Interaction between ROS and p38MAPK contributes to chemical hypoxia-induced injuries in PC12 cells

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Abstract. The present study investigated whether there is an interaction between reactive oxygen species (ROS) and p38 mitogen-activated protein kinase (MAPK) during chemical hypoxia-induced injury in PC12 cells. The results of the present study showed that cobalt chloride ($CoCl_2$), a chemical hypoxia agent, markedly induced ROS generation and phosphorylation of p38MAPK, as well as neuronal injuries. N-acetylcysteine (NAC), a ROS scavenger, blocked $CoCl_2$-induced phosphorylation of p38MAPK. In addition, SB203580, an inhibitor of p38MAPK attenuated not only $CoCl_2$-induced activation of p38MAPK, but also ROS production. These results suggest that ROS and p38MAPK are capable of interacting positively during chemical hypoxia. Furthermore, NAC and SB203580 markedly prevented $CoCl_2$-induced cytotoxicity, apoptosis and a loss of mitochondrial membrane potential. Taken together, our findings suggest that the positive interaction between $CoCl_2$ induction of ROS and p38MAPK activation may play a significant role in $CoCl_2$-induced neuronal injuries. We provide new insights into the mechanisms responsible for $CoCl_2$-induced injuries in PC12 cells.

Introduction

Hypoxia-induced cell insult is a major concern in a variety of clinical entities, for example, ischemic disease, organ transplantation, and other diseases. Cobalt chloride ($CoCl_2$) is a well-known hypoxia mimetic agent. There are numerous reports that $CoCl_2$ mimics the hypoxia response in a number of aspects in various cultured cells, including production of reactive oxygen species (ROS), a loss of mitochondrial membrane potential (MMP), activation of hypoxia inducible factor 1α (HIF-1α) and the expression of a number of genes, such as erythropoietin (EPO), vascular endothelial growth factor (VEGF) and endothelin-2 (ET-2)/vasoactive intestinal contractor (one of the hypoxia-related factors) (1-8). Therefore, PC12 cells (a cell line derived from rat pheochromocytoma) exposed to $CoCl_2$ have been used to investigate events and mechanisms underlying neuronal cell death under conditions of hypoxia/ischemia (4,6,9).

Accumulating evidence suggests that one of the mechanisms of $CoCl_2$-induced neuronal damage is associated with its production of ROS (4,6,9). Elevated ROS is capable of attacking nucleic acids, proteins and membrane phospholipids, leading to neuronal apoptosis (10-12). Additionally, Zou *et al* reported that $CoCl_2$ activates caspase-3 and p38 mitogen-activated protein kinase (MAPK), which are involved in $CoCl_2$-induced apoptosis in PC12 cells (4). p38MAPK has been demonstrated to be one of the apoptotic markers during PC12 cell death induced by various stimuli (15-18). Notably, excessive ROS have been shown to activate members of MAPKs, including extracellular signal-regulated kinase 1/2 (ERK1/2), c-Jun-N-terminal kinase (JNK) and p38MAPK in various types of cells (19-21). Recently, studies have found that H_2O_2 induction of ROS inhibits serine/threonine protein phosphatase 2A (PP2A), a major negative regulator of phosphorylated (p)-ERK1/2, JNK and p38MAPK (22,23) and protein phosphatase 5 (PP5), a negative regulator of the JNK cascade involved in stress responses (24,25), leading to activation of the ERK1/2, JNK and p38MAPK pathways, thereby resulting in neuronal apoptosis (18). These findings suggest that ROS are involved in the activation of MAPK pathways. However, whether $CoCl_2$-induced activation of p38MAPK regulates $CoCl_2$ induction of ROS is unclear.

