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Arsenic Induces Apoptosis in Rat Cerebellar Neurons via Activation of JNK3 and p38 MAP Kinases

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Primary cultures of rat cerebellar neurons were used to study mechanisms of arsenic neurotoxicity. Exposure to 5, 10, or 15 μM sodium arsenite reduced cerebellar neuron viability and induced nuclear fragmentation and condensation as well as DNA degradation to oligonucleosome fragments. Exposure to 1 or 5 mM dimethylarsinic acid caused similar changes. Therefore, both inorganic arsenite and organic dimethylarsinic acid induce apoptosis in cerebellar neurons, with the inorganic form being more toxic. Cotreatment with cycloheximide or actinomycin D, inhibitors of protein or RNA synthesis, respectively, or with the caspase inhibitor zVAD, completely blocked arsenite-induced cerebellar neuron apoptosis. This implies that arsenite-induced cerebellar neuron apoptosis requires new gene expression and caspase activation. Interestingly, sodium arsenite selectively activated p38 and JNK3, but not JNK1 or JNK2 in cerebellar neurons. Blocking the p38 or JNK signaling pathways using the inhibitors SB203580 or CEP-1347 protected cerebellar neurons against arsenite-induced apoptosis. These data suggest that arsenite neurotoxicity may be due to apoptosis caused by activation of p38 and JNK3 MAP kinases. © 2001 Academic Press

Key Words: **apoptosis; cell death; signal transduction; JNK; p38; MAP kinase; CNS; neurons; cerebellar neurons; arsenite; arsenic; arsenical; dimethylarsinic acid; DMAA.**

Arsenic is an environmental toxicant found naturally in ground water. Arsenic contamination of the environment also results from industrial and agricultural uses. Inorganic arsenic, the predominant form identified in drinking water, is more acutely toxic than organic forms. Multiple valency states of inorganic arsenic including pentavalent arsenate (As^{5+}) and trivalent arsenite (As^{3+}) are found in the environment. As^{3+} is more acutely toxic than As^{5+} (Goyer, 1996) and a significant portion of the toxicity of arsenate is thought to be secondary to its *in vivo* reduction to arsenite (Winski and Carter, 1995). On the molecular level, arsenic causes cellular toxicity via multiple mechanisms, including production of reactive oxygen species and alterations in the activity of key enzymes (Bernstam and Nriagu, 2000).

Epidemiology studies have suggested a correlation between arsenic exposure and potential neurotoxicity. For example, acute arsenic intoxication in human patients can cause severe polyneuropathy with prolonged sensory and motor deficits (Murphy *et al.*, 1981). Encephalopathy in human patients after chronic low level occupational exposure or acute massive arsenic ingestion has also been reported, with symptoms including cognitive impairment and even delirium (Beckett *et al.*, 1986; Fincher and Koerker, 1987; Morton and Caron, 1989). Furthermore, chronic consumption of inorganic arsenic in both adult and young rats causes defects in operant learning (Nagaraja and Desiraju, 1994).

Arsenic may also be a teratogen (Golub *et al.*, 1998; Shalat *et al.*, 1996). Both pentavalent and trivalent arsenic have been shown to cross the placental barrier readily and selectively accumulate in the fetal neuroepithelium in early gestation (Lindgren *et al.*, 1984; Nagaraja and Desiraju, 1993; Valkonen *et al.*, 1983). Elevated arsenic levels in drinking water are associated with spontaneous abortions (Aschengrau *et al.*, 1989; Borzsonyi *et al.*, 1992). Developmental exposure to arsenate or arsenite in animal models causes exencephaly and neural tube defects (Beaudoin, 1974; Chaineau *et al.*, 1990; Ferm and Carpenter, 1968; Hood and Bishop, 1972; Morrissey and Mottet, 1983; Tabocova *et al.*, 1996; Wlodarczyk *et al.*, 1996). Arsenite is about ten times more toxic than arsenate in causing neural tube defects (Chaineau *et al.*, 1990). However, recent studies showed that, when arsenite is administered orally, cranial neural tube defects are observed only after exposure to doses that are high enough to cause maternal toxicity (DeSesso *et al.*, 1998). These results led to the conclusion that inorganic arsenic is unlikely to be a human teratogen as a result of environmental exposure (DeSesso *et al.*, 1998). However, the end point of the studies by DeSesso *et al.* (1998) was the frequency of gross structural abnormalities during embryogenesis, while the effect of arsenite on motor activity, learning, and memory was not examined. Arsenic may cause cellular and biochemical defects that are not manifested

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as gross structural abnormalities. Further investigation of arsenite neurotoxicity is needed to address these issues.

The pentavalent, dimethylarsinic acid (DMAA) is the principle *in vivo* transformation product of arsenics (Goyer, 1996). It is rapidly formed and excreted in urine (Goyer, 1996; Lovell and Farmer, 1985). Although the formation of dimethylarsinic acid is presumed to be a process of detoxification of the more toxic inorganic arsenics and the trivalent, monomethylarsonous acid (MMA(III)), dimethylarsinic acid has been implicated as a carcinogen in rodents (Brown *et al.*, 1997; Hayashi *et al.*, 1998; Li *et al.*, 1998; Morikawa *et al.*, 2000; Wei *et al.*, 1999; Yamamoto *et al.*, 1997; Yamanaka *et al.*, 1996, 2000). Furthermore, dimethylarsinic acid is lethal to cultured human hepatocytes, though its toxicity is two to three orders of magnitude lower than inorganic arsenite or monomethylated MMA(III) (Petrick *et al.*, 2000).

