Inorganic arsenic causes cell apoptosis in mouse cerebrum through an oxidative stress-regulated signaling pathway

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Abbreviations

LPO: lipid peroxidation; GSH: glutathione; NQO1: NAD(P)H:quinone oxidoreductase-1; GPx: glutathione peroxidase; Bax, Bcl-associated X protein; Bak, Bcl-2 homologous antagonist killer; Mcl-1, myeloid cell leukemia sequence 1; ER: endoplasmic reticulum; GRP: glucose-regulated protein; CHOP; C/EBP homologous protein; ERK: extracellular signal-regulated kinase; NAC, *N*-acetylcysteine; ROS: reactive oxygen species; MDA: malondiadehyde.

Footnotes:

1. C. C. Yen, T. J. Ho, C. C. Wu are contributed equally to this work.

2. The detailed information of references of 'Chen et al., 1962' and 'Haider and Najar, 2008' are following as:

Chen KP, Wu HY, Wu TC (1962) Epidemiologic studies on blackfoot disease in Taiwan. 3. Physicochemical characteristics of drinking water in endemic blackfoot disease areas. Memoirs of College of Medicine (National Taiwan University). 8:115-129

Haider SS, Najar MS (2008) Arsenic induces oxidative stress, sphingolipidosis, depletes proteins and some antioxidants in various regions of rat brain. Kathmandu University Medical Journal (KUMJ). 6: 60-9 (PMID: 18604117)

Abstract

Arsenic pollution is a major public health problem worldwide. Inorganic arsenic (iAs) is usually more harmful than organic ones. iAs pollution increases the risk of human diseases such as peripheral vascular disease and cancer. However, the toxicological effects of iAs in the brain are mostly unclear. Here, we investigated the toxic effects and possible mechanisms of iAs in the cerebrum of mice after exposure to iAs (0.5 and 5 ppm (mg/L) As_2O_3, via the drinking water), which was the possible human exposed dose via ingestion in iAs-contaminated areas, for 6 consecutive weeks. iAs dose-dependently caused an increase of LPO in the plasma and cerebral cortex. iAs also decreased the reduced glutathione levels and the expressions of *NQO1* and *GPx* mRNA in the cerebral cortex. These impairments in the cerebral cortex caused by iAs exposure were significantly correlated with the accumulation of As. Moreover, iAs induced the production of apoptotic cells and activation of caspase-3, up-regulation of *Bax* and *Bak*, and down-regulation of *Mcl-1* in the cerebral cortex. Exposure to iAs also triggered the expression of ER stress-related genes, including *GRP78*, *GRP94*, and *CHOP*. Meanwhile, an increase of p38 activation and dephosphorylation of ERK1/2 were shown in the cerebral cortex as a result of iAs-exposed mice. These iAs-induced damages and apoptosis-related signals could be significantly reversed by NAC. Taken together, these results suggest that iAs-induced oxidative stress causes cellular apoptosis in the cerebrum, and signaling of p38 and ERK1/2, and ER stress may be involved in iAs-induced cerebral toxicity.

Keyword: Inorganic arsenic; apoptosis; oxidative stress; ER-stress; p38; ERK1/2

Introduction

Unrestrained industrialization has resulted in the exposure of humans to agents that have the potential to induce or exacerbate many diseases. Arsenic (As), a naturally occurring toxic metalloid found in both inorganic and organic forms, is ubiquitous in the environment and contaminates water as a result of geological and industrial pollution (WHO 2001). The inorganic form of arsenic (iAs) is more toxic than the organic form and predominant in surface and underground water reservoirs (Nordstrom 2002). In the United States, Japan, India, Bangladesh, and Taiwan, drinking water containing high levels of iAs supplied by natural deposits or agricultural and industrial pollution us the major source of exposure to iAs in humans. In epidemiological studies, exposure to iAs through inhalation or ingestion has been associated with increasing incidencs of various chronic diseases, including peripheral vascular disease (as blackfoot disease), cardiovascular disease, diabetes mellitus, and various cancers such as lung, skin, and bladder cancer (Chen et al. 1992; Guo et al. 1997; Meliker et al. 2007; Navas-Acien et al. 2005; Smith et al., 2000).

iAs is a potently toxic metalloid and has been shown to cause severe cytotoxicity in various types of cells, which demonstrated that iAs induces cellular death by apoptotic processes (Cai et al. 2010; Celino et al. 2009; Wang et al. 2009). Moreover, clinical case reports have indicated that patients exposed to iAs, whether accidentally or occupationally, display the severe nervous system dysfunction or signs of pathogenesis of neuropathy (Danan et al. 1984; Halatek et al. 2009; Mathew et al. 2010). In experimental animals, it has also been found iAs induces brain injuries causing behavioral alterations, changes in the development and morphology, and brain cell apoptosis (Flora et al. 2009; Rios et al. 2009). Although iAs has the potential to exert toxic effects in the brain, the detailed mechanisms of its neurotoxicity remain unclear.

Oxidative stress can trigger undesirable biological reactions and is implicated in a wide variety of pathophysiological processes, including neuronal cell death and tissue injuries within the nervous system (Inoue et al. 2004; Loh et al. 2006). Oxidative stress has a potent effect on destroying a balance in pro-oxidant/antioxidant homeostasis, and has been implicated as an important mechanism of in neurotoxicants-induced apoptosis (Chen et al. 2010; Lu et al. 2010). Moreover, the cerebral cortex of the brain is more vulnerable to damage from oxidative stress than other tissues due to its high content of polyunsaturated lipid-rich neural parenchyma, high oxygen utilization, and low levels of antioxidant enzymes (Esposito et al. 2002). Oxidative stress is known to produce reactive oxygen species (ROS), which have has been proposed to be important signaling molecules for the apoptosis caused by many neurotoxicants (Hunag et al. 2008; Rios et al. 2009). A close relationship has been suggested between the overproduction of ROS by toxic metals, including iAs, and the progression of neurodegenerative diseases such as Alzheimer's disease (Bharathi et al. 2006; Gharibzadeh and Hoseini 2008). However, the mechanisms underlying iAs-induced oxidative stress causing neurotoxicity in the brain tissue have not been established. Taken together, in the current study, we tried to explore the toxic effects of iAs on the nervous system and the mechanisms underlying these effects. To this aim, we attempted to investigate the *in vivo* effects of iAs on the production of ROS, level of reduced GSH, expression of *NQO1* and *GPx*, and expression of apoptosis-related genes and proteins in the cerebral cortex of mice exposed to iAs (0.5 and 5 ppm As_2O_3, via the drinking water). Further, the potential protective effects of NAC against apoptosis in the cerebral cortex of mice exposed to iAs were observed.

Materials and Methods

Animal preparation and study design

Seventy-two male ICR mice (4weeks old, 20-25 g) were obtained from the Animal Center of the College of Medical, National Taiwan University. The protocols used were approved by the Institutional Animal Care and Use Committee (IACUC) and the care and use of laboratory animals were conducted in accordance with the guidelines of the Animal Research Committee of China Medical University. Mice were housed six per cage under standard laboratory conditions at a constant temperature (23 ± 2 ℃), 50 ± 20 % relative humidity, given a solid diet and tap water available *ad libidum*, and 12 hrs of light-ark cycles. Mice were acclimatized to the laboratory conditions prior to the experiments and all experiments were carried out between 8:00 AM and 05:00 PM. All mice were randomly divided into six groups (each group contained 12 mice) and exposed to 0, 0.5, and 5 ppm inorganic arsenic (1 mg of As_2O_3 (iAs^{3+}) per liter distilled water (mg/L) via the drinking water available (mimic the possible exposed dose by ingestion in As-contaminated areas) for 6 consecutive weeks. In addition, the groups in NAC alon, iAs-0.5 ppm + NAC, and iAs-5 ppm + NAC were oral gavages with 150 $mg \cdot kg^{-1} \cdot day^{-1}$ NAC, which exposure to iAs was stopped one hour before to one hour after the NAC gavage.

All experimental mice were sacrificed by decapitation under pentobarbital anesthesia (80 $mg \cdot kg^{-1}$, i.p.) and the whole blood samples were collected from the peripheral vessels. Whole blood sample were centrifuged at 3,000 x *g* for 10 min, and plasma was obtained, and LPO levels was assayed immediately. At the same time, cerebral cortex were quickly removed and stored at -80℃ until use. The tissues were analysis of LPO production, GSH levels, and apoptosis-related genes and proteins

expression.

Determination of arsenic contents

300 mg of whole blood was placed in a 15 ml polyethylene tube, and 0.5 ml of a 3:1 mixture of hydrochloric acid (35%) and nitric acid (70%) was added. The tube were capped and allowed to stand overnight at 50℃ oven. After cooling, suitable dilution buffer (0.3% nitric acid and 0.1% Triton X-100 in distilled water) was added to the digested material, and the total arsenic content was determined by inductively coupled plasma mass spectrometry (ICP-MS). The detection limit for arsenic was ~0.1 ppb (μg/L).

