors such as smoking may synergistically interact with arsenic [Pershagen, 1981; Vhter and Marafante, 1987; Hertzpicciotto and Smith, 1993]. In experimental animals, arsenic exposure shows no reliable evidence of carcinogenicity [Leonard and Lauwerys, 1980; Goyer, 1986]. However, research in animal studies suggests that arsenic acts in concern with other agents to alter or enhance biological effects which are potentially involved steps in progression to carcinogenesis [Nordberg and Anderson, 1981].

Many hypotheses have been proposed to describe the possible mechanism of the action of arsenic in cancer development. It has been suggested that arsenic may interact with other genotoxic agents in humans [Beckman and Nordenson, 1986]. Arsenic has been found to enhance the UV mutagenicity in prokaryotic cells [Rossman, 1981] and to inhibit DNA ligase activity in mammalian cells [Li and Rossman, 1989]. Additionally, cytogenetic studies have showed that arsenic induces chromosome aberration [Nakamuro and Sayato, 1981; Jha et al., 1992], sister chromatid exchanges [Jha et al.,

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1992], micronuclei formation [Wang and Huang, 1994; Liu and Huang, 1996], gene amplification [Lee et al., 1988; Rossman and Wolosin, 1992], and cell transformation [Lee et al., 1985].

Recently, the genotoxic mechanism of arsenic in mammalian cells has been considered to correlate with oxidative stress. Induction of DNA breakage in vivo and production of an oxygen free radical after dimethylarsinic acid (DMAA) administration have been reported [Yamanaka et al., 1991; Kato et al., 1994]. In addition, our previous report also showed that exogenous addition of catalase can protect cells from arsenite-induced micronuclei formation [Wang and Huang, 1994]. However, generation of reactive oxygen species which involves in arsenic toxicity is still obscure.

Recently, a calcium ion involved in arsenite-induced micronuclei formation has been suggested [Liu and Huang, 1996]. A calcium ion regulates many cell functions and is involved in the cell death process [Nicotera et al., 1986; Mcconkey et al., 1991; Jiang et al., 1995]. In addition, a calcium ion is the cofactor of protein kinase C (PKC) [Nishizuka, 1988; Stabel and Parker, 1991; Azzi et al., 1992; Hug and Sarre, 1993]. PKC takes part in cellular responses to various agonists including hormones, neurotransmitters, and some growth factors [Nishizuka, 1986]. The enzyme is a family of closely related protein kinase isoenzymes characterized by their dependence on phospholipid and diacylglycerol [Dekker and Parker, 1994]. These isoenzymes can be sorted into calcium-dependent kinases such as PKC α, β, and γ or calcium-independent kinases such as PKC δ, ϵ, and ζ. PKC is of particular interest because it was found to be the receptor for the tumor-promoting phorbol esters [Ashendel, 1985]. And it is generally believed that PKC plays a vital role in regulation of cell proliferation and differentiation [Clemens et al., 1992; Nishizuka, 1992]. The activation of PKC also correlates with oncogene expression such as c-jun, c-fos, and c-myc [Hug and Sarre, 1993].

Little is known concerning the role of the calcium ion and the expression of calcium-dependent PKC on the genotoxicity of arsenic. In this study, we examined the content of intracellular calcium, the change of many intracellular defense molecules in normal and calcium-free conditions, and the activity of calcium-dependent PKC after arsenite treatment in Chinese hamster ovary (CHO-K1) cells. Also, we used well-established PKC activators and inhibitors to examine the effect of PKC on arsenite-induced genotoxicity expressed as micronuclei (MN) formation. The results indicated that arsenite exposure in CHO-K1 cells can induce calcium accumulation and activate calcium-dependent PKC activity. On the basis of these results, intracellular calcium accumulation and calcium-dependent PKC activation may play essential roles in arsenite-induced MN formation.

MATERIALS AND METHODS

Chemicals and Cell Culture

Chemicals for cell cultures were obtained from Sigma (St. Louis, MO). Sodium arsenite (SA) was purchased from Merck (Darmstadt, Federal Republic of Germany). Verapamil, 2-[(2-bis-[carboxymethyl]-amino-5-methylphenoxy)-methyl]-6-methoxy-8- bis[carboxy-methyl]aminoquinoline (quin 2), A23187, O-tetradecanoylphorbol 13-acetate (TPA), ethylene glyco-bis [beta-aminoethylether] N,N,N′,N′,-tetraacetic acid (EGTA), 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine (H7), histone type III, cytochalasin B, ATP, and calcium-free McCoy's 5A medium were from Sigma. Calcium-45 (^{45}Ca) and [γ-^{32}P]ATP were purchased from Amersham (Buckinghamshire, UK). Chinese hamster ovary (CHO-K1) cells were provided by Dr. T.C. Lee (Academia Sinica, Taipei, Taiwan, ROC). CHO-K1 cells were cultured in McCoy's 5A medium supplemented with 10% heat-inactivated fetal calf serum, 100 units/ml penicillin, 100 µg/ml streptomycin, and 0.03% L-glutamine. The cells were incubated in a water-saturated atmosphere containing 5% CO_2 at 37°C.

Micronuclei Assay

The method of Fenech [1993] was followed with some modifications. In our previous report [Wang and Huang, 1994] we showed that 1 µg/ml cytochalasin B was sufficient to inhibit cytoplasmic division in CHO-K1 cells. Accordingly, we used the concentration of 1 µg/ml cytochalasin B in our experiments. After treatment, cells were washed twice with phosphate buffered saline (PBS) and replaced with fresh standard (STD) medium containing 1 µg/ml cytochalasin B. After 24 h incubation, the cells were washed with PBS and placed in 0.5% KCl for 10 min. Then cells were fixed in 3 ml of Carnoy's solution (20:1, methanol:acetic acid)

for 5 min. The dishes were then air-dried and stained for 15 min with freshly diluted Giemsa's solution (5% in 0.1 mM sodium phosphate buffer, pH 6.8). For quin 2 treatment, cells were washed twice with PBS to remove the calcium ion from the extracellular environment. Subsequently, cells were incubated with quin 2 in PBS for 30 min. After incubation, the cells were washed and treated with arsenite in fresh STD medium for 4 h. Micronuclei were scored under a microscope at a magnification of 200×. One thousand binucleated cells per dish were examined.