The objective of this study was to determine if arsenite induces apoptosis in primary cultures of rat cerebellar neurons and to elucidate mechanisms underlying this apoptosis. Apoptosis, a form of programmed cell death, plays an important role during normal development, including the development of the central nervous system (CNS) (Jacobson *et al.*, 1997; Oppenheim, 1991). Many developmental toxicants induce apoptosis and give rise to structural malformations and/or functional abnormalities (Alison and Sarraf, 1995; Corcoran *et al.*, 1994; Li and Kaya, 1994). The development of rat cerebellum during the first 2–3 postnatal weeks is dependent upon apoptosis of selective subpopulations of neurons. Furthermore, the cerebellum is critical for learning motor skills and for developing motor coordination. Because cerebellar neuron cultures are relatively homogeneous, the majority being granule cells, they are frequently used to study neuronal apoptosis, including apoptosis induced by toxicants (Bhave and Hoffman, 1997; Zhang *et al.*, 1998a,b). Therefore, we used cerebellar neurons cultured from postnatal 7-day-old rats to investigate the neurotoxicity of arsenite.

Our data demonstrate that sodium arsenite and dimethylarsenic acid induce apoptosis in cerebellum neurons, with sodium arsenite approximately 1000-fold more toxic than dimethyl arsenic acid. Arsenite-induced apoptosis required *de novo* gene expression, caspase activation, and activation of the JNK and p38 MAP kinases. Our data suggest that arsenite can cause cellular and biochemical changes in CNS neurons, which may contribute to arsenite neurotoxicity.

METHODS

Experimental animals. Time-pregnant female Sprague–Dawley rats were purchased from B&K Universal (Kent, WA). Both male and female pups, 6–8 days old, weighing approximately 60 g each, were used for preparing cerebellar neuron cultures. Animals were kept in the central Animal Care Facility at the University of Washington. All treatment of animals met the standards of the NIH Guide for the Care and Use of Laboratory Animals and was approved by the University of Washington Animal Care Committee.

Reagents used. Sodium arsenite ($NaAsO_2$) and DMAA were dissolved in DMEM as a 1000× stock. Cycloheximide (CXM) was dissolved in water as a 1000× stock. The following chemicals were dissolved in dimethyl sulfoxide (DMSO) as a 1000× stock: zVAD-fluoromethylketone (zVAD), actinomycin D (ActD), SB203580, and CEP1347. Equal volumes of DMSO (0.1%, vol/vol) were used as vehicle control for zVAD, ActD, SB203580, and CEP1347. In our experience, addition of DMSO up to 0.5% does not alter the levels of basal cell death in cultured cerebellar neurons. In the studies reported here, we used 0.1–0.2% DMSO, which does not cause any measurable toxicity (see Figs. 5 and 9).

Primary cerebellar neuron cultures. Cerebellar granule neurons were prepared from 6–8-day-old Sprague–Dawley rats as described (D'Mello *et al.*, 1993). Briefly, dissociated cerebellar neurons were plated in 60-mm culture dishes (5×10^6 cells/60-mm plate) for biochemistry experiments or in 35-mm dishes for nuclear staining experiments (2.5×10^6 cells/35-mm plate). Plates and glass coverslips were coated with poly-D-lysine and poly-ornithine. D'Mello and colleagues (1993) described a protocol using poly-L-lysine to coat plates for culturing cerebellar neurons. However, poly-ornithine is also used by many investigators to coat plates for culturing cerebellar neurons (Datta *et al.*, 1997; Dudek *et al.*, 1997). In addition, poly-D-lysine is used for cerebellar neuron cultures to minimize digestion of the coating substrates by the cells (Hatten *et al.*, 1998). We found that poly-ornithine treatment alone was, in most cases, sufficient for cerebellar granule cell cultures plated on plastic tissue culture dishes. However, addition of poly-D-lysine improved the attachment of cells to the glass coverslips. Therefore, we used a combination of poly-ornithine (Sigma P2533, 15 μg/ml) and poly-D-lysine (Collaborative Research, 40210; 50 μg/ml) to precoat glass slides and plates.

Cells were cultured in basal medium Eagle (BME) supplemented with 10% heat-inactivated bovine calf serum, 25 mM KCl, 35 mM glucose, 1 mM L-glutamine, 100 U/ml of penicillin, and 0.1 mg/ml streptomycin and maintained in a humidified incubator with 5% CO_2 at 37°C. Cytosine-β-D arabinofuranoside (Ara-C, 10 μM final concentration, Sigma, St. Louis, MO) was added to cultures at days *in vitro* (DIV) 1 to inhibit the proliferation of non-neuronal cells. Cerebellar neurons were cultured for 7 days (DIV 7) before drug treatment.

Cerebellar neuron survival assayed by MTT metabolism. Neuronal survival was assayed by the colorimetric conversion of the yellow, water-soluble tetrazolium 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to the blue, water-insoluble formazan. This conversion is catalyzed by cellular mitochondrial dehydrogenases. Because the extent of this reaction is proportional to the number of surviving cells, MTT assay is widely used to quantify viable cells (Hansen *et al.*, 1989). MTT assays were performed in 96-well plates as described (Hansen *et al.*, 1989). Briefly, MTT (Sigma, final concentration of 1 mg/ml) was added to the cells at the end of various drug treatments. The cells were then incubated for 2 h at 37°C in a 5% CO_2 incubator and solubilized overnight with 9% SDS and 22% dimethyl formamide before determination of absorbance at 570 nm. Optical blanks, used as controls, were generated by incubating the corresponding drugs and MTT in the conditioned culture medium. Data are presented as the percentage of survival at various times relative to that at the zero time point. All MTT assays were performed in triplicate.

