Original Paper

Neuroglobin Plays a Protective Role in Arsenite-Induced Cytotoxicity by Inhibition of Cdc42 and Rac1GTPases in Rat Cerebellar Granule Neurons

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Key Words

Sodium Arsenite • Neuroglobin • Rat Cerebellar Granule Neurons • Cytotoxicity • Rac1 GTPase • Cdc42 GTPase

Abstract

Background and Aims: We have previously shown that neuroglobin (Ngb) expression can be regulated by sodium arsenite (NaAsO_2) exposure in rat cerebellar granule neurons (CGNs). However, the precise molecular mechanisms of Ngb action are largely unknown. Ras homolog (Rho) guanosine triphosphatases (Rho GTPases) are involved in the regulation of a number of cellular processes, including cell cytotoxicity. It has been reported that Ngb can act as a guanine nucleotide dissociation inhibitor (GDI) role to inactivate Rho GTPases. Therefore, we investigated Rho GTPases activation induced by NaAsO_2 exposure in rat CGNs and effects of Rho GTPases activation on the cells. We also investigated the role of Ngb in this process.

Methods: Primary cultures of CGNs were prepared from 7-day-old Wistar rat pups. The cytotoxic effects of NaAsO_2 on CGNs were evaluated using the Cell Counting Kit-8 assay and TUNEL staining. RNA interference technology was used to silence Ngb, and the subsequent effects were evaluated by quantitative RT-PCR and Western blot. Cdc42 and Rac1 activation were measured by pull-down assay and Western blot. **Results:** NaAsO_2 induced cytotoxicity in rat CGNs, increased GTP-bound form of Cdc42 and Rac1 GTPases in the cells. Furthermore, inhibition of Cdc42 or Rac1 activity using the inhibitor ZCL278 or NSC23766 decreased apoptosis and increased cell viability in the cells exposed to NaAsO_2 . Using siRNA-mediated knockdown, we show that NaAsO_2 -induced cytotoxicity was exacerbated, activation of Cdc42 (GTP-Cdc42) and Rac1 (GTP-Rac1) was increased in Ngb RNA silencing cells. **Conclusions:** cytotoxic effects of NaAsO_2 on rat CGNs is induced at least partly by Cdc42 and Rac1 activation, and Ngb can inhibit Cdc42 and Rac1 activation to play protective role in rat CGNs exposed to NaAsO_2 .

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Introduction

Arsenic (As) is a widespread toxicant found in water, food, and air. Chronic As toxicity is a growing public health problem that affects more than 30 million people worldwide, and China is one of the most severely affected countries. Inorganic As, the predominant form identified in drinking water, is more toxic than the organic forms. Arsenite [As(III)], the trivalent state of inorganic As, is able to bind to macromolecular thiols and is more toxic than the pentavalent form (arsenate, As V) [1, 2]. Long-term health effects of inorganic As in drinking water may result in neurological abnormalities [3]. Nervous system damage due to As exposure has been widely studied and several studies have shown that people consuming water contaminated with high-level As particularly experience cerebellar symptoms [4-6]. Our previous study has demonstrated that rat cerebellum is one of the critical target organs of inorganic arsenite exposure [7, 8]. The involved mechanism research of As toxic effects on cerebellum is of importance.

It has been widely reported that arsenite results in cytotoxicity and mitogen-activated protein kinases (MAPKs) activation [9, 10]. Cdc42 and Rac1, the most extensively studied members of Ras homolog (Rho) guanosine triphosphatases (GTPases), are upstream signals of MAPKs [11, 12]. In mammals, the Rho GTPase family consists of 5 subfamilies, Rho, Rac, Cdc42, Rnd, and Rho BTB, based on their sequence identity, domain structure, and function [13]. Each of Rho GTPases is an important regulator of many biological processes including cytoskeletal reorganization, membrane trafficking, cell cycle progression, and transcription [14-16]. As a molecular switch, Rho family GTPases alternate between an inactive GDP-bound and an active GTP-bound state [17, 18]. The activation status of Rho family GTPases is supported by three regulatory proteins: Guanine nucleotide exchange factors (GEFs), GTPase activation proteins (GAPs), and guanine nucleotide dissociation inhibitors (GDIs). GEFs facilitate the exchange of GDP for GTP, whereas GAPs promote the hydrolysis of GTP to GDP. GDI proteins exert additional negative regulation and thus keep Rho GTPases from being activated [19]. It has been reported that activated Rho-like GTPase Cdc42 can induce apoptosis of neurons, whereas expression of dominant negative mutants of Cdc42 blocked apoptosis [20]. Also, one study has shown that injured axon can regrow on complex inhibitor substrates when Rho GTPases are inactivated [21]. All mentioned above support the fact that inhibition of Rho GTPases can play a protective role in these biological processes.