Arsenic Determination

Inorganic arsenic was measured by an atomic absorption spectrophotometer using a vapor generation accessory (VGA-76 device from Varian, Victoria, Australia). The method of Moffett [1988] was followed. In brief, about 2×10^6 cells were washed twice with PBS and twice with 10 mM EDTA, trypsinized, and collected in 2 ml PBS. The cells were digested with 0.2 ml 65% HNO_3 for 24 h at room temperature. Then the debris was removed by centrifugation, and the supernatant was collected for arsenic determination. Calibration solutions were prepared from the Sigma standard.

Calcium Determination

Calcium accumulation after arsenite exposure was measured by ^{45}Ca accumulation. Briefly, cells were plated in dishes at the density of 3×10^5/60 mm dish for 12 h. Cells were treated with arsenite in the presence of 2 µCi/ml ^{45}Ca (2 mCi/ml) for 4 h. Subsequently, cells were fixed with buffer containing 12.5% formaldehyde and 2% oxalate acid for 10 min. After washing with distilled water, cells were scraped in the presence of 2% SDS. Finally, radioactivities were determined by liquid scintillation.

Glutathione Determination

The cellular GSH levels were determined according to the procedures described by Cohn and Lyle [1966]. The cells of logarithmic phase were harvested and washed twice with PBS. The cell pellets were resuspended in 0.2 ml ice-cold distilled water and vortexed for 5 min, and 50 µl of 25% metaphosphoric acid was then slowly added. After centrifugation at 10,000 rpm for 30 min, 20 µl of clear supernatant was transferred into a small tube containing 2 ml distilled water and 0.5 ml 0.1 M sodium phosphate (pH 8.0). Then 0.1 ml of 0.1% o-phthaldehyde (dissolved in methanol) was added. The mixture was kept in darkness at room temperature for 25 min. The fluorescence (excitation at 350 nm, emission at 420 nm) was measured with Hitachi F-4000 fluorescence spectrophotometer.

Glutathione S-Transferase Activity Determination

Glutathione S-transferase (GST) activity was measured by the method of Habig et al. [1974] using 1-chloro-2,4-dinitrobenzene (CDNB) and glutathione as substrates. The cell pellets were resuspended in 300 µl of 100 mM phosphate buffer, pH 6.8, and sonicated four times with a Sonifer Model 200 (Branson Sonic Power Co., Danbury, CT) at setting 4 for 10 s at 4°C. The cell debris was removed by centrifugation at 10,000 rpm for 30 min at 4°C. Assay was performed at room temperature in 850 µl reaction mixture containing 0.1 M sodium phosphate and 1 mM ethylenediamine-tetraacetic acid (EDTA) (pH 6.5), 50 µl glutathione, 50 µl of 20 mM CDNB, and 50 µl of clear cell lysate. The absorbance at 340 nm was continuously recorded for 3 min with Hitachi model U-3210 spectrophotometer.

Catalase Activity Determination

Catalase activity was measured as described by Aebi [1984] with minor modifications. Briefly, cells were suspended in 50 mM potassium phosphate buffer (pH 7.0) and sonicated by the same procedure as in glutathione S-transferase activity assay. After centrifugation, the clear supernatant was mixed with 10 mM hydrogen peroxide, and the decomposition of hydrogen peroxide was followed directly by a decrease in absorbance at 240 nm. A Hitachi model U-3210 spectrophotometer was used for this assay. Proteins were determined by the Bio-Rad (Bio-Rad laboratories, Richmond, CA) protein assay using bovine serum albumin as a standard.

Measurement of Cellular ATP Content

Cellular ATP was extracted by trichloroacetic acid and estimated by firefly bioluminescent assay according to the method of Junod et al. [1989] with some modifications. Approximately 1×10^6 cells were suspended in 100 µl of 3% TCA solution. The mixture was keep in room temperature for 15 min. After centrifugation at

3,000 rpm for 10 min, the supernatant was diluted with distilled water (1:9). The luciferin-luciferase reaction buffer was prepared by adding 0.4 ml firefly lantern extract to 2.4 ml Hepes buffer (40 mM Hepes, 2 mM EDTA, and 7.5 mM $MgSO_4$, pH 8.0). In a polystyrene cuvette, 0.2 ml reaction buffer and 0.2 ml diluted cell extract were mixed. Finally, the emitted light of the mixture was counted for 60 s in a Lumac/3M model 2021 biocounter. The pure ATP purchased from Sigma (sodium salt, MW = 605.2) was prepared as a standard.

Subcellular Distribution of Protein Kinase C (PKC) Activity

Briefly, cells were suspended in homogenization buffer (20 mM Tris/HCl, pH 7.5, 2 mM EGTA, 2 mM EDTA, 0.25 M sucrose, 2 mM mercaptolethanol, 0.5 mM PMSF) and were homogenized four times with a Sonifier model 200 at setting output level 4 (~50 W) for 10 s at 4°C. The homogenates were then centrifuged for 1 h at 105,000*g*. The pellets were resuspended in homogenization buffer containing 0.5% Triton X-100 as membrane fractions. The whole cell lysates were prepared by homogenization of cells in 0.5% Triton X-100 containing homogenization buffer.

Calcium-dependent PKC activity was determined by histone phosphorylation [Kikkawa et al., 1986]. Fifty micrograms of whole cell lysates and membrane fractions were incubated with reaction mixture (final volume = 250 µl) containing 20 µM Tris/HCl, pH 7.5, 5 mM $MgCl_2$, 200 µg/ml histone type III-ss, 8 µg/ml phosphatidylserine, 10 ng/ml TPA, 10 µM ATP, and [γ-^{32}P]ATP (50–100 cpm/pmol) in the presence of 0.5 mM $CaCl_2$ or 1 mM EGTA. The reactions were carried out at 30°C for 5 min and then stopped by addition of ice-cold acetone. After incubation at −20°C for 30 min, the proteins were precipitated by centrifugation and subsequently applied to SDS-PAGE to separate histone from cellular proteins. The radioactivity of phosphorylated histone was determined by autoradiography.