Quantitation of apoptosis. To visualize nuclear morphology, cells were fixed with 4% paraformaldehyde/4% sucrose for 1 h, permeabilized with 0.1% Triton X-100, and stained with 2.5 μg/ml of the DNA dye Hoechst 33258 (bis-benzimide) (Xia *et al.*, 1995). Apoptosis was quantitated by scoring the percentage of apoptotic cells in the adherent cell population. Uniformly stained nuclei were scored as healthy, viable neurons. Condensed or fragmented nuclei were scored as apoptotic. Statistical analysis of the data was performed using one-way ANOVA and Fisher's predicted least square determination (PLSD) *post hoc* test.

DNA ladder assay. To examine DNA cleavage, soluble cytoplasmic DNA was isolated and subjected to agarose gel electrophoresis (Hockenbery *et al.*, 1990).

Western blot analysis. Cell lysates were prepared as described (Dérijard *et al.,* 1994) and 100 μg proteins were used for each Western blot. The p38 activation was assayed by Western analysis using an anti-phospho-p38 antibody (New England Biolabs, Beverly, MA). Western analysis using an anti-p38 antibody (Santa Cruz Biotech, Santa Cruz, CA) was conducted as a control for normalization. The intensity of the bands on Western blots was quantitated by scanning the Western blots and was analyzed by ImageQuant analysis. The relative phospho-p38 was normalized to the total p38 from anti-p38 Western blots and data are presented as fold activation. The relative phospho-p38 level at time 0 was arbitrarily set as 1.

Kinase assays. Cell lysates were prepared as described (Dérijard *et al.,* 1994) and 150 μg proteins were used for each kinase assay. The p38 activity was measured as described using an immune complex kinase assay and glutathione *S*-transferase (GST)-ATF-2 as substrates (Xia *et al.,* 1995). The JNK1 and 2 MAP kinase activity was quantitated by an immune complex kinase assay as described (Namgung and Xia, 2000) using GST-cJun (1-79) as substrates and a polyclonal antibody to JNK that recognizes both JNK1 and 2 (Dérijard *et al.,* 1994) to immunoprecipitate JNK1/2 together. JNK3 activity was assayed as described (Namgung and Xia, 2000). Briefly, cell lysates were immunoprecipitated with a mixture of a polyclonal antibody that recognizes both JNK1 and 2 (Dérijard *et al.,* 1994) and a monoclonal antibody that recognizes JNK1 (Pharmingen, San Diego, CA) in order to remove both JNK1 and 2 from the lysates. The remaining JNK3 kinase activity in the supernatant was assayed by the JNK capture assay. To ensure that JNK1 and 2 were completely removed from the supernatant, cell lysates (30 μg) before and after JNK1/2 immunodepletion were analyzed by Western blotting using a monoclonal antibody that recognizes both JNK1 and 2 (Pharmingen). Relative increases (fold activation) in kinase activity were determined by ImageQuant analysis of the autoradiographic images. The band intensity at time 0 was arbitrarily set as 1.

Statistical analysis. Data are presented as means ± SEM. We used StatView512+ computer software for statistical analysis of the data, employing one-way ANOVA and Fisher's PLSD *post hoc* test. Statistically significant differences were reported as $*p < 0.05$, $**p < 0.01$, or $***p < 0.001$. Data with statistical values of $p < 0.05$ are generally accepted as statistically significant (Zar, 1996). Data with values of $p < 0.01$ or $p < 0.001$ are statistically more significant than those with $p < 0.05$ (Zar, 1996).

RESULTS

Both Sodium Arsenite and Dimethylarsinic Acid Induce Cerebellar Neuron Apoptosis

To examine the toxic effects of sodium arsenite, cerebellar neurons were treated with varying concentrations of sodium arsenite (0, 5, 10, and 15 μM) and assayed for cell viability 3, 12, 24, and 48 h after treatment using the MTT metabolism assay (Fig. 1A). Sodium arsenite-mediated reduction of cell viability was dose- and time-dependent. Cerebellar neurons were also treated with the pentavalent dimethylarsinic acid, a biotransformation product of arsenic whose excretion in urine is used as an indicator of arsenic metabolism (Goyer, 1996). Dimethylarsinic acid also reduced cerebellar neuron viability (Fig. 1B). However, concentrations of dimethylarsinic acid approximately 1000-fold higher than those for sodium arsenite were required to reach similar levels of toxicity. For example, at 48 h post treatment, 5 μM sodium arsenite reduced cell viability to 70%, but 5 mM of dimethylarsinic acid was required to obtain a similar effect. This is consistent with the notion that inorganic trivalent arsenic is more toxic than pentavalent dimethylated arsenic (Goyer, 1996).