Neuroglobin (Ngb), discovered in 2000, is widely expressed in vertebrate's central and peripheral nervous systems [22]. Accumulating evidence has clearly demonstrated that Ngb is an endogenous molecule that exhibits neuroprotective role against hypoxic/ischemic injuries *in vivo* and *in vitro* [23-25]. Furthermore, several reports have shown that Ngb can act as a signal transducer and exert GDI role inhibiting the rate of exchange of GDP for GTP, thus playing against neuronal injuries [26-28]. We have previously demonstrated neuroglobin (Ngb) expression can be regulated by NaAsO₂ exposure in rat cerebellar granule neurons (CGNs) [29]. We speculate Ngb may be an important regulator in the toxic process of NaAsO₂ exposure.

In this study, we set out to determine the specific role of Ngb in the NaAsO₂-induced neurotoxicity and Rho GTPases activation in rat CGNs. Our data suggest Ngb can inhibit Rho-GTPases activation to play protective role in rat CGNs exposed to NaAsO₂.

Materials and Methods

Chemicals and Reagents

NeurobasalA and B27 supplement were purchased from Gibco (Grand Island, NY, USA). Fetal bovine serum (FBS) and DMEM/F12 were from Hyclone Laboratories (Logan, UT, USA). Poly-L-lysine hydrobromide, Cytosine arabinoside and DNase I were purchased from Sigma-Aldrich (St. Louis, MO, USA). Hanks' Balanced Salt Solution (without CaCl₂, MgCl₂ or MgSO₄) and L-glutamine were purchased from Solarbio Science &

Technology (Shanghai, China). Cell Counting Kit-8 and BCA protein assay kit were from Beyotime Institute of Biotechnology (Shanghai, China). Neuroglobin primary antibody was from abcam (Cambridge, UK). Secondary antibody was from Rockland Immunochemicals Inc. (Gilbertsville, PA, USA). Complete Inhibitor Cocktail and *In Situ* cell death detection kit were purchased from Roche (Indianapolis, IN, USA). Rac1 inhibitor NSC23766 was purchased from Santa Cruz Biotechnology Inc. (CA, USA). Cdc42 inhibitor ZCL278 was purchased from Selleckchem (Houston, USA). Cdc42 Activation Assay Biochem Kit and Rac1 Activation Assay Biochem Kit were purchased from Cytoskeleton, Inc. (Denver, CO, USA). Lipofectamine RNAiMAX, Ngb siRNA and Opti-MEM were purchased from Life Technologies (Carlsbad, Cal, USA). Trizol reagent and PrimeScript RT reagent Kit was purchased from Takara Bio Inc. (Dalian, China).

Primary Culture of Rat CGNs

Primary cultures of rat cerebellar granule neurons (CGNs) were prepared from 7-day-old Wistar rat pups (provided by Animal Experimental Center of the Second Affiliated Hospital of Harbin Medical University, China), as described elsewhere [30]. Briefly, cerebella were dissected, meninges were removed, and tissues were digested with 0.5% trypsin at 37°C for 10 min and treated with 50 mg/L DNase I at room temperature for 3 min. Cells were dissociated by repeated pipetting, and separated from nondissociated tissue by sedimentation. Cell density was adjusted to 2×10^6 cells ml^{-1} and cells were plated on poly-L-lysine-coated plates at a density of 4×10^5 cells cm^{-2} . Cultures were grown in Neurobasal A containing 2% B27 NeuroMIX supplement, 2 mM L-glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin. Cytosine arabinoside (5 μM) was added in the media 16–18 h after plating to inhibit the growth of non-neuronal cells.

siRNA Transfection

The Ngb specific siRNA (Ambion, Life Technologies) was transfected with Lipofectamine RNAiMAX (Life Technologies) in six-well plates. The target sequence against Ngb was sense 5'-GGACCACAUUAGGAAGGUUtt-3' and anti-sense 5'-AACCUUCCUAAUGUGGUCCag-3' (NCBI UniGene ID: Rn.64645). The negative control siRNA was used as non-targeting control for all siRNA experiment. Briefly, Lipofectamine RNAiMAX diluted in Opti-MEM (9 μL /150 μL) was incubated for 5 min. Control siRNA and Ngb siRNA (30 pmol) were diluted in Opti-MEM (150 μL) and added to the Lipofectamine RNAiMAX mixture. The incubation was continued for an additional 20 min before addition to cultures and transfection. Following 48 h transfection, the rat CGNs were treated with NaAsO_2 (0 μM , 10 μM) for 45 min or 24 h and subject to western blotting, Quantitative Real-Time PCR and TUNEL assay.