RESULTS

Role of Intracellular Calcium Homeostasis on Arsenite-Induced Genotoxicity

Recently, the phenomenon of arsenite-induced calcium accumulation in nucleus was observed [Liu and Huang, 1996]. In this study, further quantitation of intracellular calcium was presented. Figure 1 shows the dose-dependent elevation of cellular calcium in CHO-K1 cells after 0–80 µM arsenite treatment for 4 h. Since arsenic was reported to induce micronuclei (MN) formation (the biomarker of genotoxicity) in mammalian cells [Wang and Huang, 1994; Liu and Huang, 1996; Heddle et al., 1991], the effect of calcium modifiers on arsenite-induced MN production was investigated. Table I shows that a calcium-free condition can protect cells against arsenite-induced MN. The MN formation from cells treated with 80 µM arsenite in a calcium-free condition was not different from that of a control group (50 MN per 1,000 binucleated cells). However, the same concentration of arsenite under normal conditions could induce 155 MN per 1,000 binucleated cells.

On the other hand, 100 µM verapamil (a calcium channel blocker) and arsenite cotreatment could increase MN production in a dose- and time-dependent manner (Fig. 2). In order to rule out that the extracellular calcium content may affect cellular uptake and excretion of arsenic, atomic absorption spectrophotometry was applied to quantify the intracellular arsenic. The results showed that there was no significant difference in arsenic accumulation

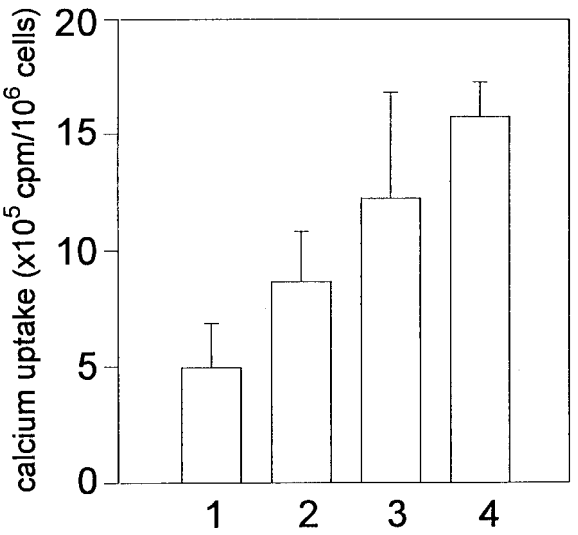

Fig. 1. Calcium accumulation from the extracellular environment after arsenite treatment. CHO-K1 cells were treated with arsenite for 4 h in the presence of 2 µCi/ml ^{45}Ca. Subsequently, cells were fixed with solution containing 12.5% formaldehyde and 2% oxalate acid for 10 min. After being washed, cells were scraped in 2% SDS buffer. Then the radioactivity within cells was determined by liquid scintillation. Bars represent the standard deviation of three determinations. *Lane 1:* Control. *Lane 2:* Arsenite (40 µM). *Lane 3:* Arsenite (80 µM). *Lane 4:* Arsenite (160 µM).

TABLE I. Arsenite-Induced Micronuclei (MN) Formation in CHO-K1 Cells After Sodium Arsenite (SA) Treatment*

	MN/1,000 BN cells
STD condition (4 h)	
Control	50 ± 10
SA 80 μM	155 ± 12
CAF condition (4 h)	
Control	45 ± 7
SA 80 μM	50 ± 10

*CHO-K1 cells were treated with arsenite in standard medium (STD) or calcium-free medium (CAF) for 4 h. STD medium contains 1.4 mM calcium. BN, binucleated.

or excretion in CHO-K1 cells either in calcium-free or normal medium after 80 μM arsenite treatment for 4 h and further incubation in fresh medium for 4 h (Fig. 3). Since cotreatment of verapamil and arsenite could also enhance arsenite-induced calcium accumulation in the nuclei of CHO-K1 cells [Liu and Huang, 1996], these results confirmed that arsenite-induced intracellular calcium elevation plays an important role in arsenite-induced genotoxicity. And the increased intracellular calcium ion after arsenite treatment may come, at least in part from the extracellular environment.

Effects of a Calcium-Free Condition on Arsenic Detoxification Molecules

Intracellular molecules such as glutathione (GSH), glutathione S-transferase (GST), catalase, and ATP level have been reported to protect against arsenic toxicity in mammalian cells [Huang et al., 1993; Lee et al., 1989b; Yin et al., 1991; Wang and Huang, 1994]. In this study, we compared the changes of these defense molecules in cells before and after arsenite treatment. In normal medium, the levels or the activities of these molecules within cells showed no significant difference between control and arsenite-treated groups (Table II). In a calcium-free condition, arsenite treatment did not affect catalase activity and cellular ATP level (Table II). On the other hand, the GSH level and the GST activity from cells cultured in a calcium-free condition for 4 h were suppressed to 70% and 63%, respectively, of those from cells grown in normal medium. After 80 μM arsenite treatment for 4 h in a calcium-free condition, the cellular GSH level and the GST activity were suppressed to 70% and 15%, respectively, of those in control cells. In addition, cells after