With this background, we investigated whether there is an interaction between $CoCl_2$-induced ROS and the activation of p38MAPK, in particular, the effect of p38MAPK activation by $CoCl_2$ on ROS production during $CoCl_2$-induced neuronal injuries, and attempted to gain new insights into the mechanisms behind $CoCl_2$-induced injuries in PC12

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cells. Here we showed that: i) $CoCl_2$ induced ROS production which enhanced p38MAPK activation induced by $CoCl_2$; ii) SB203580, an inhibitor of p38MAPK, inhibited not only $CoCl_2$-induced phosphorylation of p38MAPK, but also ROS production; and iii) NAC, a ROS scavenger, and SB203580 protected PC12 cells against $CoCl_2$-induced injuries evidenced by an increase in cell viability and a decrease in apoptotic cells as well as a loss of MMP. The findings of the present study suggest that a positive interaction between $CoCl_2$ induction of ROS and activation of the p38MAPK may exist in the $CoCl_2$-induced neuronal injury process, which may be one of the key mechanisms responsible for $CoCl_2$-induced injuries in PC12 cells.

Materials and methods

Materials. Sodium hydrogen sulfide (NaHS), SB203580, $CoCl_2$, N-acetyl-L-cysteine (NAC), dichlorofluorescein diacetate (DCFH-DA) and Rhodamine 123 (Rh123) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The Cell Counter kit-8 (CCK-8) was purchased from Dojindo Lab (Japan). DMEM medium and fetal bovine serum (FBS) were supplied by Gibco BRL (Grand Island, NY, USA). p-p38 and p38 antibody were purchased from Cell Signaling Technology (Boston, MA, USA). HRP-conjugated secondary antibody and BCA protein assay kit were purchased from Kangchen Bio-Tech (Shanghai, China). ECL solution was supplied by KeyGen Biotech (Nanjing, China).

Cell culture and treatments. Rat pheochromocytoma PC12 cells were obtained from Sun Yat-sen University Experimental Animal Centre (Guangzhou, China) and were grown in DMEM medium supplemented with 10% FBS at 37˚C under an atmosphere of 5% CO_2 and 95% air.

Cell viability assay. Cell viability was detected by using the CCK-8 kit. PC12 cells were cultured in 96-well plates, with 4 duplicate wells in each group. When 70-80% confluence was reached, the cells were treated with conditioned media as indicated. Following the indicated treatments, 10 μl CCK-8 solution was added into each well and then the plates were incubated for 3 h in the incubator. Absorbance at 450 nm was measured with a microplate reader (Molecular Devices, Sunnyvale, CA, USA). The means of the optical density (OD) measurements from 4 wells of the indicated groups were used to calculate the percentage of cell viability according to the formula: Percentage of cell viability = (OD treatment group/OD control group) x 100%. The experiment was repeated three times.

Nuclear staining for assessment of apoptosis. Chromosomal condensation and morphological changes in the nuclei of PC12 cells were observed with the chromatin dye Hoechst 33258 followed by photofluorography. The cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 10 min. After three washes with PBS, cells were stained with 5 mg/l Hoechst 33258 for 10 min, and then washed briefly with PBS and air-dried again. The cells were visualized under a fluorescence microscope (BX50-FLA, Olympus). Viable cells displayed normal nuclear size and uniform fluorescence, whereas apoptotic cells showed condensed, fractured or distorted nuclei.

Measurement of intracellular ROS generation. Intracellular ROS content was determined by 2',7'-dichlorofluorescin (DCF) staining followed by photofluorography. DCF is a fluorescent substance derived from cell-permeable DCFH-DA (Sigma-Aldrich). PC12 cells were cultured on a slide in DMEM. DCFH-DA in FBS-free DMEM was added at a final concentration of 10 μmol/l to the PC12 cells. Cells were then incubated at 37˚C for 60 min and the indicated treatments were performed. After all the treatments were accomplished, slides were washed three times with FBS-free DMEM, and DCF-fluorescence was measured over the entire field of vision with a fluorescent microscope connected to an imaging system (BX50-FLA; Olympus, Tokyo). Mean fluorescence intensity (MFI) (which represents the amount of intracellular ROS content) from 3 random fields was analyzed using ImageJ 1.41o software (National Institute of Health, Bethesda, MD, USA).