TUNEL Assay

We performed the TUNEL assay using an *In Situ* cell death detection kit (Roche) following the instructions. Briefly, rat CGNs exposed to NaAsO_2 (0 μM , 10 μM) were grown in 96-well plates for 24 h with or without 20 μM ZCL278 (an inhibitor of Cdc42) pretreatment for 1 h, 50 μM NSC23766 (an inhibitor of Rac1) pretreatment for 12 h, Ngb-siRNA transfection for 48 h. The cells were washed with PBS for 3 times, fixed with 4% paraformaldehyde and permeabilized in a solution of 0.1% Triton X-100 and 0.1% sodium citrate at a room temperature. Cells were incubated with 50 μL TUNEL reaction mixture. Nuclei of both normal and apoptotic cells were stained with 100 nM 4', 6-diamidino-2-phenylindole dihydrochloride (DAPI). TUNEL positive cells were observed using fluorescence microscope (C1-plus, Nikon, Japan). Imagepro plus6.0.0.260 software was used to quantify. The percentage of apoptotic cells was determined in five randomly chosen fields.

Cell Viability Assay

Viability of rat CGNs was assessed by a CCK-8 [31]. Cells were seeded in 96-well plates at a density of 4×10^5 cells cm^{-2} . The plates were pre-incubated for 24 h, followed by the treatments of NaAsO_2 (0 μM , 10 μM) for 24 h with varying concentrations of ZCL278 or NSC23766 (0, 10, 20, 50 and 100 μM) for 1 h or 12 h, or with 20 μM ZCL278 (an inhibitor of Cdc42) pretreatment for 1 h, 50 μM NSC23766 (an inhibitor of Rac1) pretreatment for 12 h, Ngb-siRNA transfection for 48 h. Then, 10 μL CCK-8 solution was added to each well of the plates and cells were incubated for 4 h in the incubator (37°C and 5% CO_2). The optical density values at a wavelength of 450 nm were measured on an imaging reader (Cytation3, BioTek, USA). Viability results were expressed as: Cell viability = $(A_{\text{experimental}} - A_{\text{blank}}) / (A_{\text{control}} - A_{\text{blank}}) \times 100\%$. The absorbance measured from non-treated cells was taken to be 100%.

Quantitative Real-Time PCR

Total RNA was extracted from primary-cultured rat CGNs with Trizol Reagent. Reverse-transcription (37°C for 15 min and 85°C for 30 s) and the qRT-PCR were performed by using PrimeScript RT reagent Kit according to the manufacturer's instructions. The qRT-PCR for Ngb and the housekeeping gene β -actin was performed in a total volume of 20 μ L in each well using 1 μ L cDNA. Primers were the same as those reported before [32]. The forward primer for rat Ngb was 5'-CCTGGACCACATTAGGAAGG-3' and reverse 5'-TCACCTACTGTCGAGAAGGA-3' (GenBank accession number is NM_033359). The amplification fragment length of Ngb was 150 bp; forward primer for rat β -actin was 5'-TGACGGTCAGGTCATCACTATC-3' and reverse 5'-GGCATAGAGGTCTTTACGGATG-3' (Genbank accession number is NM_031144). The amplification fragment length of β -actin was 247 bp. The PCR condition consisted of initial denaturation step of 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s, 56 °C for 15 s and 72 °C for 15 s. Extension was at 72 °C for 60 s. Specificity of amplified products was monitored by performing melting curves at the end of each amplification and finally a cooling step to 30 °C. Melting curves were acquired by stepwise increase of the temperature from 55 °C to 95 °C. All the amplicons generated a single peak, thus reflecting the specificity of the primers. Experiments were performed on Chromo4 real time PCR detector (Bio-Rad, America). Results were calculated using the Delta Ct method normalizing to β -actin expression for each sample [33, 34].

Western Blot Analysis of Neuroglobin Expression

Primary cultured cells were washed with cold phosphate-buffered saline (PBS) and extracted in a lysis buffer including RIPA buffer and Complete Mini. The protein concentrations were determined by an enhanced BCA Protein Assay Kit. An equal amount of protein for each sample in loading buffer was heated at 100°C for 5 min and loaded onto 12% polyacrylamide gels. After electrophoresis, proteins were transferred onto polyvinylidene difluoride membranes. Subsequently, the membrane was blocked by incubating in TBST plus 5% fat-free milk at room temperature for 2 h and then was probed with Ngb primary antibody (Mouse monoclonal mAb, 1:1000) overnight at 4 °C. After incubation for 1 h with the secondary antibody (Anti-Mouse IgG, 1:5000), signals were detected by Odyssey Infrared Imaging System (Li-COR, USA). Data are expressed as normalized ratios to GAPDH.