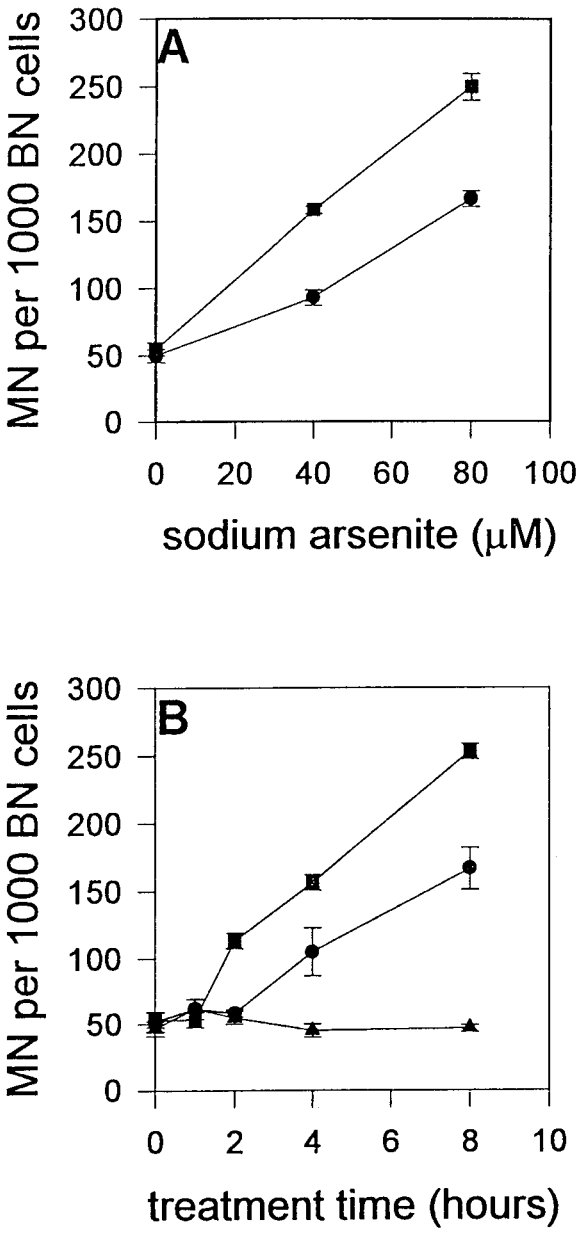

Fig. 2. Effect of calcium channel blocker on arsenite-induced micronuclei formation in CHO-K1 cells. **A:** CHO-K1 cells were treated with arsenite in the presence (■) or absence of 100 μM verapamil (●) for 4 h. **B:** CHO-K1 cells were treated with 40 μM arsenite alone (●), 100 μM verapamil alone (▲), or 40 μM arsenite plus 100 μM verapamil (■) for different time periods. Then cells were washed and incubated for 20 h in the presence of 1 μg/ml cytochalasine B. Finally, cells were swelled with 0.5% KCl and stained with Giemsa's solution. Micronuclei were scored under microscope at a magnification of 200×. Bars represent standard deviation of three experiments. BN, binucleated; MN, micronuclei.

arsenite treatment in a calcium-free condition possessed only one-tenth of the GST activity compared to cells cultured in normal medium. However, the catalase activity and the ATP level did not change significantly either in the

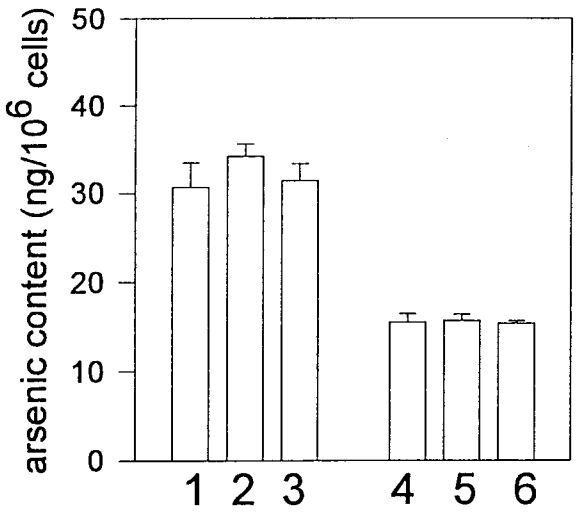

Fig. 3. Effect of extracellular calcium concentration on arsenic accumulation and release in CHO-K1 cells. In determination of arsenic accumulation, CHO-K1 cells were incubated with 80 μM arsenite for 4 h in different conditions (*lane 1:* normal McCoy's 5A medium containing 1.4 mM calcium ion; *lane 2:* serum-free MaCoy's 5A medium containing 1.4 mM calcium ion; *lane 3:* calcium-free McCoy's 5A medium). In determination of arsenic release, CHO-K1 cells were treated with 80 μM arsenite for 4 h in normal conditions; then cells were washed and released in different conditions (*lane 4:* normal McCoy's 5A medium containing 1.4 mM calcium ion; *lane 5:* serum-free McCoy's 5A medium containing 1.4 mM calcium ion; *lane 6:* calcium-free McCoy's 5A medium) for 4 h. Finally, cells were harvested for cellular arsenic determination by atomic absorption spectrophotometry. Bars represent the standard deviation of three experiments.

normal or calcium-free condition. Many reports have indicated that GST can cooperate with GSH to perform cellular defense against toxican injury [Kim et al., 1992]. However, our results showed that less arsenite-induced MN formation from cells cultured in a calcium-free medium is not correlated with an increase of any of the above mentioned detoxification molecules (Tables I, II). The calcium-free condition, however, could not enhance the levels of GSH, GST, catalase, and ATP within cells. These results suggest that these molecules may have little benefit in preventing MN formation in arsenite treatment for 4 h.

Activation of Calcium-Dependent Protein Kinase C After Arsenite Treatment

Recently, arsenite has been found to activate mitogen-activated protein (MAP) kinase activities in HeLa cells [Trigon and Morange, 1995]. In this study, we demonstrated that arsenite treatment in normal medium can increase cellular calcium content (Fig. 1) and a calcium-free condition can protect cells against arsenite-induced MN formation (Table I). One would expect that intracellular calcium-dependent events are involved in arsenite-induced MN in CHO-K1 cells. Since PKC acts as an upstream regulator of MAP kinase and a calcium ion is an important cofactor for activating PKC, the relationship between arsenite treatment and calcium-dependent PKC was investigated. As shown in Figure 4, after arsenite treatment for 4 h, the calcium-dependent PKC activities significantly increased in membrane fractions of cells in a dose-dependent manner. In addition, no obvious increase of PKC activities was found in whole cell lysates after arsenite treatment (Fig. 4). As known, the PKC proteins exist mainly in cytosol before activation. After being triggered by stimuli, the PKC proteins may translocate from cytosol to the cell membrane and nucleus. These results indicated that arsenite administration for 4 h in CHO-K1 cells causes calcium-dependent PKC translocation and activation.

Since arsenite treatment could activate calcium-dependent PKC in CHO-K1 cells, it is interesting to see the effect of PKC modifiers on arsenite-induced MN formation. CHO-K1 cells were coadministered with arsenite and a PKC inhibitor, H7, or the phorbol ester TPA or a calcium ionophore, A23187. TPA and A23187 are well-known activators of PKC [Castagna et al., 1982; Nishizuka, 1984]. The results, in agreement with PKC inhibition, demonstrated that 500 μM H7 cotreated with arsenite can inhibit MN formation (Fig. 5A). However, 5 ng/ml TPA and 5 μg/ml A23187 could synergisticly enhance arsenite-induced micronuclei formation in CHO-K1 cells (Fig. 5B). In order to further confirm the role of the calcium ion in arsenite-induced MN formation, EGTA (the extracellular calcium chelator) and quin 2 (the intracellular calcium chelator) were applied to cells. The results, in agreement with the calcium-free condition, showed that 2 mM EGTA cotreatment and 40 μM quin 2 preloading can suppress arsenite-induced MN completely (Fig. 5C,D). And our results also showed that EGTA and quin 2 treatment can inhibit calcium-dependent PKC activity in CHO-K1 cells (data not shown). These data firmly supported the idea that arsenite can perturb intracellular calcium homeostasis and activate PKC to induce micronuclei in mammalian cells.