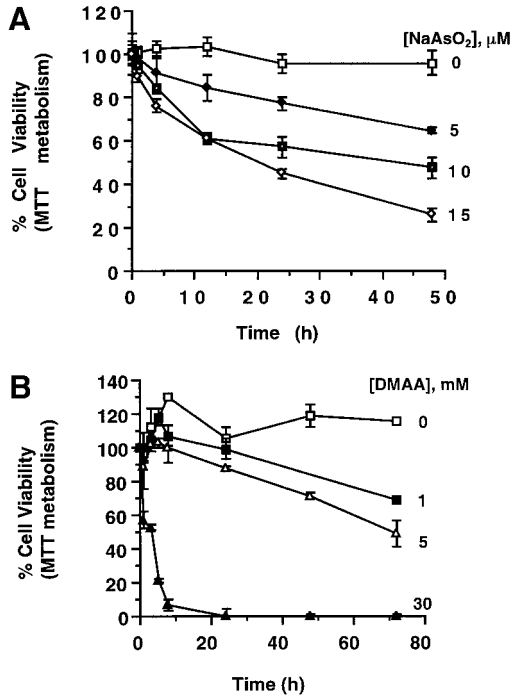

FIG. 1. Sodium arsenite and dimethylarsinic acid reduce viability of cerebellar neurons. Rat cerebellar neurons were treated with 0, 5, 10, and 15 μM sodium arsenite ($NaASO_2$) (A) or 0, 1, 5, and 30 mM dimethylarsinic acid (DMAA) (B) for various times as indicated, and cell viability was measured with MTT metabolism assay. Error bars indicate SEM ($n = 3$).

To determine if the reduced cell viability is due to apoptosis, cerebellar neurons were treated with 10 μM sodium arsenite or 5 mM dimethylarsinic acid, and cytoplasmic DNA was isolated and analyzed by agarose gel electrophoresis. Both sodium arsenite and dimethylarsinic acid caused DNA cleavage into oligonucleosome fragments manifested as "DNA laddering," a hallmark of apoptosis (Fig. 2). Cerebellar neurons were also stained with the DNA dye Hoechst 33258 (bis-benzimide) to visualize nuclear morphology. Both sodium arsenite and dimethylarsinic acid caused morphological changes characteristic of apoptosis, including degeneration of neurites, shrinkage of cell bodies, as well as fragmentation and condensation of nuclei (Fig. 3). Induction of the apoptotic phenotype was dependent on the arsenite concentration and the time of incubation (Fig. 4). Because 10 μM sodium arsenite gave a more robust induction of apoptosis than 5 μM sodium arsenite, 10 μM sodium arsenite was used for subsequent studies.

The effect of arsenite on cerebellar neuron apoptosis was inhibited by treatment with cycloheximide or actinomycin D (Fig. 5), inhibitors of protein or RNA synthesis, respectively. To determine if arsenite-induced apoptosis is caspase dependent, cerebellar neurons were preincubated with zVAD, a pan-caspase inhibitor. zVAD reduced loss of cell viability (Fig. 6A) and inhibited apoptosis (Fig. 6B) after arsenite treatment.

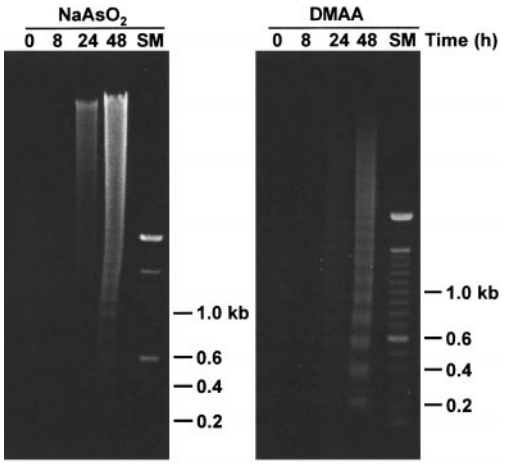

FIG. 2. Both sodium arsenite and dimethylarsinic acid induce DNA fragmentation in cerebellar neurons, manifested as a "DNA-ladder". Cytoplasmic DNA was isolated from 5×10^6 cells after treatment with 10 μM sodium arsenite ($NaASO_2$) or 5 mM dimethylarsinic acid (DMAA) for various times as indicated and subjected to 1.2% agarose gel electrophoresis. Positions of molecular size markers (SM, in kb) are indicated on the right.

Collectively, these data indicate that arsenic induces apoptosis in cerebellar neurons, which is dependent on caspase activation and gene expression.

Cerebellar Neuron Apoptosis Induced by Sodium Arsenite Requires Activation of p38 and JNK3 MAP Kinases

To define signaling pathways that mediate arsenite-induced apoptosis in cerebellar neurons, we evaluated the contribution of the p38 and JNK MAP kinases. These kinases are activated by various stress signals and mediate CNS neuron apoptosis induced by various types of injury (Ip and Davis, 1998; Kawasaki *et al.*, 1997; Kuan *et al.*, 1999; Le Niculescu *et al.*, 1999; Luo *et al.*, 1998; Maroney *et al.*, 1998; Namgung and Xia, 2000; Yang *et al.*, 1997). We first determined if p38 and JNK are activated by arsenite treatment in cerebellar neurons. p38 activity was measured with an immune complex kinase assay at various times after arsenite treatment (Fig. 7A). p38 activity was elevated by 10 μM sodium arsenite at 4 h and remained activated for 24 h. Activation of p38 was confirmed by Western analysis using an anti-phospho-p38 antibody that specifically recognizes phosphorylated and activated p38 (Fig. 7B). Treatment of cerebellar neurons with 5 mM dimethylarsinic acid also activated p38 (Fig. 7C).