Pull-Down Assay

Cdc42 and Rac1 were measured using a Cdc42 and Rac1 pull-down kit according to the manufacturer's protocols, respectively [35, 36]. Briefly, rat CGNs cultures were starved with serum-free medium for 24 hours, then exposed to NaAsO₂ before an ice-cold PBS rinse and lysis in 500 μ L of the lysis buffer including Complete Mini. Cell lysates were clarified by centrifugation at 10,000×g for 15 min. The supernatant was collected; equal volumes of supernatant were incubated with PAK-PBD affinity beads for 1 hour at 4 °C, followed by two washes in the supplied wash buffer. Bound proteins were boiled in 5×1% SDS sample buffer at 95°C for 5 minutes and detected by 12% polyacrylamide gels and Western blot with anti-Cdc42 (Mouse monoclonal mAb, 1:250) and Rac1 (Mouse monoclonal mAb, 1:500) analysis. Aliquots of total lysate were also analyzed for the amount of Cdc42 and Rac1 present, respectively. Relative Cdc42 and Rac1 activity is the ratio of active Cdc42 and Rac1 divided by total Cdc42 and Rac1 normalized to the untreated control, respectively.

Statistical Analysis

All experiments were repeated for at least 3 times independently. Results were presented as mean $\pm$ standard deviation and evaluated with one-way-ANOVA followed by Least Significant Difference test. All statistical evaluations were carried out using the SPSS17.0 software package. *P* values less than 0.05 were considered statistically significant.

Results

Induction of Neurotoxicity in Rat CGNs by Treatment with NaAsO₂

We evaluated the neurotoxic effects, including apoptosis and cell viability, of NaAsO₂ on rat CGNs using TUNEL and CCK-8 assays. Apoptosis was detected by TUNEL assay. TUNEL-positive rat CGNs exposed to NaAsO₂ were rounder and smaller than the healthy

Fig. 1. Detection of apoptotic rat CGNs by TUNEL assay. A. Representative fluorescence images of cells exposed to 0 and 10 μM NaAsO_2 for 24 h. After treatment of NaAsO_2 , specimens were labeled with TUNEL (green). Nuclei were stained blue with DAPI. Original Magnifications: $\times 400$. B. The graph indicates that TUNEL-positive rat CGNs exposed to NaAsO_2 (10 μM) increased significantly compared with those of normal cells. Data are expressed as mean $\pm$ SD from at least 5 visual fields. C. Rat CGNs in 96-well plates were treated with NaAsO_2 (0 μM and 10 μM) for 24h, and cell viability was determined using the CCK-8 assay (10 μL /well CCK-8 solution for 4 h). NaAsO_2 decreased the viability of CGNs. All statistical results are expressed as means $\pm$ SD from at least three independent experiments. *** $P < 0.001$ VS control group.

cells (Fig. 1A). Both rounding-up of the cell and reduction of the cell volume are displayed, typical features of cells undergoing apoptosis [37]. In this case, after treated with 0 and 10 μM NaAsO_2 for 24 h, percentages of TUNEL-positive rat CGNs were $20.61 \pm 6.35\%$ and $67.77 \pm 5.37\%$, respectively. Apoptotic cells increased significantly in 10 μM exposure groups compared with the control groups ($P < 0.001$; Fig. 1B). Furthermore, as shown in Fig. 1C, the CCK-8 assay revealed significantly decreased cell viability in NaAsO_2 exposure groups compared with the control group ($p < 0.001$; Fig. 1C). This means that arsenite induced neurotoxicity in rat CGNs.

Activation of Cdc42 and Rac1 GTPases by NaAsO_2 Exposure in Rat CGNs

As there is a growing body of evidence that inorganic As can activate the Rho family of GTPases [9, 38, 39], we therefore wished to study whether NaAsO_2 regulates the activation states of major members of the Rho family, namely Cdc42 and Rac1 in rat CGNs. Rat CGNs were treated with NaAsO_2 at a concentration of 10 μM for 0, 30, 60, 120, 240 min and the activation of Cdc42 and Rac1 was assessed by pull-down assay and western blot with anti-Cdc42 and anti-Rac1 antibody analysis. Results (Fig. 2) show that Cdc42 and Rac1 were activated significantly by NaAsO_2 exposure. Relative Cdc42 and Rac1 activity is the ratio of active Cdc42 and Rac1 divided by total Cdc42 and Rac1 normalized to the untreated control, respectively. Ratios of active Cdc42 in the five groups are 1.00 ± 0.14 , 2.11 ± 0.21 , 1.49 ± 0.26 , 1.23 ± 0.25 , 1.03 ± 0.14 , respectively (Fig. 2A). Significant increase occurred in 30 and 60 min NaAsO_2 treatment groups compared with the control group ($p < 0.01$ in 60 min group, $p < 0.001$ in 30 min group). Ratios of active Rac1 in the five groups are 1.00 ± 0.26 , 2.49 ± 0.58 , 2.03 ± 0.70 , 2.05 ± 0.18 , 1.45 ± 0.29 , respectively (Fig. 2B). active Rac1 ratio in the 30, 60 and 120 min