TABLE II. Contents of Glutathione (GSH), Glutathione S-Transferase (GST), Catalase, and ATP Level in CHO-K1 Cells After Sodium Arsenite (SA) Treatment*

	GSH (nmol/mg protein)	GST (nmol CDNB/min/mg protein)	Catalase (nmol H_2O_2 decomposition/min/mg protein)	ATP (μM/mg protein)
STD condition (4 h)				
Control	8.8 ± 0.9	108 ± 8.0	41 ± 8.0	48.5 ± 1.6
SA 80 μM	8.5 ± 1.2	110 ± 9.0	32 ± 5.9	42.6 ± 1.1
CAF condition (4 h)				
Control	6.3 ± 0.1	68 ± 2.4	45 ± 8.1	39.7 ± 1.4
SA 80 μM	4.4 ± 0.5	11 ± 1.4	46 ± 1.2	37.2 ± 2.1

*CAF, calcium-free medium; STD, standard medium.

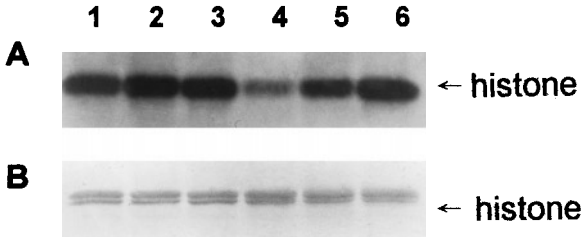

Fig. 4. Effect of arsenite on calcium-dependent PKC activity. CHO-K1 cells were treated with arsenite for 4 h in normal McCoy's 5A medium. Subsequently, whole cell lysates and membrane fractions were separated. Aliquots of 50 μg protein from whole cell lysates and membrane fractions were applied to assay calcium-dependent PKC activity by the histone III-S phosphorylation method described in Materials and Methods. *Lanes 1–3:* Whole cell lysates. *lanes 4–6:* Membrane fractions. **A:** Autoradiograph of histone III-SS. **B:** Coomassie blue staining of histone III-SS. Lane 1: Control. 2: Arsenite (40 μM). Lane 3: Arsenite (80 μM). Lane 4: Control. Lane 5: Arsenite (40 μM). Lane 6: Arsenite (80 μM).

DISCUSSION

Generally, micronuclei are formed as extranuclear bodies when acentric chromosomal fragments or whole chromosomes are not incorporated into daughter nuclei during mitosis [Vine, 1990]. There are understood mechanisms by which micronuclei can arise, such as mitotic loss of acentric fragments, chromosome breakage and exchange, mitotic loss of whole chromosome, spindle disruption, and apoptosis [Heddle et al., 1991]. However, the further molecular insight is not yet explored. Micronuclei production is also thought to be associated with some cancers [Reali et al., 1987; Rosin and Anwar, 1992; Livingston et al., 1990]. In respect to arsenic-induced micronuclei, the mechanism by which arsenic exerts its genotoxicity was obscure. However, arsenic was reported to disturb the assembly of spindle fiber in a variety of cell lines [Sharma and Talukder, 1987; Chou, 1989; Huang et al., 1995]. In our laboratory, the arsenite-induced DNA fragmentation and apoptosis were also observed in CHO-K1 cells [Wang et al., 1996].

Verapamil can block the conduct of the L-type calcium channel in many cell types, especially neuron cells [Henry, 1980]. Verapamil is lipophilic and is reported to protect cells against free-radical damage [Tong-Mak et al., 1992; Floersheim, 1993]. However, the report by Lee et al. [1989a] showed that verapamil can potentiate arsenite-induced cytotoxicity in CHO cells. They proposed that cells may use a calcium channel to excrete arsenic from cytosol. However, our unpublished data showed that verapamil cotreatment cannot reinforce arsenic accumulation in CHO-K1 cells (data not shown). It seemed that a calcium channel may not be used to excrete arsenic from the intracellular environment. In this study, we found that verapamil and A23187 can enhance but a calcium-free condition, EGTA, and quin 2 can significantly suppress arsenite-induced micronuclei in CHO-K1 cells (Figs. 1, 5; Table I). These results suggest that a calcium ion plays an important role in arsenite-induced genotoxicity. In fact, many toxicants have been reported to disturb cellular calcium homeostasis to exert their toxicity [Atchison et al., 1986; Simons and Pocock, 1987; Hinkle et al., 1987]. In addition, factors such as GSH, GST, catalase, and ATP level were reported to protect cells against arsenic toxicity [Huang et al., 1993; Lee et al., 1989b; Yin et al., 1991; Wang and Huang, 1994]. However, the protective effect of a calcium-free condition against arsenite-induced MN formation was not correlated with increase of these factors (Table II). Moreover, after arsenite treatment, elevation of intracellular calcium was observed (Fig. 1). As a result, arsenite-induced