Lipid peroxidation assay for plasma and cerebral cortex

LPO was colorimetrically measured by the generation of malondiadehyde (MDA) with commercial LPO assay kit (Calbiochem, USA) according to the manufacturer's instructions. In briefly, the cerebral cortex was homogenized with 20 mM Tris-HCl buffer (pH7.4) and then homogenized sample were assayed immediately. Both plasma and tissue homogenized were added 3.25 volumes of diluted R1 reagent (10.3 mM N-methyl-2-phenylindole in acetonitrile). After mixing, the mixture was added with 0.75 volumes of 37% HCl was added to the mixtures, which was then incubated at 45°C for 60 min. After cooling, the absorbance of the clear supernatant was read by an enzyme-liked immunosorbent assay (ELISA) microplate reader (Bio-Rad, model 550, Hercules, CA) at 586 nm. The linearity of the standard curve was confirmed with 0, 5, 10, 20, 40 μM MDA (1, 1, 3, 3-tetramethoxypropane). The protein concentration was determined using the bicinchoninic acid protein assay kit with an absorption band

of 570 nm (Pierce, Rockford, IL, UAS). LPO level was expressed as nanomoles (nmol) MDA per milligram protein and estimated from the standard curve.

Western blot analysis

Western blotting was performed using standard protocols, as previously described (Chen et al. 2010). In brief, the cerebral cortexes were homogenized with Protein Extraction Solution (iNtRON Biotechnology). The protein concentration was determined using the bicinchoninic acid protein assay kit (Pierce, Rockford, IL, UAS). Equal amounts of proteins (50 μg per lane) was subjected to electrophoresis on 10% (W/V) SDS-polyacrylamide gels and transferred to polyvinylidene difluoride membranes. The membranes were blocked for 1 h in PBST (PBS, 0.05% Tween-20) containing 5% nonfat dry milk. After blocking, the membranes were incubated with anti-cleaved caspase-3, phosphor-p38 and phosphp-ERK1/2 (Cell Signaling Technology, Inc.), and anti-p38 and anti-ERK1/2 (Santa Cruz Biotechnology, Inc.) antibodies in 0.1% PBST for 1 h. After they were washed in 0.1% PBST followed by two washes (15 min each), the respective secondary antibodies conjugated to horseradish peroxidase were applied for 1 h. The antibody-reactive bands were revealed by enhanced chemiluminescence reagents (Perkin-Elmer^{TM}, Life Sciences) and were exposed on the Fuji radiographic film.

Assessment of apoptosis in mouse cerebral cortex

Apoptosis production of the cerebral cortex was detected by DeadEnd^{TM} Colorimetric terminal deoxynucleotidyl transferase mediated dUTP nick end labelling (TUNEL) system (TUNEL assay kit, Promega Corporation, Pty. Ltd.) according to the

manufacturer's instructions. The TUNEL positive cells presenting a deep brown coloration were imaged under the Nikon ECLIPSE 80i upright microscope equipped with a charge-coupled device camera (with ×200 magnification).

Real-time quantitative reverse-transcribed polymerase chain reaction (RT-PCR) analysis

The expression of apoptosis-related genes was evaluated by the real-time quantitative RT-PCR, as previously described (Lu et al. 2010). Briefly, intracellular total RNA was extracted from cerebral cortex using RNeasy kits (Qiagen) according to the instructions provided, was heated to 90 ˚C for 5 min to remove any secondary structures, then placed rapidly on ice. Samples were reverse transcribed into cDNA using the AMV RTase system (Promega Corporation, Pty. Ltd.) according to the manufacture's instructions. Each sample (2 μ1) was tested with Real-time Sybr Green PCR reagent (Invitrogen, USA) with mouse species primers (PCR primers for the examined genes were listed in Table 1.) in a 25 μl reaction volume, and amplification and Real-time fluorescence detection were performed using an ABI StepOnePlus sequence detection system (PE, Applied Biosystems). The fold difference in mRNA expression between treatment groups was determined using the relative quantification method utilizing real-time PCR efficiencies and normalized to the housekeeping gene, *β*-actin, thus comparing relative C_T changes between control and experimental samples. Prior to conducting statistical analyses, the fold change from the mean of the control group was calculated for each individual sample (including individual control samples to assess variability in this group).

Measurement of reduced glutathione (GSH) levels

Reduced glutathione (GSH), a tripeptide (γ-gultamyl-cysteinylglycine), is the major free thiol in most living cells and the key antioxidant in cells or tissues. Reduced GSH levels of the cerebral cortex were measured the fluorimetric thiol probe (monichlorobimane) by Glutathione assay kit (Sigma, USA). In brief, the cerebral cortex was homogenated with an isotonic buffer (25 mM Hepes. pH 7.4, containing 250 mM sucrose) and then centrifuge at 1,000 x *g* at 4 ℃. For each sample, the pellet was discarded, the relative volume (200 μl) of 10X lysis buffer added to the supernatant and placed on ice for 10 mins. After centrifuged, the supernatant was used for the assay. Equal volume sample (30 μ1) was placed in microplate and added assay buffer (82.5 μ1), GST reagents (5 μ1) and the substrate solution (2.5 μ1); and then the plate was incubated at 37 °C for 60 min. The fluorescence intensity of sample was read by using a fluorescent microplate reader with an excitation and emission wavelength of 360 and 485 nm, respectively. Reduced GSH level was expressed as percentage of control group and estimated from the standard curve.

Statistical analysis

Data are presented as means ± SEM. The significance of difference was evaluated by the Student's t-test. When more than one group was compared with one control, significance was evaluated according to one-way analysis of variance (ANOVA) was used for analysis, and the Duncans's post hoc test was applied to identify group differences. The P value less than 0.05 was considered to be significant. The statistical package SPSS, version 11.0 for Windows (SPSS Inc., Chicago, IL, USA) was used for the statistical analysis.

Results

iAs exposure caused arsenic accumulation in the whole blood of mice

Inorganic arsenic (iAs) is the predominant form of As in surface and underground water. Drinking water containing high levels of iAs and industrial pollution are the major sources of people exposure to iAs throughout the world (Nordstrom 2002). Therefore, iAs (As_2O_3) was chosen to investigate the toxic effects of arsenic in the brain. To examine whether iAs could be absorbed by the gastrointestinal (G-I) tract and accumulated in mammals, we first analyzed the total As content in the whole blood of mice exposed to iAs by ICP-MS. As shown in Figure 1, the total As concentration in the whole blood of mice after daily exposure to iAs (0.5 and 5 ppm, via the drinking water) for 6 consecutive weeks was significantly elevated as compared with that in the whole blood of age-matched control mice; the concentration reached up to 19.59 ± 0.87 and 38.35 ± 2.96 ppb (μg/L) in the 0.5 and 5 ppm groups, respectively, as compared with 9.08 ± 0.22 ppb in the control ($^{*}P$ <0.05). These results provide evidence that As can be absorbed by the G-I tract and significantly accumulated in the whole blood after exposure to iAs for 6 consecutive weeks.

iAs induced ROS production and GSH depletion in exposed mice

To further investigate the involvement of oxidative stress in the mechanism underlying iAs-induced neurotoxicity, we analyzed LPO production (as an indicator of oxidative stress damage) in the plasma and cerebral cortex of mice. After the exposure of mice to iAs, MDA levels were significantly increased in the plasma (Figure 2A). Markedly increased MDA levels were also observed in the cerebral

cortex of mice exposed to iAs (Figure 2A). GSH is the first line of defense against oxidative stress; its depletion allows the excess and deleterious ROS to attack various subcellular or intracellular structures and induces apoptosis (Chandar et al. 2000). We, therefore, examined whether iAs-induced oxidative stress led to GSH depletion in the cerebral cortex of iAs-exposed mice. As shown in Figure 2B, reduced GSH levels in the cerebral cortex were significantly decreased after exposure of mice to iAs at both doses (0.5 ppm group, 66.76 ± 6.42 % of control; 5 ppm group, 49.71 ± 2.82 % of control, $n = 12$, $^{*}P < 0.05$ as compared with an age-matched control group).