Fig. 2. Treatment with NaAsO₂ increased levels of activated Cdc42 and Rac1 in rat CGNs. Representative images of western blots and corresponding densitometric quantification of the level of activated (A) Cdc42 and (B) Rac1 in rat CGNs. Relative Cdc42 and Rac1 activity is the ratio of active Cdc42 and Rac1 divided by total Cdc42 and Rac1 normalized to the untreated control, respectively. All statistical results are expressed as means±SD from three independent experiments. ** $P < 0.01$, *** $P < 0.001$ VS control group.

Fig. 3. NSC23766 or ZCL278 ameliorated NaAsO₂-induced inviability of rat CGNs in a dose-dependent manner. Rat CGNs in 96-well plates were pretreated with different concentrations of NSC23766 or ZCL278 for 12 h or 1 h, followed by treatment with NaAsO₂ for 24 h. Cell viability was expressed as a percentage of untreated cells. All statistical results are expressed as means±SD from three independent experiments. * $P < 0.05$ as compared with control group alone, # $P < 0.05$ as compared with NaAsO₂ treatment group alone.

NaAsO₂ treatment groups increased significantly compared with the control group ($p < 0.01$ in 60 and 120 min groups, $p < 0.001$ in 30 min group). This indicates NaAsO₂ increased the levels of the active GTP-bound form of Cdc42 and Rac1 in rat CGNs.

Fig. 4. Rho GTPases were involved in NaAsO₂ induced apoptosis in rat CGNs. **A.** Representative fluorescence images of cells exposed to 0 and 10 μ M NaAsO₂ for 24 h with or without ZCL278 (an inhibitor of Cdc42), or a Rac1 inhibitor NSC23766. Both ZCL278 and NSC23766 reduced the apoptotic cells. **B.** The graph indicates TUNEL-positive rat CGNs exposed to NaAsO₂ (10 μ M) with ZCL278 or NSC23766 groups decreased significantly compared with those of NaAsO₂ treatment group. Data are expressed as mean $\pm$ SD from at least 5 visual fields. **C.** Rat CGNs in 96-well plates were treated by 0 and 10 μ M NaAsO₂ for 24h with or without ZCL278 (an inhibitor of Cdc42) pretreatment for 1 h, or a Rac1 inhibitor NSC23766 pretreatment for 12 h, and assessed by a CCK-8. ZCL278 and NSC23766 increased rat CGNs viability exposed to NaAsO₂. All statistical results are expressed as means $\pm$ SD from at least three independent experiments. *** P <0.001 vs control, # P <0.05, ## P <0.01, ### P <0.001 VS NaAsO₂ treatment group.

Effects of different concentrations of ZCL278 or NSC23766 on viability of rat CGNs exposed to NaAsO₂

In this report, we examined the effect of NSC23766 or ZCL278 on the viability of rat CGNs exposed by NaAsO₂. Concentration-dependent viability was clearly observed when rat CGNs were incubated with increasing concentrations of NSC23766 or ZCL278 in NaAsO₂ treatment groups (Fig. 3) in this study. The viability of NaAsO₂ exposure rat CGNs increased significantly with 20, 50 and 100 μ M ZCL278 pretreatment for 1 h (p <0.05), when compared with NaAsO₂ treatment group alone. NSC23766, similar to ZCL278, also increased the viability of the cells exposed to NaAsO₂ significantly in dose-dependent manner. Based on the above results, 20 μ M ZCL278 and 50 μ M NSC23766 were selected for further study.

Amelioration of NaAsO₂-Induced Cytotoxicity by Inhibition of Cdc42 or Rac1 GTPases in Rat CGNs

To understand whether Rho GTPases are involved in NaAsO₂-induced cytotoxicity, the effects of Cdc42 inhibitor ZCL278 or the Rac1 inhibitor NSC23766 on NaAsO₂-induced apoptosis and cell viability in rat CGNs were examined (Fig. 4). As shown in Fig. 4A and quantified in Fig. 4B, Cdc42 or Rac1 inhibition resulted in a significant decrease (56.91 $\pm$ 3.86%, 53.24 $\pm$ 8.17%) of apoptotic cells in comparison with NaAsO₂ exposure group (67.77 $\pm$ 5.37%). Furthermore, inhibition of Cdc42 or Rac1 ameliorated NaAsO₂-induced inviability of rat

Fig. 5. Knock-down of Ngb in rat CGNs. A. Ngb-siRNA and Negative-siRNA were transfected into rat CGNs. A representative qRT-PCR result shows that the expression level of Ngb mRNA decreased in Ngb-siRNA transfected rat CGNs compared with Control group. B. A representative Western blot analysis shows decreased expression of Ngb protein in Ngb-siRNA cells. All statistical results are expressed as means $\pm$ SD from three independent experiments. * $P < 0.05$, *** $P < 0.001$ VS control group.