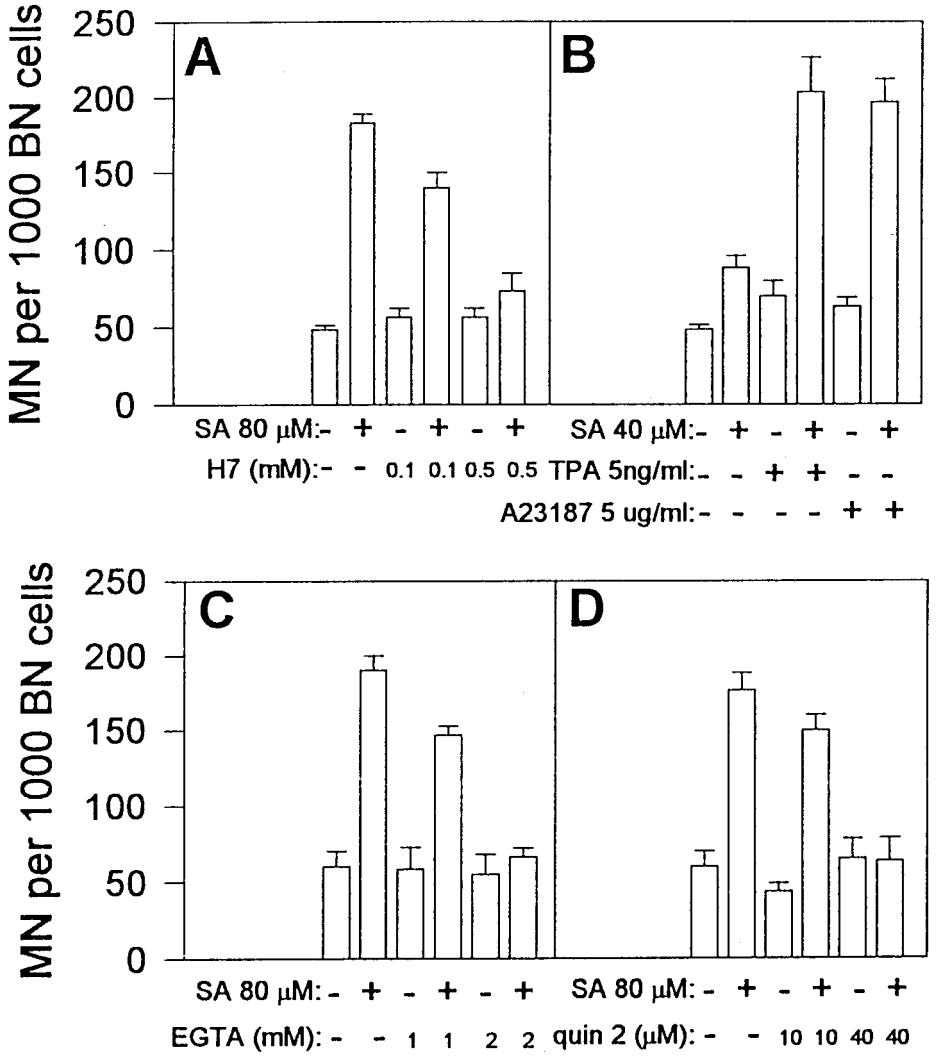

Fig. 5. Effects of calcium chelator, PKC inhibitors, and activators on arsenite-induced micronuclei in CHO-K1 cells. **A:** Cells were cotreated with H7 and arsenite for 4 h. **B:** Cells were cotreated with arsenite plus A23187 or TPA for 4 h. **C:** Cells were cotreated with EGTA and arsenite for 4 h. **D:** Cells were preloaded with quin 2 (intracellular calcium chelator) in PBS for 30 min and then treated with arsenite for 4 h. Bars represent the standard deviation of three experiments. BN, binucleated; MN, micronuclei; SA, sodium arsenite.

MN formation in CHO-K1 cells strongly correlated with disturbance of intracellular calcium homeostasis but not with disturbance of intracellular defense molecules (Fig. 1; Table II).

Many reports have been published noting that elevation of intracellular calcium can activate calcium-dependent PKC [Dorio et al., 1987; Christiansen et al., 1988]. PKC is ubiquitously expressed in mammalian cells and can be classified into three types: the classical PKC (cPKC α, β I/II, and γ), the novel PKC (nPKC δ, ϵ, η, θ, μ), and the atypical PKC (aPKC ξ, ι, λ). Several hypotheses were established that PKC can deliver the signals toward and within cell nucleus directly or indirectly [Buchner, 1995]. Direct activation of PKC by phorbol ester, the well-known tumor promoting agent, resulted in activation of AP-1 (complex of c-fos and c-jun) by transcriptional activation of c-fos and altered phosphorylation of c-jun [Hug and Sarre, 1993]. In this study, we found that PKC activity increases in membrane fractions of cells after arsenite treatment for 4 h. Abnormal activation of PKC was reported to regulate and phosphorylate some oncogene products such as c-fos and c-myc to cause irregulated proliferation and the promotion of carcinogenesis [Nishizuka, 1986]. Recently, arsenite was found to induce the expression of c-myc and c-fos but had no effect on the expression of erbB and c-H-ras in quiescent

C3H/10T1/2 cells [Li et al., 1992]. Except for arsenic-induced genotoxicity such as chromosome aberration, sister chromatid exchange, and micronuclei, more recently arsenic has been found to induce chromosome endoreduplication in Syrian hamster embryo cells [Lee et al., 1985]. Whether arsenic-induced genotoxicity is mediated through activation of calcium-dependent PKC is not yet clear. Our results, however, showed that the PKC modifiers such as H7 and TPA can obviously interfere with arsenite-induced MN formation (Fig. 5A,B). In fact, the dose of H7 (500 μM) used in this study may also inhibit other kinase activity such as cAMP-dependent protein kinase (PKA) [Hidaka et al., 1984]. However, our preliminary results showed that arsenite treatment does not affect PKA activity. In addition, a PKA activator such as cAMP did not potentiate arsenite-induced MN formation (data not shown). Furthermore, just 5 ng/ml of TPA, a PKC activator, did enhance arsenite-dependent MN induction. It was well established that PKC is the intracellular receptor for TPA. Although the involvement of other protein kinases in arsenite-induced MN formation could not be completely excluded, these results indicated that PKC activation may play a pivotal role in arsenite-induced genotoxicity.

Arsenic is a well-documented human carcinogen [Ishinishi et al., 1986]. However, the carcinogenic mechanism has not been established. In this study, we found that arsenite treatment can activate calcium-dependent PKC (Fig. 4). It was reported that phorbol esters such as TPA exert their tumor promotion effects by activation of PKC [Nishizuka, 1986]. These suggested that arsenite may act as tumor promoting agent to activate PKC. The activation of PKC perhaps played a role in arsenite-induced oncogene expression [Li et al., 1992] and cancer promotion [IARC, 1980].

In regard to MN formation, our results indicated that calcium-dependent PKC plays an essential role in arsenite-induced MN formation (Fig. 5A,B). However, there was no evidence that PKC can directly affect the stability of the genome. It is possible that arsenite-induced MN formation was operated by some PKC-regulated proteins. In fact, chromosomal proteins such as histone and DNA methylase were potent substrates of PKC [Kikkawa et al., 1986; Nishizuka, 1986]. The function of these proteins was thought to affect genome stability. Therefore, further examination of PKC-interacted proteins would help to explore the mechanism of arsenite-induced genotoxicity in mammalian cells.