There are three genes encoding the JNKs, JNK1, JNK2, and JNK3. mRNAs for all three genes are expressed in the brain (Gupta *et al.*, 1996). However, JNK3 is the only neural-specific isoform (Martin *et al.*, 1996). The relative contribution of

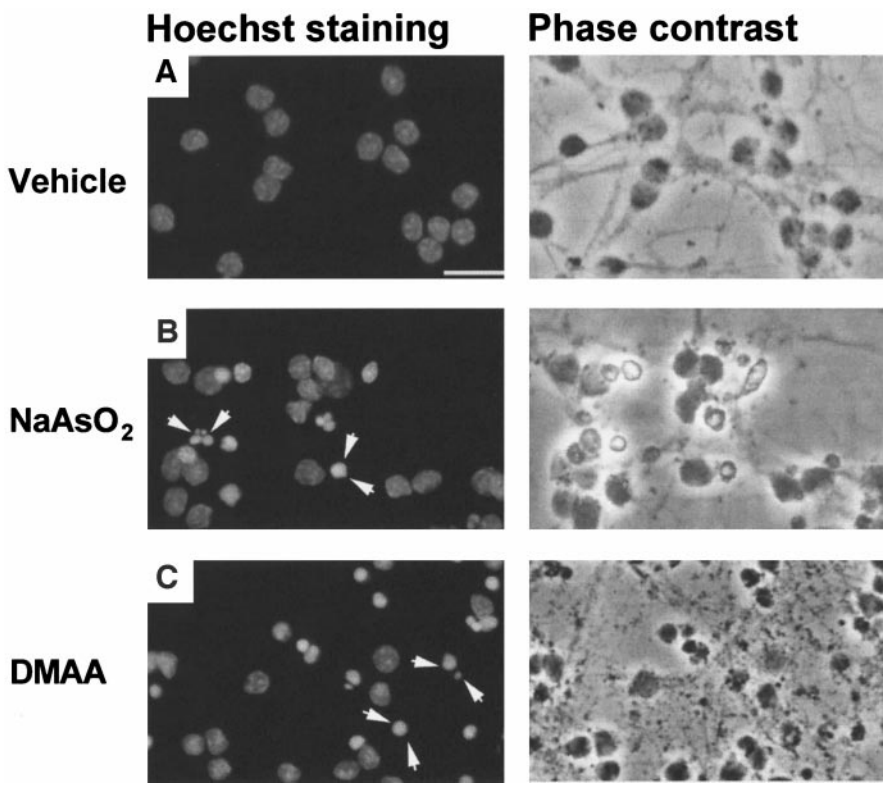

FIG. 3. Sodium arsenite and dimethylarsinic acid cause nuclear fragmentation and condensation in cerebellar neurons, characteristics of apoptosis. Cerebellar neurons were treated with vehicle control (DMEM), 10 μM sodium arsenite, or 5 mM dimethylarsinic acid (DMAA). Cultures were fixed 48 h after treatment and stained with Hoechst dye to visualize nuclei morphology. Pictures shown are representative Hoechst nuclei staining or phase-contrast photomicrographs. Arrowheads indicate apoptotic nuclei that are fragmented and/or condensed. Bar in A represents 15 μm, which applies to all panels.

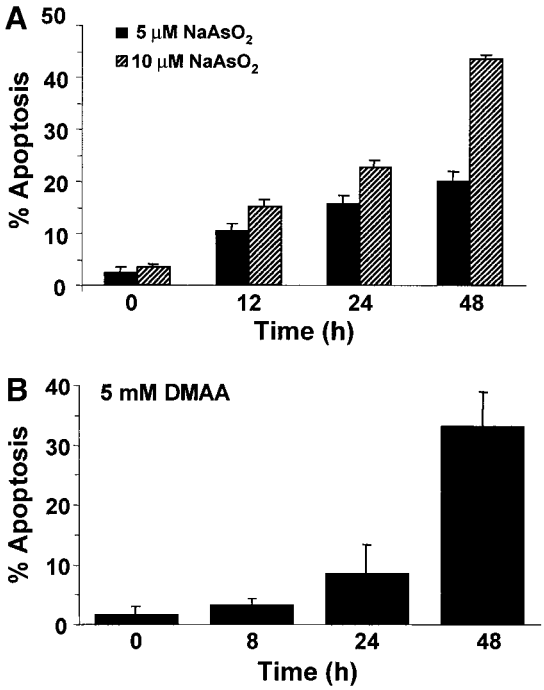

FIG. 4. Quantitation of cerebellar neuron apoptosis induced by sodium arsenite or dimethylarsinic acid. Apoptosis was scored by nuclear fragmentation and/or condensation. (A) Treatment with 5 or 10 μM sodium arsenite ($NaASO_2$). (B) Treatment with 5 mM dimethylarsinic acid (DMAA). At least 2000 cells were counted for each data point. Data are the mean ± SEM (n = 3).

different isoforms of JNK was evaluated by assaying JNK1/2 and JNK3 activities separately. JNK1/2 showed the high basal activity that was not stimulated by arsenite (Fig. 8A). To test if JNK3 is activated by arsenite, JNK1 and JNK2 were depleted from the cell lysates using a mixture of a polyclonal antibody that recognizes both JNK1 and 2 (Dérijard *et al.*, 1994) and a monoclonal antibody that recognizes JNK1 only. This treatment completely removed JNK1 and 2 from cell lysates (Fig. 8C). The remaining JNK3 kinase activity in the supernatant was assayed by a JNK capture assay (Fig. 8B). In contrast to JNK1 and 2, the basal activity of JNK3 was not high and JNK3 was activated threefold by 10 μM sodium arsenite (Fig. 8B). The JNK3 activation was apparent 1 h after arsenite treatment and persisted for at least 24 h. These data indicate that arsenite activates JNK3 but not JNK1 or JNK2 in cerebellar neurons.