Moreover, the mRNA expression levels of *NQO1* and *GPx*, which play an important role in the antioxidant system, were analyzed by real-time quantitative RT-PCR. The cerebral cortex of mice exposed to iAs showed marked decrease in the expression of *NQO1* and GP_x in a dose-dependent manner (Figure 3). These iAs-induced responses could be reversed by NAC (150 $mg \cdot kg^{-1} \cdot day^{-1}$, oral application by gavage) (Figures 2, and 3). These findings indicate that exposure to iAs induces oxidative stress damage in the cerebral cortex.

iAs caused apoptosis in the cerebral cortex of exposed mice

To investigate whether iAs-induced neurotoxicity is expressed an apoptotic mechanism; we detected the production of apoptotic cells by TUNEL assay and analyzed the activation of caspase-3 by using western blot. The deep brown of TUNEL-positive neuronal cells (Figure 4A) and caspase-3 protein activation (Figure 4B) in the cerebral cortex of mice were significantly increased by exposure to iAs in a concentration-dependent manner. We further investigated whether iAs could induce the expression of apoptosis-related genes. As shown in Figure 5, the expression of *Bax* and *Bak* (pro-apoptotic genes) significantly increased (approximatelly 2-6 fold)

(Figure 5A, *a*) and that of *Mcl-1* (anti-apoptotic gene) markedly decreased (Figure 5A, *b*) in the cerebral cortex of mice after exposure to iAs. A concomitant and significant increase in the levels of *GRP78*, *GRP94*, and *CHOP*, which are involved in ER stress-induced apoptosis (Szegezdi et al., 2006), were also observed (Figure 5B). NAC could effectively reverse these iAs-induced responses. These results indicated that exposure to iAs via the drinking water can cause the cerebral cortex injury (iAs-induced neurotoxicity), leading to a physiological state associated with apoptosis.

iAs altered the phosphorylation of p38 MAPK and ERK1/2 in the cerebral cortex of exposed mice

Mitogen-activated protein kinases (MAPKs) play an important role in inducing apoptosis in several cell types. The next aim of the investigation was to ascertain whether the activation of MAPKs might be involved in iAs-induced apoptosis in the cerebral cortex of mice. As shown in Figure 6, the level of phosphorylation of p38 MAPK in the cerebral cortex was significantly increased after exposure of mice to iAs (0.5 and 5 ppm, in drinking water) for 6 consecutive weeks. In contrast, the level of phosphorylation of ERK1/2 in the cerebral cortex was markedly decreased in the iAs-exposed mice. NAC (150 $mg \cdot kg^{-1} \cdot day^{-1}$) effectively reversed these iAs-induced responses.

Discussion

Arsenic (As) is a common and important environmental pollutant worldwide. Epidemiological and clinical studies have indicated that chronic iAs exposure causes severe nervous system dysfunctions, including the deterioration of pattern memory, impairment of learning and concentration, disorders of psychic functions, and polyneuropathies (Nagaraja and Desiraju 1994; Sinczuk-Walczak 2009; Tsai et al. 2003). In As-contaminated areas, As concentration in drinking water or groundwater ranges from 0.25 to 2.1 ppm (mg/L), and even reaches >3.0 ppm in some severely contaminated areas (Chen et al. 1962; Chowdhury et al. 2000; Rahman et al. 1998). Thus, the doses of iAs used in this study was relevant to the human exposure levels in iAs-contaminated areas, as opposed to high doses, which might not be useful for inference to human exposure conditions. In addition, oxidative stress plays a key role in the pathogenesis of several diseases, including neurodegenerative diseases, in humans. Neurotoxicant-induced oxidative stress can cause neuronal injury or neuronal apoptosis, which is closely associated with neurodegenerative disorders (Lin and Beal 2006; Loh et al. 2006). Mammalian cerebral cortex is an important region of the brain, involved in learning, memory, and behavioral responses, and particularly vulnerable to oxidative injury leading to neuronal cell apoptosis (Arendt 2001; Loh et al. 2006). Recent studies have shown that chronic exposure to As increases apoptosis in the neuronal cells of the brain and that this neurotoxic effect can be mediated by oxidative stress (Haider and Najar 2008; Flora et al. 2009). Despite several studies showing that iAs can induce neurotoxic effects in mammals, the precise action and molecular mechanisms of iAs-induced neurotoxicity in the brain is still unknown, especially in the cerebral cortex. In the present work, exposure of mice to iAs (0.5 and 5 ppm, via the drinking water) for 6 consecutive weeks was capable of inducing LPO production in the plasma and the cerebral cortex of mice. Meanwhile, increased activation of

caspase-3, increased expression of pro-apoptotic genes (*Bax* and *Bak*), and decreased expression of an anti-apoptotic gene (*Mcl-1*) were observed in the cerebral cortex of iAs-exposed mice. Furthermore, the encoded protein NQO1 plays a classical direct antioxidant role in the detoxification of ROS, which can be induced under the conditions of free radical overproduction due to oxidative stress caused by exposure to toxic compounds (Siegel et al. 2004). GPx is also an important antioxidant enzyme that catalyzes the reduction of hydrogen peroxide. However, it has been indicated that As-induced ROS production, which leads to apoptosis, is accompanied with the decrease in the mRNA expression level of *NQO1* and he depletion of antioxidant enzymes, such as GPx, in mammalian (Flora et al. 2009; Shi et al. 2010). In this study, we found that the cerebral cortex of mice exposed to iAs showed significantly decreased the reduced GSH levels and mRNA expressions of *NQO1* and *GPx*. These iAs-induced responses could be effectively prevented by antioxidant NAC. These findings indicate that oxidative stress is involved in iAs-induced neurotoxicity in the cerebrum.

Apoptosis, also known as programmed cell death, plays an important role not only in controlling the development of multicellular organisms and maintaining tissue homeostasis but also in a growing number of disease processes ranging from inflammatory to neurodegenerative diseases (Loh et al. 2006; Marsden and Strasser 2003). Caspases are cysteine aspartate proteases, which represent a hallmark of the apoptotic process and both initiators and executors of the process of cell death. Recent studies investigating the effects of exposure to toxic metals such as arsenic and mercury have shown that caspase-3 activation is involved in the apoptotic pathway induced by toxic metals (Chen et al. 2010; Tang et al. 2009). Furthermore, exposure to xenobiotics disturbs cellular energy levels, induces Ca^{2+} overloading, and causes failed protein synthesis, folding, transport, and degradation, thus disturbing ER

function and resulting in ER stress (Soboloff and Berger 2002; Szegezdi et al. 2006). GRPs (e.g., GRP 78 and GRP 94), the most abundant glycoproteins in ER, and CHOP, a member of the C/EBP transcription factor family, are the major ER chaperones involved in the ER stress-induced apoptotic pathway, and play a critical role to be involved in the pathogenesis of several diseases, including diabetes mellitus and neurodegenerative diseases (Kaufmam et al. 2002; Zinszner et al. 1998). Herein, we exposed of mice to iAs of relevance to human exposure levels in As-contaminated areas. It was found that iAs significantly increased the deep brown of TUNEL-positive neuronal cells and caspase-3 activation in the cerebral cortex of mice exposed to iAs for 6 consecutive weeks. Moreover, significant up-regulation of pro-apoptotic genes (*Bax* and *Bak*) and down-regulation of an anti-apoptotic gene (*Mcl-1*) were observed. These findings indicate that iAs-induced neurotoxicity occurred through apoptotic cell death in the cerebral cortex of mice. In addition, our study showed that iAs induced marked increase in the mRNA expression levels of *GRP78*, *GRP94*, and *CHOP* in the cerebral cortex of mice exposed to iAs. NAC could prevent these iAs-induced responses. Taken together, these results imply that iAs-induced oxidative stress contributing to neurotoxic damage in the cerebral cortex may be related with the ER stress-mediated apoptotic pathway. The detailed mechanisms of the involvement of ER stress in iAs-induced damage in the cerebral cortex may needs to be clarified in the future.

Recent studies have shown that ROS generation induced by toxic metals (including arsenic) causes neuronal apoptosis, which is closely associated with the progression of neurodegenerative diseases (Bharathi et al. 2006; Flora et al. 2009; Gharibzadeh and Hoseini 2008). In addition, MAPK signaling pathways have been indicated to play a pivotal role in the development of many human diseases, including neurodegenerative diseases. Activated MAPK signaling pathways are suggested to

contribute to the pathogenesis of neurodegenerative diseases through the overproduction of oxidative stress, inducing neuronal apoptosis (Kim and Choi 2010; Miloso et al. 2008). MAPKs (e.g., ERK1/2, p38, and JNK) are a family of serine-threonine kinases that play a critical role in intracellular signaling associated with a variety of cellular functions, including proliferation, differentiation, response to environmental stimuli, and apoptosis (Chang and Karin 2001; Cowan and Storey 2003). In mammalian cells, alterations in p38 MAPK and ERK1/2 activation have been reported to be involved in toxic metal-induced apoptosis (Agarwal et al. 2009; Chen et al. 2009). However, the role of p38 MAPK and ERK1/2 signaling pathways in iAs-induced oxidative stress causing apoptosis in the cerebrum remains unclear. The present work showed that p38 MAPK was significantly activated in the cerebral cortex of mice exposed to iAs and accompanied with ERK1/2 dephosphorylation in the cerebral cortex of iAs-exposed mice, which could be prevented by treatment with antioxidant NAC. These findings implicate that p38 MAPK and ERK1/2 signaling are related, at last in part, to iAs-induced oxidative stress causing apoptosis in the cerebral cortex.