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REFERENCES

Aebi H (1984): Catalase in vitro. Methods Enzymol 105:121–126.

Ashendel CL (1985): The porbol ester recetor: A phospholipid-regulated protein kinase. Biochim Biophys Acta 822: 219–242.

Atchison WD, Joshi U, Thornburg JE (1986): Irreversible suppression of calcium entry into nerve terminals by methylmercury. J Pharmacol Exp Ther 238:618–624.

Azzi A, Boscoboinik D, Hensey C (1992): The protein kinase C family. Eur J Biochem 208:547–557.

Bates MN, Smith AH, Hopenhayn-Rich C (1992): Arsenic ingestion and internal cancer—a review. Am J Epidemiol 135:462–467.

Beckman L, Nordenson I (1986): Interaction between some common genotoxic agents. Hum Hered 36:397–401.

Buchner K (1995): Protein kinase C in the transduction of signals toward and within the cell nucleus. Eur J Biochem 228:211–221.

Castagna M, Takai Y, Kaibuchi H, Sano K, Kikkawa U, Nishizuka Y (1982): Direct activation of calcium-activated, phospholipid-dependent protein kinase by tumor-promoting phorbol esters. J Biol Chem 257:7847–7851.

Chou IN (1989): Distinct cytoskeletal injuries induced by As, Cd, Co, Cr, and Ni compounds. Biomed Environ Sci 2:358–365.

Christiansen NO, Larsen CS, Esmann V (1988): A study on the role of protein kinase C and intracellular calcium in the activation of superoxide generation. Biochim Biophys Acta 971:317–324.

Clemens MJ, Trayner I, Menaya J (1992): The role of protein kinase C isozymes in the regulation of cell proliferation and differentiation. J Cell Sci 103:881–887.

Cohn VH, Lyle J (1966): A fluorometric assay for glutathione. Anal Biochem 14:434–440.

Dekker LV, Parker PJ (1994): Protein kinase C—a question of specificity. Trends Biochem Sci 19:73–77.

Dorio RJ, Nelson J, Forman HJ (1987): A dual role for calcium in regulation of superoxide generation by stimulated rat alveolar macrophages. Biochim Biophys Acta 928:137–143.

Fenech M (1993): The cytokinesis-block micronucleus technique—a detailed description of the method and its application to genotoxicity studies in human populations. Mutat Res 285:35–44.

Floersheim GL (1993): Radioprotective effects of calcium-antagonists used alone or with other type of radioprotectors. Radiat Res 133:80–87.

Goyer RA (1986): Toxic effects of metals. In Klaassen CD, Amdur MO, Doull J (eds): "Casarett and Doull's Toxicology." New York: Macmillan, pp 582–635.

Habig WH, Pabst MJ, Jakoby WB (1974): Glutathione S-transferase. J Biol Chem 249:7130–7139.

Heddle JA, Cimino MC, HayAshi M, Romagna F, Shelby MD, Tucker JD, Vanparys Ph, MacGregor JT (1991): Micronuclei as an index of cytogenetic damage: Past, present, and future. Environ Mol Mutagen 18:277–291.

Henry PD (1980): Comparative pharmacology of calcium natagonists—nifedipine, verapamil and diltiazem. Am J Cardiol 46:1047–1058.

Hertzpicciotto I, Smith AH (1993): Observations on the dose-response curve for arsenic exposure and lung cancer. Scand J Work Environ Health 19:217–226.

Hidaka H, Inagaki M, Kawamoto S, Sasaki Y (1984): Isoquinolinesulfonamides, novel and potent inhibitors of cyclic nucleotide-dependent protein kinase and protein kinase C. Biochemistry 23:5036–5041.

Hinkle PM, Kinsella PA, Osterhoudt KC (1987): Cadmium uptake and toxicity via voltage-sensitive calcium channels. J Biol Chem 262:16333–16337.

Huang H, Huang CF, Wu DR, Jinn CM, Jan KY (1993): Glutathione as a cellular defense against arsenite toxicity in cultured Chinese hamster ovary cells. Toxicology 79:195–204.

Huang RN, Ho IC, Yih LH, Lee TC (1995): Sodium arsenite induces chromosome endoreduplication and inhibits protein phosphatase activity in human fibroblasts. Environ Mol Mutagen 25:188–196.

Hug H, Sarre TF (1993): Protein kinase C isozymes: Diergence in signal transduction? Biochem J 291:329–343.

IARC (1980): Carcinogenesis of arsenic and arsenic compounds. In: "IARC Monographs on Evaluation of Carcinogenic Risk to Humans," Vol. 23. Lyon: International Agency for Research on Cancer, pp 37–141.

Ishinishi N, Tsuchiya K, Vahter M, Fowler BA (1986): In Friberg L, Nordberg GF, Vouk V (eds): "Handbook on the Toxicology of Metals." Amsterdam: Elsevier, pp 43–83.

Jha AN, Noditi M, Nilsson R, Natarajan AT (1992): Genotoxic effects of sodium arsenite on human cells. Mutat Res 284:215–221.

Jiang S, Chow SC, Mccabe MJ, Orrenius S (1995): Lack of Ca2+ involvement in thymocyte apoptosis induced by chelation of intracellular Zn2+. Lab Invest 73:111–117.

Junod AF, Petersen H (1989): Different effects of hyperoxia and hydrogen peroxide on DNA damage, polyadenosine diphosphate-ribose polymerase activity and nicotinamide adenine dinucleotide and adenosine tripophosphate contents in cultured endothelial cells and fibroblast. J Cell Physiol 140:177–185.

Kato K, Hayashi H, Hasegawa A, Yamanaka K, Okada S (1994): DNA damage induced in cultured human alveolar (L-132) cells by exposure to dimethylarsenic acid. Environ Health Perspect 102:285–288.

Kikkawa U, Go M, Koumoto J, Nishizuka Y (1986): Rapid purification of protein kinase C by high performance liquid chromatography. Biochem Biophys Res Commun 135:636–643.

Kim R, Hirabayashi N, Nishiyama M, Jinushi K, Toge T, Okada K (1992): Factors contributing to adriamycin sensitivity in human xenograft tumors: the relationship between expression of the MDR1, GST-π and topoisomerase II genes and tumor sensitivity to adriamycin. Anticancer Res 12:241–246.