To evaluate the functional consequence of p38 and JNK3 activation in arsenite-induced cerebellar neuron apoptosis, we utilized inhibitors of these signaling pathways, including SB203580 and CEP-1347. SB203580 is a specific inhibitor of p38 (Clifton *et al.*, 1996; Cuenda *et al.*, 1995) and CEP1347 is an inhibitor of the JNK pathway (Maroney *et al.*, 1998). Arsenite-induced apoptosis was significantly reduced when cells were treated with SB203580 ($*p < 0.05$), CEP1347 ($**p < 0.01$), or SB203580 and CEP1347 together ($***p < 0.001$) (Fig. 9). Furthermore, incubation with both inhibitors was even more protective than either one alone ($*p < 0.05$) (Fig. 9). These data suggest that activation of both p38 and JNK3 MAP kinases contributes to arsenite-induced cerebellar neuron apoptosis.

DISCUSSION

The objectives of this study were to determine if arsenic induces apoptosis in primary cultured rat cerebellar neurons and to elucidate the underlying mechanisms for this apoptosis. Treatment of cerebellar neurons with micromolar concentrations of sodium arsenite decreased cell viability and caused cellular changes typical of apoptosis. Dimethylarsinic acid also caused similar changes, although it was about three orders of magnitude less potent than arsenite. Arsenite-induced cerebellar neuron apoptosis was blocked by inhibitors of protein synthesis, RNA synthesis, and caspases, implicating a role for *de novo* gene expression and caspase activity. Interestingly, JNK3 and p38, but not JNK1 or 2, were activated by arsenite at concentrations that induced apoptosis. Furthermore, we demonstrated that inhibition of either JNK or p38 signaling using inhibitors protected cerebellar neurons from arsenite toxicity.

The discovery that the JNK3 isoform of JNKs is differentially regulated by arsenite is interesting. Messenger RNAs for all of the JNK genes are expressed in the brain (Gupta *et al.*, 1996) and our data indicate that cerebellar neurons express kinase activities of all three isoforms. JNK1 and 2 exhibited high basal activity in cerebellar neurons that was not stimulated by arsenite. The high basal JNK1/2 activity is consistent with published observations concerning basal levels of JNK in whole rat brain (Xu *et al.*, 1997) and in rat cortical neurons

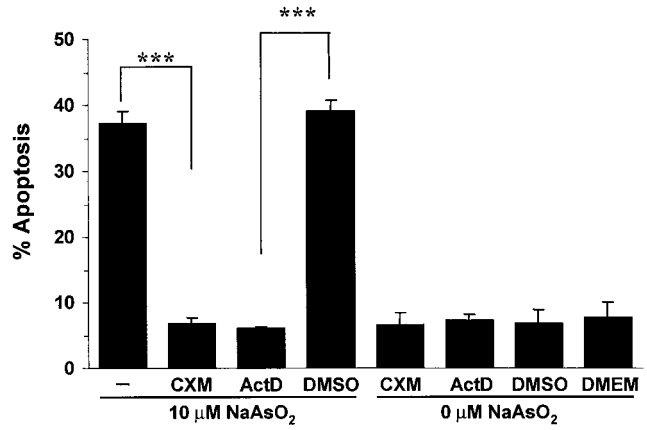

FIG. 5. Arsenite-induced cerebellar neuron apoptosis is blocked by cycloheximide (CXM) or actinomycin D (Act D), inhibitors of protein or RNA synthesis, respectively. Cerebellar neurons were pretreated for 30 min with 10 μg/ml cycloheximide, 1 μg/ml actinomycin D, or equal volumes of DMEM or DMSO (0.1%, vol/vol) as vehicle control for CXM or ActD, respectively. —, no pretreatment. The cells were then stimulated with 10 μM sodium arsenite ($NaAsO_2$) or with DMEM vehicle (0 μM $NaAsO_2$). Apoptosis was scored 48 h later. Data are the mean ± SEM (n = 3). At least 2000 cells were scored for each data point. $***p < 0.001$.