Conclusion

In conclusion, the results of this study provide evidence that iAs induces apoptosis in the cerebral cortex and, more importantly, indicates the underlying possible mechanisms for its effect. The present study demonstrates that iAs induces neurotoxicity in the cerebral cortex through alteration of the oxidative stress-mediated p38 MAPK and ERK1/2 activation, changes in the expression of pro- and anti-apoptotic genes, and activation of caspase-3. Furthermore, our study also reveals that the ER stress-mediated apoptotic signaling pathway is, at last in part, involved in iAs-induced cerebral toxicity. These observations will be beneficial for confirming the possibility that iAs is an environmental risk factor for neurotoxicity.

Conflict of interest statement

All authors declare that they have no conflicts of interest in this study.

Acknowledgments

This study was supported by research grants form the China Medical University Hospital, Taichung, Taiwan (DMR-100-074) and the Central Region Hospital Alliance, Department of Health (P10017), and also supported in part by Taiwan Department of Health Clinical Trial and Research Center of Excellence (DOH100-TD-B-111-004).

References

Agarwal S, Roy S, Ray A, Mazumder S, Bhattacharya S (2009) Arsenic trioxide and lead acetate induce apoptosis in adult rat hepatic stem cells. Cell Biol Toxicol 25:403-13

Arendt T (2001) Alzheimer's disease as a disorder of mechanisms underlying structural brain self-organization. Neuroscience 102:723-765

Baskin-Bey ES, Huang W, Ishimura N, Isomoto H, Bronk SF, Braley K, Craig RW, Moore, DD, Gores, GJ (2006) Constitutive androstane receptor (CAR) ligand, TCPOBOP, attenuates Fas-induced murine liver injury by altering Bcl-2 proteins. Hepatology 44:252-62.

Bharathi Ravid R, Rao KS (2006) Role of metals in neuronal apoptosis: challenges associated with neurodegeneration. Curr Alzheimer Res 3:311-26

Cai BZ, Meng FY, Zhu SL, Zhao J, Liu JQ, Liu CJ, Chen N, Ye ML, Li ZY, Ai J, Lu YJ. Yang BF, (2010) Arsenic trioxide induces the apoptosis in bone marrow mesenchymal stem cells by intracellular calcium signal and caspase-3 pathways. Toxicol Lett 193:173-8

Celino FT, Yamaguchi S, Miura C, Miura T (2009) Arsenic inhibits in vitro spermatogenesis and induces germ cell apoptosis in Japanese eel (Anguilla japonica). Reproduction 138:279-87

Chang L, Karin M (2001) Mammalian MAP kinase signalling cascades. Nature 410:37-40.

Chen CJ, Chen CW, Wu MM, KuoTL (1992) Cancer potential in liver, lung, bladder and kidney due to ingested inorganic arsenic in drinking water. Br J Cancer 66:888-92

Chen KP, Wu HY, Wu TC (1962) Epidemiologic studies on blackfoot disease in Taiwan. 3. Physicochemical characteristics of drinking water in endemic

blackfoot disease areas. Mem Coll Med Natl Taiwan Univ. 8:115-129

Chen X, Lan X, Mo S, Qin J, Li W, Liu P, Han Y, Pi R (2009) p38 and ERK, but not JNK, are involved in copper-induced apoptosis in cultured cerebellar granule neurons. Biochem Biophys Res Commun 379:944-8

Chen YW, Huang CF, Yang CY, Yen CC, Tsai KS, Liu SH (2010) Inorganic mercury causes pancreatic beta-cell death via the oxidative stress-induced apoptotic and necrotic pathways. Toxicol Appl Pharmacol 243: 323-31

Chowdhury UK, Biswas BK, Chowdhury TR, Samanta G, Mandal BK, Basu GC, Chanda CR, Lodh D, Saha KC, Mukherjee SK, Roy S, Kabir S, Quamruzzaman Q, Chakraborti D (2000) Groundwater arsenic contamination in Bangladesh and West Bengal, India. Environ Health Perspect 108:393-7.

Cowan KJ, Storey KB (2003) Mitogen-activated protein kinases: new signaling pathways functioning in cellular responses to environmental stress. J Exp Biol 206:1107-15

Danan M, Dally S, Conso F (1984) Arsenic-induced encephalopathy. Neurology 34:1524

Esposito E, Rotilio D, Di Matteo V, Di Giulio C, Cacchio M, Algeri S (2002) A review of specific dietary antioxidants and the effects on biochemical mechanisms related to neurodegenerative processes. Neurobiol Aging 23:719-735

Flora SJ, Bhatt K, Mehta A (2009) Arsenic moiety in gallium arsenide is responsible for neuronal apoptosis and behavioral alterations in rats. Toxicol Appl Pharmacol 240:236-44

Gharibzadeh S, Hoseini SS (2008) Arsenic exposure may be a risk factor for Alzheimer's disease. J Neuropsychiatry Clin Neurosci 20:501

Guo HR, Chiang HS, Hu H, Lipsitz SR, Monson RR (1997) Arsenic in drinking water

and incidence of urinary cancers. Epidemiology 8:545-50

Haider SS, Najar MS (2008) Arsenic induces oxidative stress, sphingolipidosis, depletes proteins and some antioxidants in various regions of rat brain. Kathmandu Univ Med J (KUMJ) 6: 60-9

Halatek T, Sinczuk-Walczak H, Rabieh S, Wasowicz W (2009) Association between occupational exposure to arsenic and neurological, respiratory and renal effects. Toxicol Appl Pharmacol 239:193-9

Hoijman E, Rocha Viegas L, Keller Sarmiento MI, Rosenstein RE, Pecci A (2004) Involvement of Bax protein in the prevention of glucocorticoid-induced thymocytes apoptosis by melatonin. Endocrinology 145: 418-25

Huang CF, Hsu CJ, Liu SH, Lin-Shiau SY (2008) Neurotoxicological mechanism of methylmercury induced by low-dose and long-term exposure in mice: oxidative stress and down-regulated Na+/K(+)-ATPase involved. Toxicol Lett 176:188-97

Inoue M, Sato EF, Nishikawa M, Hiramoto K, Kashiwagi A, Utsumi K (2004) Free radical theory of apoptosis and metamorphosis. Redox Rep 9:237-47

Kaufman RJ (2002) Orchestrating the unfolded protein response in health and disease. J Clin Invest 110:1389-98

Kim EK, Choi EJ (2010) Pathological roles of MAPK signaling pathways in human diseases. Biochim Biophys Acta 1802:396-405

Kim SH, Kim SC, Kho YJ, Kwak SW, Lee HG, You SK, Woo JH, Choi YJ (2004) C2-ceramide as a cell death inducer in HC11 mouse mammary epithelial cells. Cancer Lett 203:191-7

Lin MT, Beal MF (2006) Mitochondrial dysfunction and oxidative stress in neurodegenerative diseases. Nature 443:787-95

Kuwano K, Hagimoto N, Kawasaki M, Yatomi T, Nakamura N, Nagata S, Suda T, Kunitake R, Maeyama T, Miyazaki H, Hara N (1999) Essential roles of the

Fas-Fas ligand pathway in the development of pulmonary fibrosis. J Clin Invest 104:13-19

Loh KP, Huang SH, De Silva R, Tan BK, Zhu YZ (2006) Oxidative stress: apoptosis in neuronal injury. Curr Alzheimer Res 3:327-37

Lu TH, Chen CH, Lee MJ, Ho TJ, Leung YM, Hung DZ, Yen CC, He TY, Chen YW (2010) Methylmercury chloride induces alveolar type II epithelial cell damage through an oxidative stress-related mitochondrial cell death pathway. Toxicol Lett 194:70-8

Marsden VS, Strasser A (2003) Control of apoptosis in the immune system: Bcl-2, BH3-only proteins and more. Annu Rev Immunol 21:71-105

Martindale JJ, Fernandez R, Thuerauf D, Whittaker R, Gude N, Sussman MA, Glembotski CC (2006) Endoplasmic reticulum stress gene induction and protection from ischemia/reperfusion injury in the hearts of transgenic mice with a tamoxifen-regulated form of ATF6. Circ Res 98:1186-93

Mathew L, Vale A, Adcock JE (2010) Arsenical peripheral neuropathy. Pract Neurol 10:34-8

Meliker JR, Wahl RL, Cameron LL, Nriagu JO (2007) Arsenic in drinking water and cerebrovascular disease, diabetes mellitus, and kidney disease in Michigan: a standardized mortality ratio analysis. Environ Health 6:4

Miloso M, Scuteri A, Foudah D, Tredici G (2008) MAPKs as mediators of cell fate determination: an approach to neurodegenerative diseases. Curr Med Chem 15:538-48