CGNs ($p < 0.05$, $p < 0.01$ VS NaAsO₂ group; Fig. 4C). These results indicate that NaAsO₂ might induce neuron neurotoxicity by activating Cdc42 or Rac1.

Levels of Ngb mRNA and Protein expression by Treatment with Ngb-siRNA

An effective way of RNA interference, siRNA transfection, has been used for gene silencing and gene therapy applications [40]. Here, we used a siRNA system for knockdown of Ngb in rat CGNs. As shown in Fig. 5A, Ngb siRNA transfection resulted in significant decrease of Ngb mRNA levels in comparison with the untreated control ($p < 0.001$), negative siRNA ($p < 0.001$) and transfection reagent group ($p < 0.001$). Ngb protein levels in siRNA-transfected rat CGNs were lower than in control-transfected cells by western blot analysis (Fig. 5B). These results confirmed that the Ngb siRNA is specific and efficient, and Ngb mRNA and protein expressions were both remarkably knocked down in rat CGNs after siRNA transfection.

Effect of Ngb siRNA on Arsenite-induced Neurotoxicity

To investigate whether silencing Ngb expression could affect the vulnerability caused by NaAsO₂, rat CGNs were transfected with or without Ngb siRNA for 48 h, in the presence of NaAsO₂ (10 μ M group) or same volume of PBS (Control group). Here, we evaluated the effect of Ngb siRNA on Arsenite-induced neurotoxicity in the cells using TUNEL and CCK-8 assays. Results showed apoptosis was obviously induced by NaAsO₂ treatment in rat CGNs. Apoptotic cells were 20.61 $\pm$ 6.35%, 67.77 $\pm$ 5.37%, 24.75 $\pm$ 4.80% and 80.67 $\pm$ 5.39%, respectively in the four groups (control, NaAsO₂ treatment, Ngb siRNA, Ngb siRNA with NaAsO₂ treatment group). Apoptotic cells increased significantly in Ngb siRNA-NaAsO₂ ($p < 0.001$) and NaAsO₂ ($p < 0.001$) exposure groups compared with the control group (Fig. 6A & 6B). Meanwhile, Ngb siRNA transfection enhanced arsenite-induced apoptosis ($p < 0.01$ VS NaAsO₂ group; Fig. 6B).

Fig. 6. Silencing of Ngf enhances cell death following NaAsO₂ treatment. A. Ngf-siRNA and normal cells were exposed to NaAsO₂ (0 and 10 μ M) for 24 h. After treatment, specimens were labeled with TUNEL (green) and nuclei were stained blue with DAPI (fluorescence microscopy, original magnification $\times$ 400). B. The graph indicates TUNEL-positive Ngf-siRNA transfected rat CGNs exposed to NaAsO₂ (10 μ M) increased significantly compared with those of normal cells. Ngf siRNA transfection enhanced arsenite-induced apoptosis compared with NaAsO₂ treatment group. Data are expressed as mean $\pm$ SD from at least 5 visual fields. C. Cell viability was measured using CCK-8 assay. Ngf siRNA transfection worsened arsenite-decreased viability of CGNs. *** $P < 0.001$ VS control, # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$ VS NaAsO₂ treatment group.

Fig. 7. Levels of activated Cdc42 and Rac1 in Ngf-siRNA transfected rat cerebellar granule neurons (CGNs). Representative images of western blot and corresponding densitometric quantification of the level of activated (A) Cdc42 and (B) Rac1 in Ngf-siRNA transfected rat CGNs. Ngf knockdown cells demonstrate higher levels of both activated Cdc42 and Rac1 than control. All statistical results are expressed as means $\pm$ SD from three independent experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ VS control group, # $P < 0.05$ VS NaAsO₂ treatment group.

Furthermore, transfection with Ngf siRNA potentiated arsenite-induced neuronal inviability ($p < 0.05$ VS NaAsO₂ group; Fig. 6C). These data suggest that Ngf plays a neuroprotective role in the NaAsO₂-induced neurotoxicity in primary cultured rat cerebellar granule neurons.