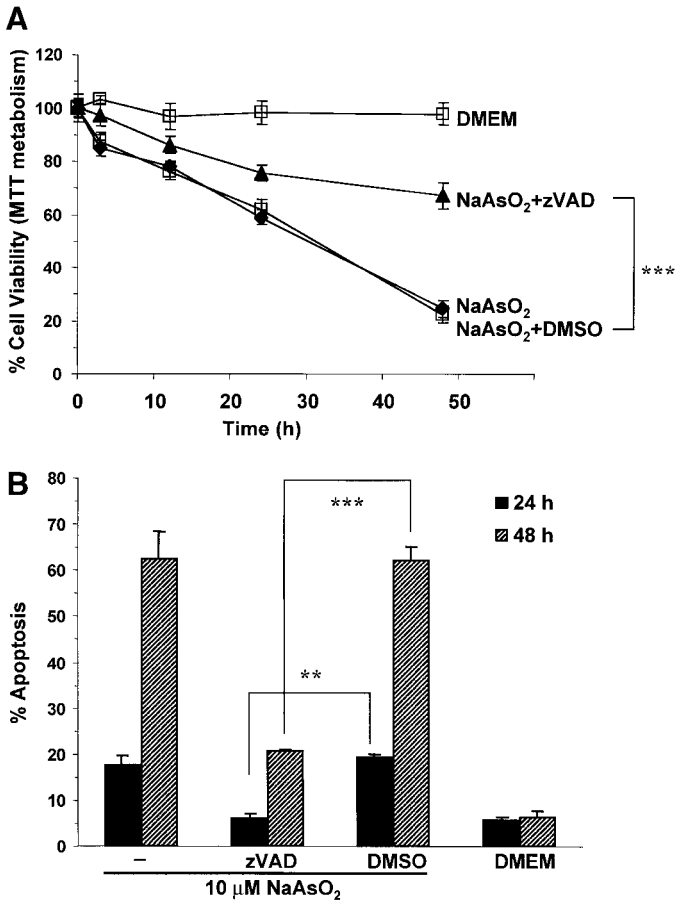

FIG. 6. Cerebellar neuron apoptosis induced by arsenite requires caspase activation. Cerebellar neurons were pretreated for 30 min with 100 μM of the caspase inhibitor zVAD or equal volumes of DMSO (0.1%, vol/vol) as vehicle control. —, no pretreatment. The cells were then stimulated with 10 μM sodium arsenite ($NaAsO_2$) or with DMEM vehicle (0 μM $NaAsO_2$) for various times as indicated. (A) Cell viability determined by MTT metabolism. $***p < 0.001$ (the group treated with $NaAsO_2$ + DMSO compared to the group treated with $NaAsO_2$ + zVAD). (B) Apoptosis scored by nuclear fragmentation and/or condensation. $**p < 0.01$; $***p < 0.001$. Data are the mean ± SEM ($n = 3$ for A and $n = 4$ for B). At least 1500 cells were scored for each data point.

(Namgung and Xia, 2000). In contrast, the basal activity of JNK3 was not high and JNK3 was activated by arsenite. These results are consistent with recent data implicating JNK3 in cortical neuron apoptosis (Namgung and Xia, 2000).

Although JNK and p38 contribute to apoptosis in PC12 cells and non-neuronal cells, their role in apoptosis in primary CNS neurons has not been well defined. There are reports both supporting and arguing against a role for these kinases in neuronal apoptosis (Gunn-Moore and Tavare, 1998; Mielke and Herdegen, 2000; Namgung and Xia, 2000; Yang *et al.*, 1997). Our data support a role for JNK and p38 MAP kinases in cerebellar neuron apoptosis. Together with recent studies in cortical neurons (Namgung and Xia, 2000), our results suggest the possibility that JNK3 but not JNK1/2 may be important for mature CNS neurons to respond to stress signals.

The finding that sodium arsenite induces apoptosis in both cortical and cerebellar neurons suggests a general mechanism underlying arsenic neurotoxicity. Our data with CNS neurons are also consistent with reports that arsenite induces apoptosis in nonneuronal cells (Chen *et al.*, 1996; Larochette *et al.*, 1999; Ochi *et al.*, 1996; Wang *et al.*, 1996, 1997; Watson *et al.*, 1996). However, in nonneuronal cells, it generally requires greater than 40 μM sodium arsenite to cause apoptosis (Wang *et al.*, 1996, 1997; Watson *et al.*, 1996). We discovered that 5 μM sodium arsenite is sufficient to induce a significant increase in cerebellar neuron apoptosis at 48 h (20% apoptosis with sodium arsenite in comparison to 3% apoptosis without arsenite). This suggests that CNS neurons are more sensitive to arsenite than non-neuronal cells.

The amount of arsenic that accumulates in the human brain after environmental exposure is not known and whether arsenic is a teratogen for CNS neuron development is still controversial. However, the fact that low micromolar concentrations of

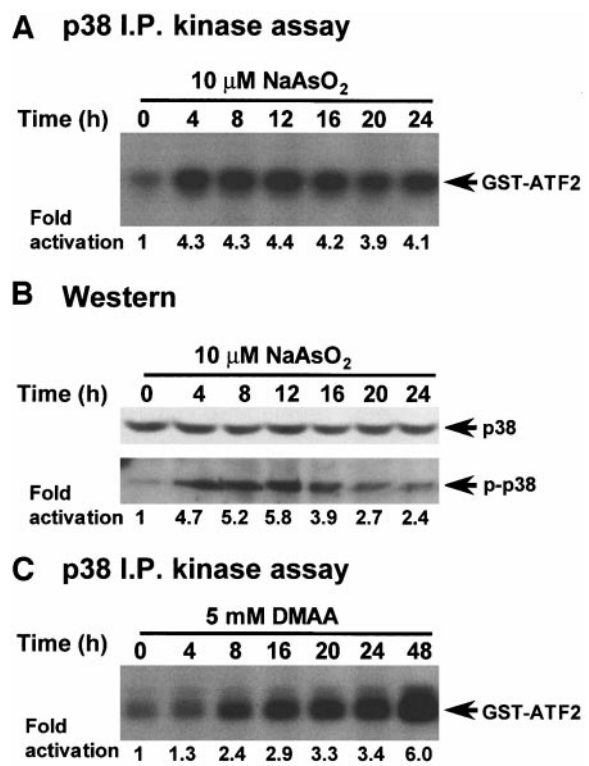

FIG. 7. Sodium arsenite activates p38 MAP kinase in cerebellar neurons. (A) Cerebellar neurons were stimulated with 10 μM sodium arsenite for the indicated times and p38 activity was determined with an immune complex kinase assay. (B) Cerebellar neurons were stimulated with 10 μM sodium arsenite for the indicated times and p38 activation was determined by Western analysis using an antibody recognizing phosphorylated and activated p-p38 (bottom). The anti-p38 Western was used to confirm an equal amount of protein loading in each gel lane and that changes of p-p38 did not result from changes in protein levels of p38. (C) Cerebellar neurons were stimulated with 5 mM DMAA for the indicated times and p38 activity was determined with an immune complex kinase assay. Similar results were obtained in three (A) or two (B and C) independent experiments.