Min HY, Kim MS, Jang DS, Park EJ, Seo EK, Lee SK (2009) Suppression of lipopolysaccharide-stimulated inducible nitric oxide synthase (iNOS) expression by a novel humulene derivative in macrophage cells. Int Immunopharmacol 9:844-9

Nagaraja TN, Desiraju T (1994) Effects on operant learning and brain acetylcholine esterase activity in rats following chronic inorganic arsenic intake. Hum Exp Toxicol 13:353-356

Navas-Acien A, Sharrett AR, Silbergeld EK, Schwartz BS, Nachman KE, Burke TA, Guallar E (2005) Arsenic exposure and cardiovascular disease: a systematic review of the epidemiologic evidence. Am J Epidemiol 162:1037-49

Nordstrom DK (2002) Public health. Worldwide occurrences of arsenic in ground water. Science 296:2143-5

Prachasilchai W, Sonoda H, Yokota-Ikeda N, Oshikawa S, Aikawa C, Uchida K, Ito K, Kudo T, Imaizumi K, Ikeda M (2008) A protective role of unfolded protein response in mouse ischemic acute kidney injury. Eur J Pharmacol 592:138-45

Rahman M, Tondel M, Ahmad SA, Axelson O (1998) Diabetes mellitus associated with arsenic exposure in Bangladesh. Am J Epidemiol 148:198-203

Rios R, Zarazua S, Santoyo ME, Sepulveda-Saavedra J, Romero-Diaz V, Jimenez V, Perez-Severiano F, Vidal-Cantu G, Delgado JM, Jimenez-Capdeville ME (2009) Decreased nitric oxide markers and morphological changes in the brain of arsenic-exposed rats. Toxicology 261:68-75

Shi Y, Wei Y, Qu S, Wang Y, Li Y, Li R (2010) Arsenic Induces Apoptosis of Human Umbilical Vein Endothelial Cells Through Mitochondrial Pathways. Cardiovasc Toxicol 10:153-60

Siegel D, Gustafson DL, Dehn DL, Han JY, Boonchoong P, Berliner LJ, Ross D (2004) NAD(P)H:quinone oxidoreductase 1: role as a superoxide scavenger. Mol Pharmacol 65:1238-47

Sinczuk-Walczak H (2009) [Nervous system disorders induced by occupational exposure to arsenic and its inorganic compounds: a literature review]. Med Pr 60:519-22

Smith AH, Lingas EO, Rahman M (2000) Contamination of drinking-water by arsenic in Bangladesh: a public health emergency. Bull World Health Organ 78:1093-103

Soboloff J, Berger SA (2002) Sustained ER Ca2+ depletion suppresses protein synthesis and induces activation-enhanced cell death in mast cells. J Biol Chem 277:13812-20

Szegezdi E, Logue SE, Gorman AM, Samali A (2006) Mediators of endoplasmic reticulum stress-induced apoptosis. EMBO Rep 7:880-5

Tang CH, Chiu YC, Huang CF, Chen YW, Chen PC (2009) Arsenic induces cell apoptosis in cultured osteoblasts through endoplasmic reticulum stress. Toxicol Appl Pharmacol 241:173-81

Tsai SY, Chou HY, The HW, Chen CM, Chen CJ (2003) The effects of chronic arsenic exposure from drinking water on the neurobehavioral development in adolescence. Neurotoxicology 24:747-53

Wang Y, Xu Y, Wang H, Xue P, Li X, Li B, Zheng Q, Sun G (2009) Arsenic induces mitochondria-dependent apoptosis by reactive oxygen species generation rather than glutathione depletion in Chang human hepatocytes. Arch Toxicol 83:899-908

WHO Factsheet. Arsenic in drinking water. 2001. [Accessed: October 1, 2006]. Available from: http://www.who.int/mediacentre/factsheets/fs210/en/index.html

Yao N, Lan F, He RR, Kurihara H (2010) Protective effects of bilberry (Vaccinium myrtillus L.) extract against endotoxin-induced uveitis in mice. J Agric Food Chem 58:4731-6

Zhu H, Itoh K, Yamamoto M, Zweier JL, Li Y (2005) Role of Nrf2 signaling in regulation of antioxidants and phase 2 enzymes in cardiac fibroblasts: protection against reactive oxygen and nitrogen species-induced cell injury. FEBS Lett 579:3029-36

Zinszner H, Kuroda M, Wang X, Batchvarova N, Lightfoot, RT, Remotti H, Stevens JL, Ron D (1998) CHOP is implicated in programmed cell death in response to impaired function of the endoplasmic reticulum. Genes Dev 12:982-95

Figure Legends

Figure 1. Concentration of total arsenic in whole blood of mice treated with 0, 0.5, and 5 ppm iAs via the drinking water for 6 consecutive weeks. Blood arsenic levels of mice exposed to iAs were determined as described in Materials and Methods section. All data are expressed as mean ± S.E. (n = 12 for each group). *****P <0.05 as compared with vehicle control group.

Figure 2. Effects of iAs on lipid peroxidation (LPO)(A) and reduced glutathione (GSH) levels (B) in iAs-exposed mice. Mice were exposed to 0, 0.5, and 5 ppm iAs (via the drinking water) for 6 consecutive weeks in the presence or absence of NAC (150 mg/kg/day). (A) Malondialdehyde (MDA) levels of the plasma and cerebral cortex, and (B) reduced GSH levels of the cerebral cortex were determined using the commercial manufacturer's assay kit as described in Materials and Methods section. All data are expressed as mean ± S.E. (n = 12 for each group). *P < 0.05 as compared with vehicle control group. $^{\#}P$<0.05 as compared with iAs group alone.

Figure 3. Relative anti-oxidant gene expression in the cerebral cortex of mice exposed to iAs (0, 0.5, and 5 ppm As_2O_3, via the drinking water) for 6 consecutive weeks in the presence or absence of NAC (150 mg/kg/day). The expression of *NQO1* and *GPx* genes were determined by quantitative real-time PCR using SYBR Green. Target gene expression was normalized to β-actin, and the results are expressed as fold change from vehicle control. All data are expressed as mean ± S.E. (n = 12 for each group). *P <0.05 as compared with vehicle control group. $^{\#}P$<0.05 as compared with iAs group alone.

Figure 4. iAs-induced the apoptotic cells production and caspase-3 activation in the

cerebral cortex of mice. Mice were exposed to iAs (0, 0.5, and 5 ppm As_2O_3, via the drinking water) for 6 consecutive weeks in the presence or absence of NAC (150 mg/kg/day). (A) Apoptotic cell productions of the cerebral cortex were detected by TUNEL assay. (B) The cleaved form of caspase 3 protein expression of the cerebral cortex were analyzed by western blot, and the intensities of bands on western blots were quantified. Results shown are represented for three independent experiments. *****P<0.05 as compared with vehicle control group. $^{\#}P$ <0.05 as compared with iAs group alone.

Figure 5. iAs treatment regulated gene expression in the cerebral cortex of mice for apoptotic factors. Mice were exposed to iAs (0, 0.5, and 5 ppm As_2O_3, via the drinking water) for 6 consecutive weeks in the presence or absence of NAC (150 mg/kg/day) and the expression of pro-apoptotic (*Bax, Bak*)(A, *a*) and anti-apoptotic (M*cl-1*)(A, *b*), and ER-stress (*GRP78, GRP94, CHOP*)(B) genes in the cerebral cortex were analyzed by quantitative real-time PCR using SYBR Green. Target gene expression was normalized toβ-actin, and the results are expressed as fold change from vehicle control. Results are expressed as mean ± S.E. (n = 12 for each group). *P < 0.05 as compared with vehicle control group. $^{\#}P$<0.05 as compared with iAs group alone.

Figure 6. Analysis of the phosphorylation of p38 MAPK and ERK1/2 in the cerebral cortex of mice exposed to iAs. Mice were exposed to 0, 0.5, and 5 ppm iAs (via the drinking water) for 6 consecutive weeks in the presence or absence of NAC (150 mg/kg/day). The phosphorylation of p38 MAPK and ERK1/2 protein in the cerebral cortex were detected by western blot and quantified of protein expression. All data are represented for three independent experiments. *****P < 0.05 as compared with vehicle

control group. $^{\#}P<0.05$ as compared with MAPK group alone.