Fig. 8. Effects of ZCL278 and NSC23766 on viability of Ngb-siRNA transfected rat CGNs exposed to NaAsO₂. ZCL278 and NSC23766 increased significantly cell viability compared with Ngb-siRNA transfected rat CGNs exposed to NaAsO₂, respectively. All statistical results are expressed as means±SD from at least three independent experiments. * $P < 0.05$ as compared with NaAsO₂ treatment group alone, # $P < 0.05$ as compared with Ngb-siRNA transfected NaAsO₂ exposure group alone.

Effect of Ngb siRNA on Arsenite-induced Activation of Cdc42 or Rac1 GTPase

To examine the effects of Ngb siRNA on NaAsO₂-induced activation of Cdc42 or Rac1, rates of Cdc42 or Rac1 activation were measured in the absence or presence of NaAsO₂ (10 μ M) with or without Ngb siRNA transfection. Results show that activation of Cdc42 did not change significantly in Ngb siRNA group in comparison with the untreated control. However, activation of Cdc42 increased significantly in NaAsO₂ group ($p < 0.05$ vs control) and Ngb siRNA with NaAsO₂ group ($p < 0.001$ vs control; Fig. 7A). Similar to Cdc42, activation of Rac1, given the same treatment, showed active Rac1 also increased significantly in NaAsO₂ group ($p < 0.01$ vs control) and Ngb siRNA with NaAsO₂ group ($p < 0.001$ vs control; Fig. 7B). Furthermore, Ngb siRNA transfection significantly ($p < 0.01$ vs NaAsO₂ group) increased arsenite-elevated levels of Cdc42 or Rac1 activation.

Amelioration of NaAsO₂-Induced Cytotoxicity by ZCL278 and NSC23766 in Ngb siRNA-Silenced Rat CGNs

To understand whether ZCL278 or NSC23766 ameliorate NaAsO₂-induced cytotoxicity in untransfected or Ngb siRNA transfected cell, CCK-8 assay was used to assess cell viability. As shown in Fig. 8, ZCL278 or NSC23766 increased cell viability significantly both in untransfected and Ngb-siRNA transfected rat CGNs exposed to NaAsO₂, respectively ($p < 0.05$). When compared with Ngb-siRNA transfected NaAsO₂ exposure group alone, ZCL278 in combination with NSC23766 also increased cell viability in the cells, but there was no statistical significance ($p > 0.05$). Similar to above result, both ZCL278 and NSC23766 in combination increased cell viability in untransfected group (Fig. 8), but there was also no statistical significance in comparison with NaAsO₂ exposure group alone ($p > 0.05$). These results indicate that using ZCL278 or NSC23766 ameliorate significantly NaAsO₂-induced cytotoxicity in untransfected or Ngb siRNA transfected rat CGNs.

Discussion

Arsenic is an environmental pollutant that affects various organs in human and experimental animals. Since cerebellum is an important target of As toxicity and Ngb is regarded as playing a GDI role to inactivate Rho GTPases, we carried out this study to

investigate whether Ngb plays a role in the arsenite-induced cytotoxicity in rat CGNs by regulating activation of Cdc42 and Rac1 GTPases.

We used 10 μM NaAsO_2 to treat these cells because in our previous findings, 10 μM was half maximal (50%) inhibitory concentration (IC_{50}) of NaAsO_2 against rat CGNs [8]. Furthermore, clinical studies have shown that the concentrations of arsenics (5.54–7.30 μM) was the plasma levels of arsenics in patients treatment with arsenic trioxide [41]. Hence, 10 μM NaAsO_2 is the clinically relevant concentration.