arsenite induce apoptosis in primary CNS neurons is interesting. In cortical neurons, 2 μM sodium arsenite is sufficient to induce a significant increase in apoptosis after 48 h treatment (Namgung and Xia, 2000). Furthermore, it was recently discovered that the teratogenic response of mice to arsenite during neurulation depends upon the genetic background and that a mutation in a single gene is responsible for the strain differences between C57BL/6J and SWV/Fnn mice (Machado *et al.*, 1999). Since the genetic background of humans is quite different from that of experimental animals, it is possible that humans may be even more sensitive to arsenite toxicity than experimental animals. The fact that arsenite is a known carcinogen for humans but not for rodents supports this notion. Consequently, although the sensitivity of human neurons to arsenic has not been examined, it is possible that chronic exposure to arsenic at low concentrations, perhaps even at a submicromolar range, may induce neuronal apoptosis in human brain. In any case, our discovery that low concentrations of arsenite induce apoptosis and activate JNK3 and p38 MAP kinases in both cortical and cerebellar neurons suggests that arsenite can cause significant biochemical and cellular changes in neurons. This may contribute to arsenic neurotoxicity in the absence of gross structural abnormality. Our data suggest that the effects of arsenic on various neuronal functions, including cognition, memory, and motor coordination, warrant further investigation.

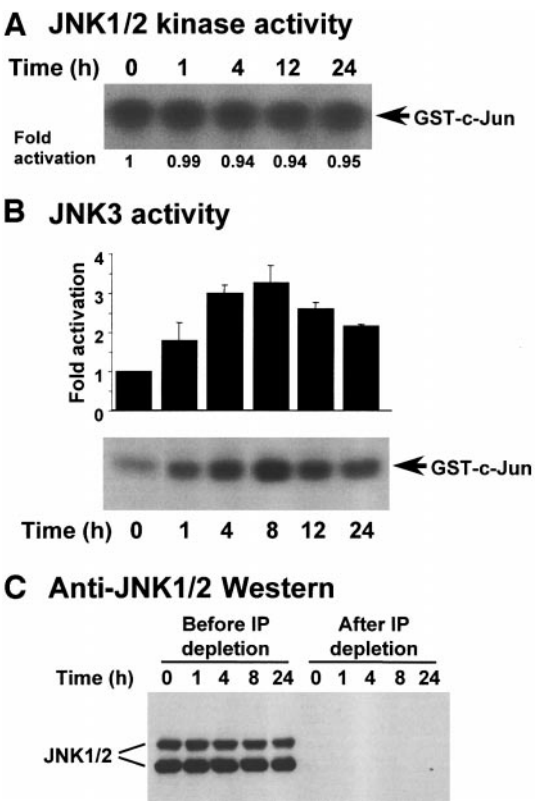

FIG. 8. JNK3, but not JNK1 or 2, is activated by arsenite. Cerebellar neurons were stimulated with 10 μM sodium arsenite for the indicated times. (A) JNK1/2 activity determined by an immune complex kinase assay. (B) JNK3 activity. JNK1 and 2 in the cell lysates (150 μg) were depleted by immunoprecipitation and the remaining JNK3 activity in the supernatant was determined with a JNK capture assay. (C) Western analysis using an antibody that recognizes both JNK1 and 2. Thirty micrograms of protein extracts before and after JNK1/2 immunodepletion were used for Western analysis. Data are the mean ± SEM ($n = 3$). Data shown are representative of two to three independent experiments.

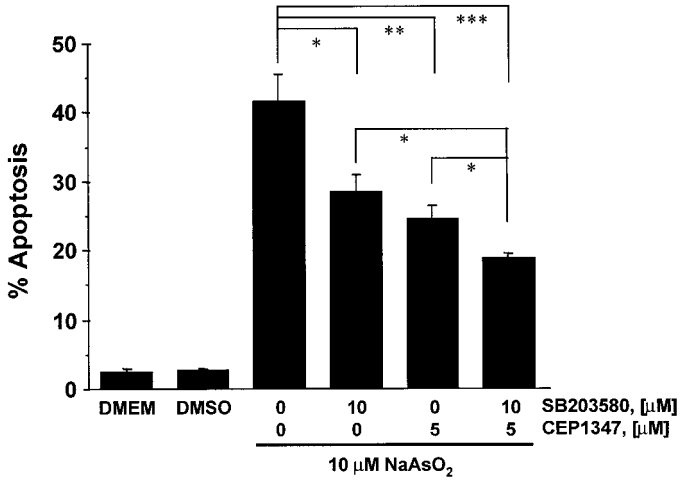

FIG. 9. Blocking the p38 and JNK signaling pathways inhibits arsenite-induced cerebellar neuron apoptosis. Cerebellar neurons were pretreated for 30 min with SB203580 (0 or 10 μM) or CEP-1347 (0 or 5 μM) as indicated and then challenged with 10 μM sodium arsenite. For additional controls, cerebellar neurons were treated with equal volumes of vehicle controls DMEM (0.1%, vol/vol) or DMSO (0.2%, vol/vol). Apoptosis was scored 48 h later. Data are the mean ± SEM ($n = 4$). At least 2000 cells were scored for each data point. $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

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