Figure. 1

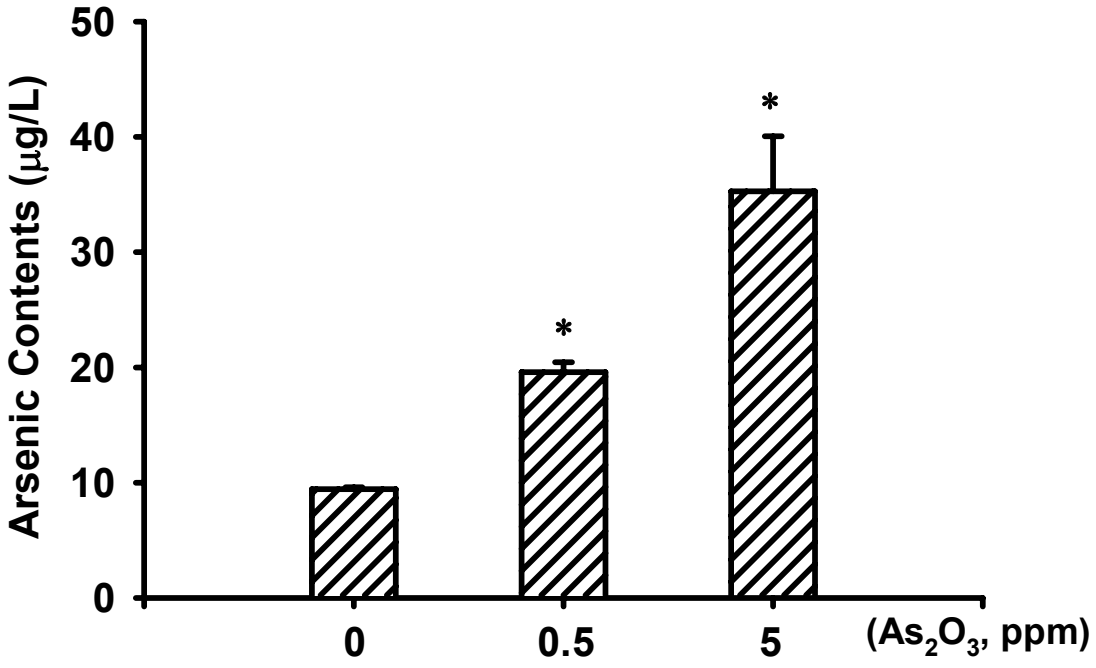


Figure. 2

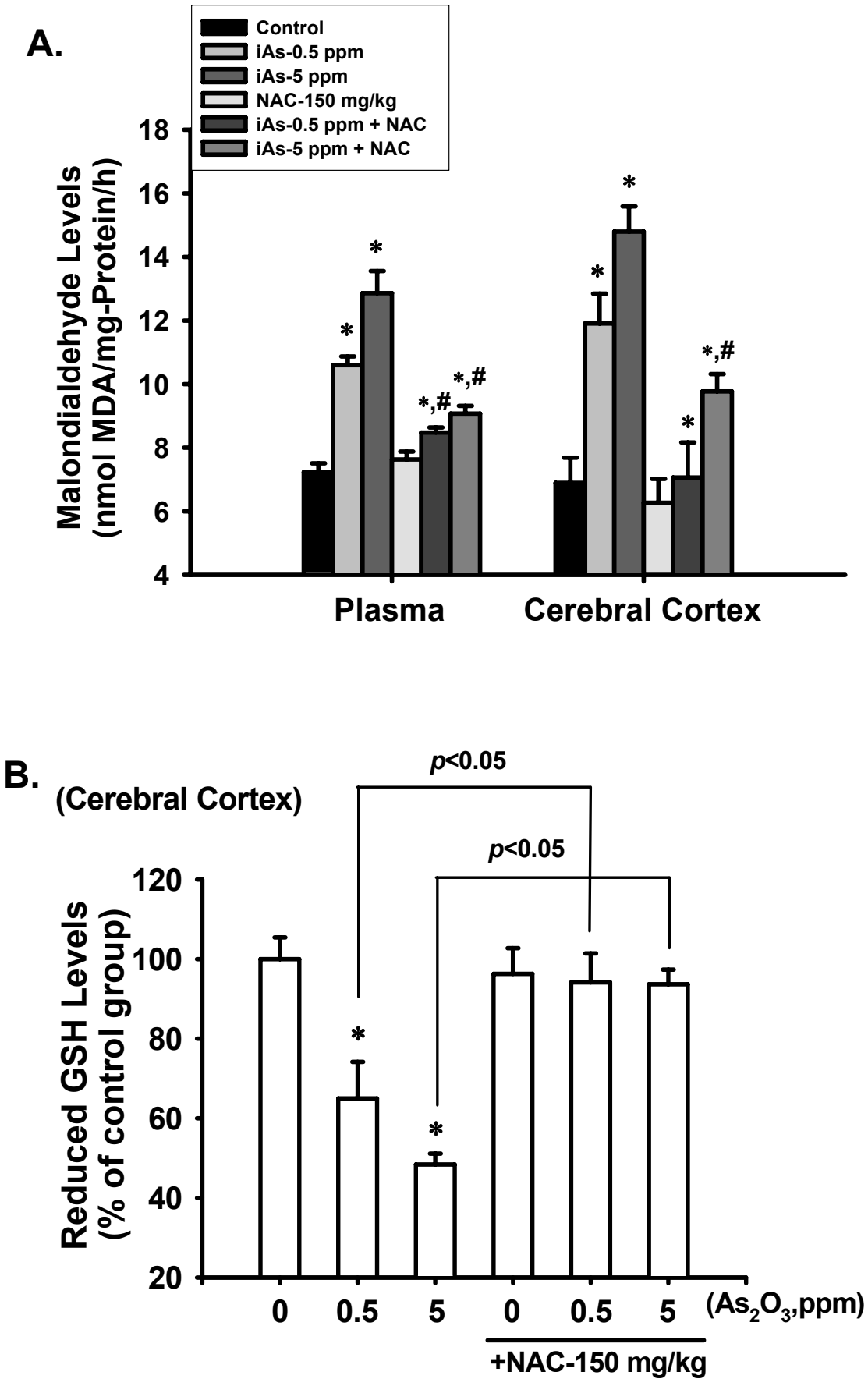

A.
Control
iAs-0.5 ppm
iAs-5 ppm
NAC-150 mg/kg
iAs-0.5 ppm + NAC
iAs-5 ppm + NAC
Malondialdehyde Levels
(nmol MDA/mg-Protein/h)
18
16
14
12
10
8
6
4
*
*,#
Plasma
Cerebral Cortex
B.
(Cerebral Cortex)
p<0.05
p<0.05
Reduced GSH Levels
(% of control group)
120
100
80
60
40
20
0
0.5
5
(As2O3,ppm)
+NAC-150 mg/kg

Figure. 3

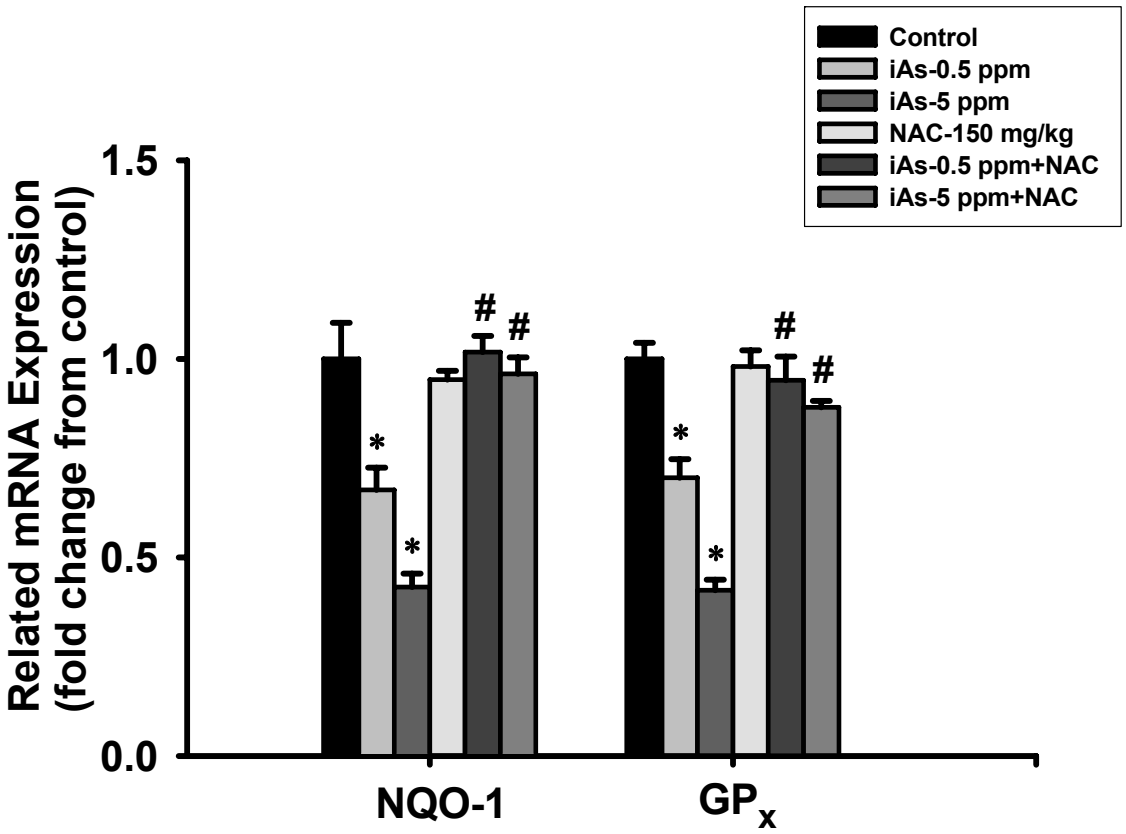

Control
iAs-0.5 ppm
iAs-5 ppm
NAC-150 mg/kg
iAs-0.5 ppm+NAC
iAs-5 ppm+NAC
Related mRNA Expression
(fold change from control)
1.5
1.0
0.5
0.0
*
*
#
#
*
*
#
#
NQO-1
GPx

Figure 4

A.