Arsenic increased apoptosis and decreased cell viability, consistent with our previous studies and other studies [8, 9, 42]. Apoptosis is induced through many ways including the disequilibrium of apoptotic/anti-apoptotic BCL₂ family members [43], reactive oxidative species generation [44], cytochrome C release, loss of mitochondrial transmembrane potential, inactivation of NF- κB [45, 46], activation of caspases [47], activation of MAPK pathways [48, 49], as well as activation of Rho GTPases pathway [9]. Among these mechanisms, the two best-characterized Rho family members Cdc42 and Rac1, have been found to play a critical role in regulating cellular processes, including cell proliferation, migration, differentiation, and apoptosis [11, 50]. Rho GTPases activation has been investigated for their involvement in neurological disorders such as spinal cord injury, traumatic brain injury, stroke and Alzheimer's disease [18, 51–53]. Although many studies show Rho GTPases activation promotes cell survival, impairing of Rho GTPases inhibits growth of tumors by inducing apoptosis [54, 55], there are still opposite conclusions supporting that activation of Rho GTPase elicits cell apoptosis, consistent with our present study [56, 57]. In the present study, NaAsO_2 induced Cdc42 or Rac1 activation. Cdc42 has been demonstrated to play an critical role in mediating apoptosis, whereas apoptosis-induction role of Rac1 was concluded differently in different studies [8, 58–63]. We used ZCL278, a Cdc42 inhibitor, or NSC23766, a Rac1 inhibitor, to inhibit Cdc42 or Rac1 activity and detected apoptosis by TUNEL assay. Results indicate that both ZCL278 and NSC23766 reduced rat CGNs apoptosis induced by NaAsO_2 . This demonstrates that apoptosis caused by NaAsO_2 exposure occurred at least partly due to Cdc42 or Rac1 activation in the present study. Furthermore, inhibition of Cdc42 or Rac1 increased the viability of the cells exposed to NaAsO_2 . These results indicate that NaAsO_2 might induce cytotoxicity by activating Cdc42 and Rac1. Many reports showed that Rho GTPases also regulate the activities of a wide range of biochemical pathways such as the MAPKs. For instance, p38 kinases and ERKs can be activated in a Rho GTPases dependent manner [64, 65]. Interestingly, our previous studies have observed that p38 and ERK 1/2 activation were induced by NaAsO_2 exposure in rat CGNs [8]. These findings indicate that Rho GTPase may either work alone by acting as signaling molecules or activate other signaling cascades to induce cytotoxicity in rat CGNs exposed to NaAsO_2 .

Damages activate the defense system. Arsenic-induced apoptosis and cell inviability trigger defense mechanisms to reduce the damages. Ngb is an endogenous neuroprotective molecule against damages [66]. However, the exact mechanism by which Ngb exert neuroprotective role still need to be clarified further. Ngb is regarded as a key mediator of protective effects in the brain including protection against oxidative stress, anti-apoptosis, and control of inflammation [66]. In addition, Ngb could be involved in a signal transduction pathway, for example, by acting as a GDI for inhibiting GDP/GTP exchange, thus exert protective effects against cell death [67]. A number of studies point out a relation between Ngb and Rho family GTPases in several tissues [26, 28, 68]. For example, Cdc42 or Rac1 activity can be regulated though Rho GDIs which are traditionally thought to inhibit Rho GTPases activation [67, 69]. Our previous studies have observed that arsenite simultaneously induced Ngb upregulation and apoptosis. Higher Ngb expression may have decreased apoptosis induced by NaAsO_2 in rat CGNs. Many studies have employed Ngb overexpression to confer an enhanced neuroprotection [24]. In contrast, we employed Ngb siRNA transfection and found that Ngb silencing significantly exacerbated NaAsO_2 -induced cytotoxicity in rat CGNs, supporting a neuroprotective role of Ngb in arsenite-induced injury of neurons. To clarify the relationship between Ngb and Rho-like Cdc42 or Rac1 GTPase, we used Ngb siRNA to inhibit Ngb followed by NaAsO_2 exposure and detect the activation of

Cdc42 or Rac1 by pull-down assay. Results show that Ngb knockdown significantly increased arsenite-elevated levels of Cdc42 or Rac1 activation. To understand whether inhibition of Cdc42 or Rac1 GTPases ameliorate NaAsO₂-induced cytotoxicity in Ngb siRNA transfected rat CGNs exposed to NaAsO₂, we used ZCL278 or NSC23766 to inhibit Cdc42 or Rac1 and detect cell viability by CCK-8 assay. Results confirmed that using ZCL278 or NSC23766 decrease NaAsO₂-induced injury in Ngb knockdown rat CGNs. These data suggest that Ngb impairs Rho GTPases pathway in the cells exposed to arsenite, providing an explanation of Ngb action in the protection of nerve cells from apoptosis and cytotoxicity [28].

In summary, NaAsO₂ induced cell cytotoxicity, activated Cdc42 and Rac1 GTPases, and inhibition of Cdc42 or Rac1 decreased NaAsO₂-induced cytotoxicity in rat CGNs. Also, Ngb knockdown significantly increased apoptosis and cell death, increased Cdc42 or Rac1 activation in rat CGNs exposed to NaAsO₂. It can be deduced Ngb plays a protective role against cytotoxicity by inhibition of Cdc42 and Rac1 GTPases in rat CGNs exposed to NaAsO₂. Our research provides insight into possible mechanisms of Ngb neuroprotection, and may provide therapeutic implication at NaAsO₂-induced injury of neurons.

Abbreviations

Ngb (neuroglobin); NaAsO₂ (sodium arsenite); CGNs (cerebellar granule neurons); Rho GTPases (Rho guanosine triphosphatases); GDI (guanine nucleotide dissociation inhibitor).

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Disclosure Statement

The authors declare no conflicts of interest.

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