Lee TC, Oshimura M, Barrett JC (1985): Comparison of arsenic-induced cell transformation, cytotoxicity, mutation and cytogenic effects in Syrian hamster embryo cells in culture. Carcinogenesis 6:1421–1426.

Lee TC, Tanaka N, Lamb WP, Gilmer TM, Barrett JC (1988): Induction of gene amplification by arsenic. Science 241:79–81.

Lee TC, Ko JL, Jan KY (1989a): Differential cytotoxicity of sodium arsenite in human fibroblasts and Chinese hamster ovary cells. Toxicology 56:289–299.

Lee TC, Wei ML, Chang WJ, Ho IC, Lo CF, Jan KY, Huang H (1989b): Elevation of glutathione levels and glutathione S-transferase activity in arsenic-resistant Chinese hamster ovary cells. In Vitro Cell Dev Biol 25:442–448.

Leonard A, Lauwerys RR (1980): Carcinogenicity, teratogenicity and mutagenicity of arsenic. Mutat Res 75: 49–62.

Li GC, Billings PC, Kennedy AR (1992): Induction of oncogene expression by sodium arsenite in C3H/10T/2 cells—inhibition of c-myc expression by the Bowman-Brik protease inhibitor-1. Cancer J 5:354–358.

Li JM, Rossman TG (1989): Inhibition of DNA ligase activity by arsenite: A possible mechanism of its comutagenesis. Mol Toxicol 2:1–9.

Liu YC, Huang H (1996): Lowering extracellular calcium content protects cells from arsenite-induced killing and micronuclei formation. Mutagenesis 11:75–78.

Livingston G, Reed R, Olson B, Lockey J (1990): Induction of nuclear aberrations by smokeless tobacco in epithelial cells of human oral mucosa. Environ Mol Mutagen 15: 136–144.

Mcconkey DJ, Aguilarsantelises M, Hartzell P, Eriksson I, Mellstedt H, Orrenius S, Jondal M (1991): Induction of DNA fragmentation in chronic B-lymphocytic leukemia cells. J Immunol 146:1072–1076.

Moffett J (1988): "The Determination of Arsenic in Non-Silicate Geological Ore Samples Using a Vapor Generation Accessory." Varian Instruments at Work, AA-78. Victoria, Australia: Varian Techtron, Pty. Limited.

Nakamuro K, Sayato Y (1981): Comparative studies of chromosomal agerration induced by trivalent and pentavalent arsenic. Mutat Res 88:73–80.

Nicotera P, Baldi C, Bellomo G, Hartzell P, Orrenius S, Svvensson SA (1986): Cystamine induced toxicity in heatocytes through the elevation of cytosolic Ca2+ and the stimulation of a nonlysosomal proteolyticcc system. J Biol Chem 261:4628–4635.

Nishizuka Y (1984): The role of protein kinase C in cell surface signal transduction and tumor promotion. Nature 308:693–698.

Nishizuka Y (1986): Studies and perspectives of protein kinase C. Science 233:305–311.

Nishizuka Y (1988): The molecular heterogeneity of protein kinase C and its implications for cellular regulation. Nature 334:661–665.

Nishizuka Y (1992): Intracellular signaling by hydrolysis of phospholipids and activation of protein kinase C. Science 258:607–614.

Nordberg G, Anderson O (1981): Metal interactions in carcinogenesis: Enhancement, inhibition. Environ Health Perspect 40:65–81.

Pershagen G (1981): The carcinogenicity of arsenic. Environ Health Perspect 40:93–100.

Reali D, DiMarino F, Bahramandpour S, Carducci A, Barale R, Loprieno N (1987): Micronuclei in exfoliated urothelial cells and urine mutagenicity in smokers. Mutat Res 192:145–149.

Rosin MP, Anwar W (1992): Chromosomal damage in urothelial cells from Egyptians with chronic schistosoma haematobium infections. Int J Cancer 50:539–543.

Rossman TG (1981): Enhancement of UV-mutagenesis by low concentrations of arsenite in *E. coli*. Mutat Res 91:207–211.

Rossman TG, Wolosin D (1992): Differential susceptibility to carcinogen-induced amplification of SV40 and dhfr sequences in SV40-transformed human keratinocytes. Mol Carcinog 6:203–213.

Sharma A, Talukder G (1987): Effects of metals on chromosomes of higher organisms. Environ Mutagen 9:191–226.

Simons TJB, Pocock G (1987): Lead enters bovine adrenal medullary cells through calcium channels. J Neurochem 48:383–389.

Stabel S, Parker PJ (1991): Protein kinase C. Pharmacol Ther 51:71–79.

Tong-Mak IT, Boehme P, Wegliciki WB (1992): Antioxidant effects of calcium-channel blockers against free-radical injury in endothelial cells—correlation of protection with preservation of glutathione levels. Circ Res 70:1099–1103.

Trigon S, Morange M (1995): Different carboxyl-terminal domain kinase activities are induced by heat-shock and arsenite. J Biol Chem 270:13091–13098.

Vhter M, Marafante E (1987): Effects of low dietary intake of methionine, choline or proteins on the biotransformation of arsenite in the rabbit. Toxicol Lett 37:41–46.

Vine MF (1990): Micronuclei. In: Hulka BS, Wilcosky TC, Griffith JD (eds): "Biological Markers in Epidemiology." pp. 125–146. New York: Oxford University Press.

Wang TS, Huang H (1994): Active oxygen species are involved in the induction of micronuclei by arsenite in Xrs-5 cells. Mutagenesis 9:253–257.

Wang TS, Kuo CF, Jan KY, Huang H (1996): Arsenite induces apoptosis in Chinese hamster ovary cells by generation of reactive oxygen species. J Cell Physiol 169:256–268.

Yamanaka K, Hasegawa A, Sawamura R, Okada S (1991): Cellular response to oxidative damage in lung induced by the administration of dimethylarsinic acid, a major metabolite of inorganic arsenics, in mice. Toxicol Appl Pharmacol 108:205–213.

Yin LH, Huang H, Jan KY, Lee TC (1991): Sodium arsenite induces ATP depletion and mitochondrial damage in HeLa cells. Cell Biol Int Rep 15:253–264.