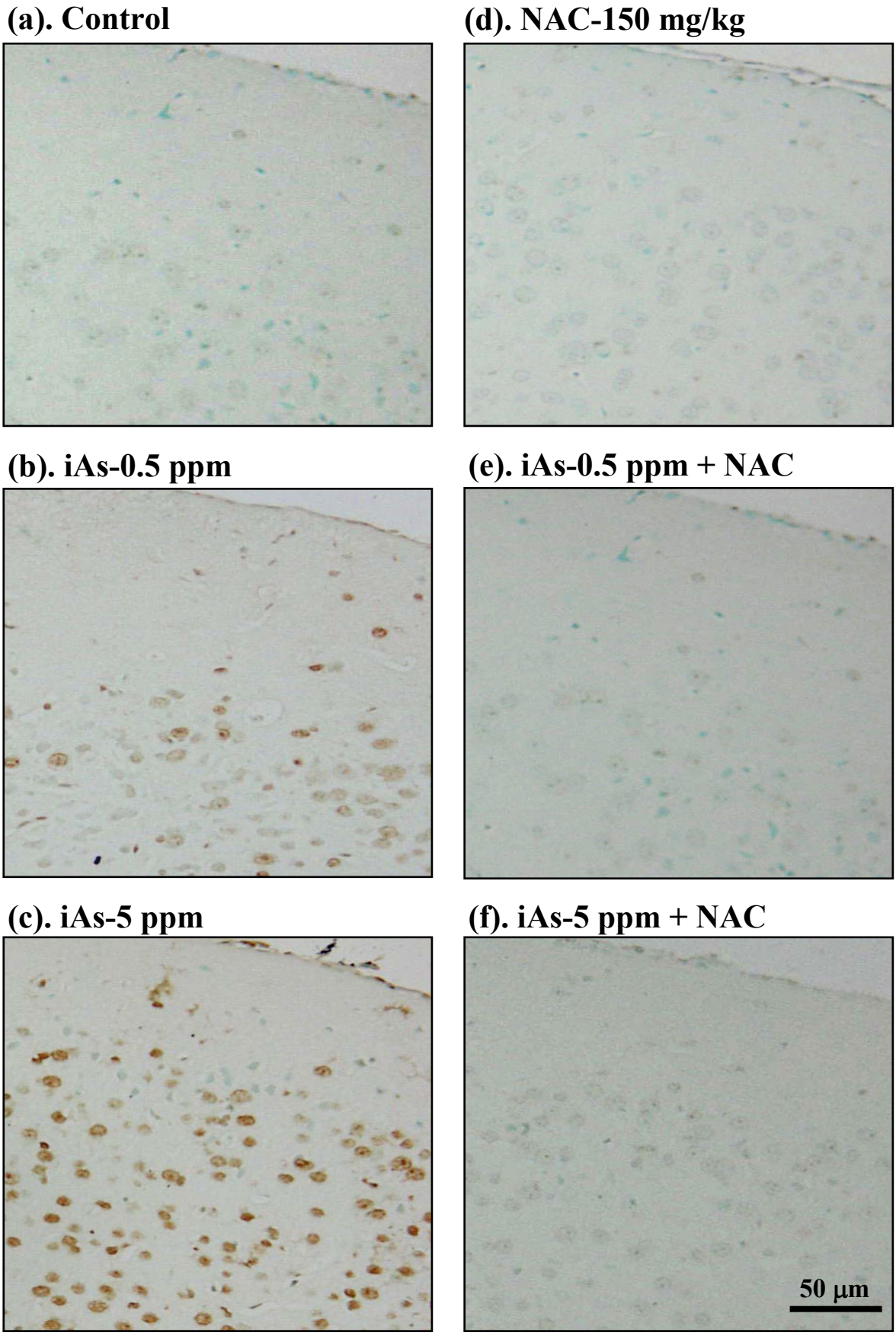

(a). Control
(d). NAC-150 mg/kg
(b). iAs-0.5 ppm
(e). iAs-0.5 ppm + NAC
(c). iAs-5 ppm
(f). iAs-5 ppm + NAC
50 μm

Figure. 4

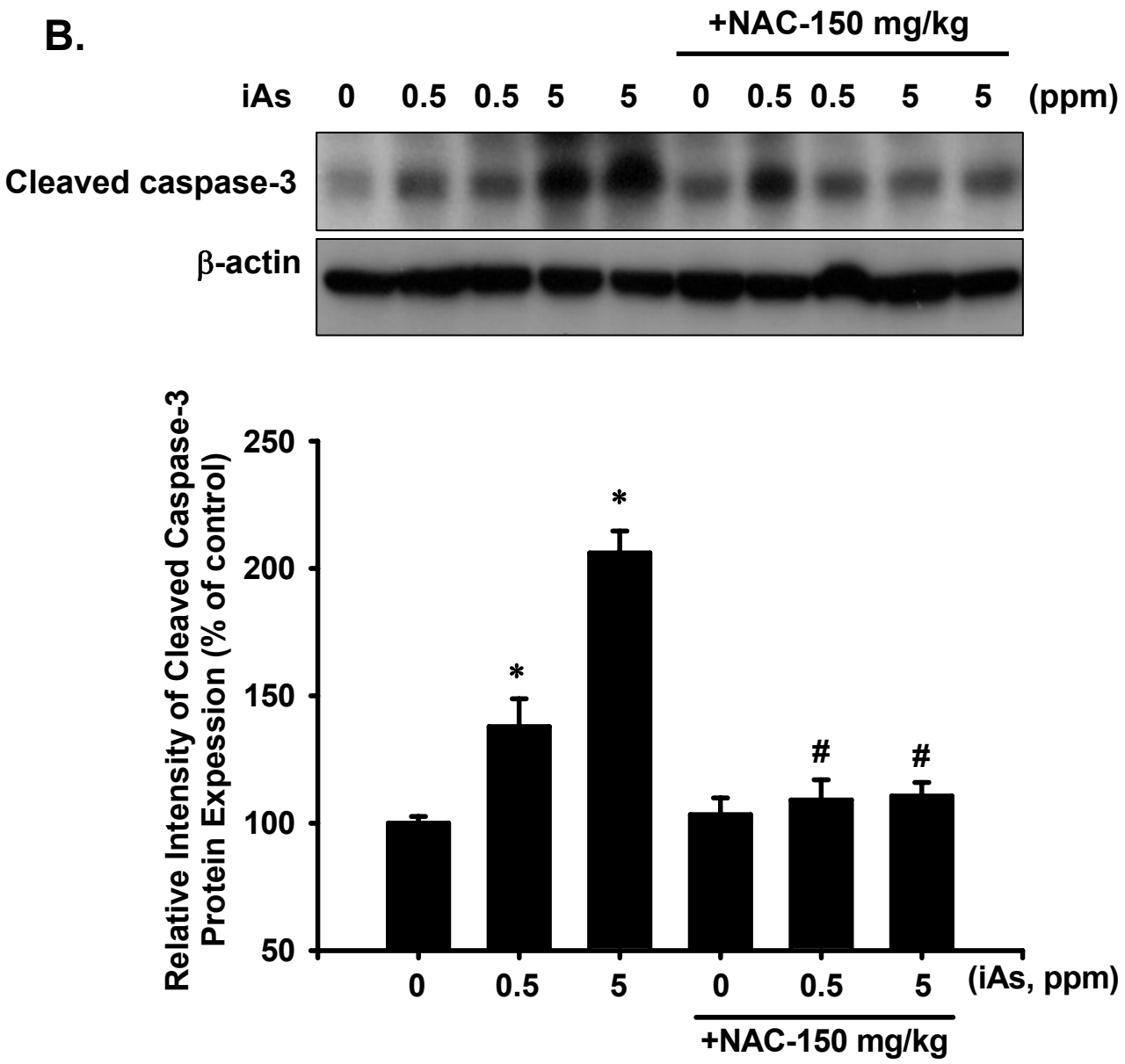

B.
+NAC-150 mg/kg
iAs 0 0.5 0.5 5 5 0 0.5 0.5 5 5 (ppm)
Cleaved caspase-3
β-actin
Relative Intensity of Cleaved Caspase-3 Protein Expession (% of control)
250
200
150
100
50
*
*
#
#
0 0.5 5 0 0.5 5 (iAs, ppm)
+NAC-150 mg/kg

Figure 5

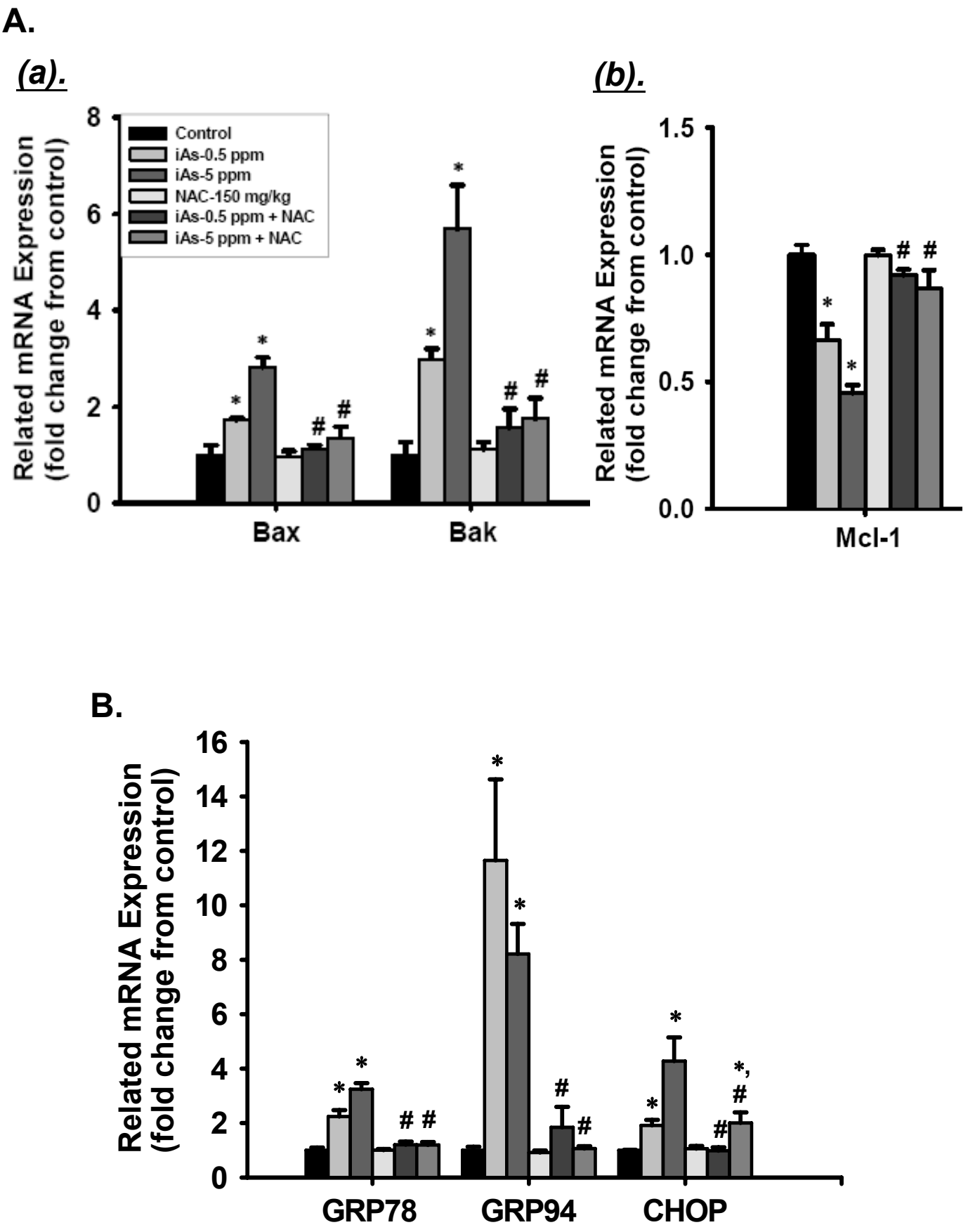
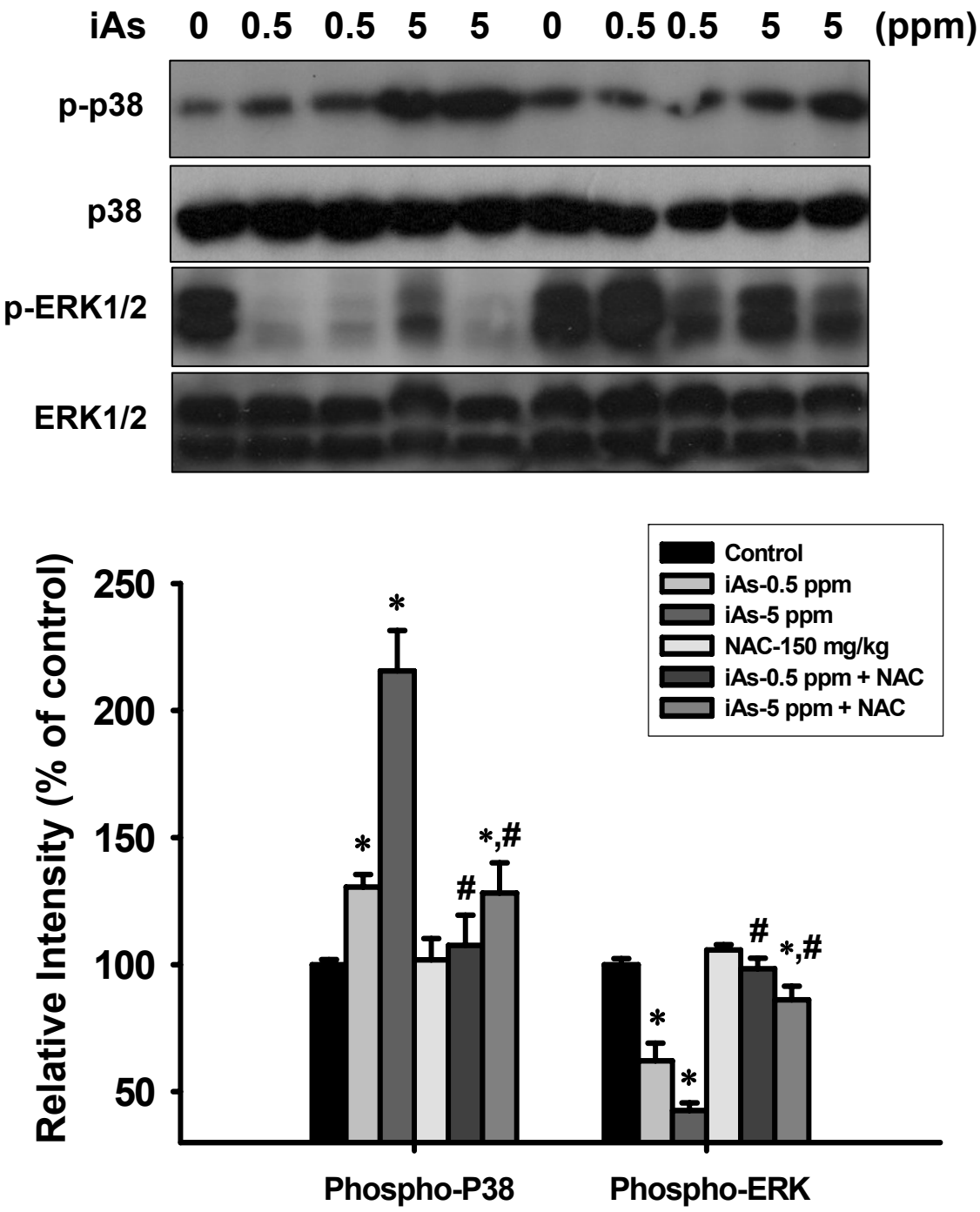

A.
(a).
(b).
Related mRNA Expression (fold change from control)
Control
iAs-0.5 ppm
iAs-5 ppm
NAC-150 mg/kg
iAs-0.5 ppm + NAC
iAs-5 ppm + NAC
Bax
Bak
Mcl-1
B.
GRP78
GRP94
CHOP

Figure. 6


iAs 0 0.5 0.5 5 5 0 0.5 0.5 5 5 (ppm)
p-p38
p38
p-ERK1/2
ERK1/2
Control
iAs-0.5 ppm
iAs-5 ppm
NAC-150 mg/kg
iAs-0.5 ppm + NAC
iAs-5 ppm + NAC
Relative Intensity (% of control)
250
200
150
100
50
*
*
*,#
#
*
*
#
*,#
Phospho-P38
Phospho-ERK