Measurement of MMP. MMP was monitored by using a fluorescent dye, Rh123, a cell-permeable cationic dye that preferentially enters into mitochondria based on the highly negative MMP. Depolarization of MMP results in a loss of Rh123 from mitochondria and a decrease in intracellular green fluorescence. In the present study, Rh123 (100 mg/l) was added into cell cultures for 60 min at 37˚C and fluorescence was measured over the entire field of vision by using a fluorescence microscope connected to the imaging system. The MFI of Rh123 from 4 random fields was analyzed using the ImageJ 1.41o software and the MFI was taken as an index of the level of MMP.

Western blot analysis for protein expression. PC12 cells were plated in 35-mm diameter Petri dishes. When grown to 70-80% confluence, the cells were treated as indicated. At the end of the treatments, PC12 cells were harvested and resuspended in ice-cold cell lysis solution and the homogenate was centrifuged at 10,000 x g for 15 min at 4˚C. Total protein in the supernatant was measured using a bicinchoninic acid (BCA) protein assay kit (Kangchen BioTech, Shanghai, China). Total protein (30 μg) from each sample was separated by 12% SDS-PAGE. The protein in the gel was transferred onto a polyvinylidene difluoride (PVDF) membrane. The membrane was blocked with 5% fat-free dry milk in TBST for 1 h at room temperature, and then incubated with the primary antibody specific to p-p38, p38 (Cell Signaling Technology, Beverly, MA, USA) overnight with gentle agitation at 4˚C. The next day, the membrane was washed and subsequently incubated with HRP-conjugated secondary antibodies for 1.5 h at room temperature. Following three washes with TBST, the membranes were developed using an enhanced chemiluminescence kit (Applygen Technologies, Beijing, China) and exposed to X-ray films. ImageJ 1.41o software was used to quantitatively analyze the protein expression level. The experiment was carried out in triplicate with similar results.

Statistical analysis. All data are presented as the mean ± SEM. The assessment of differences between groups was analyzed

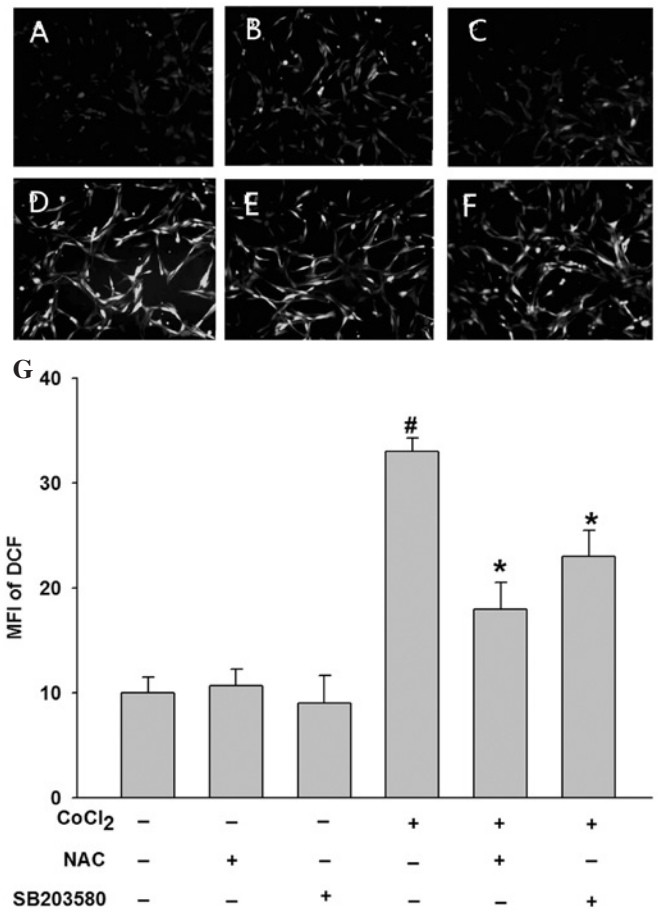

Figure 1. Effects of NAC and SB203580 on $CoCl_2$-induced ROS production in PC12 cells. (A-F) DCFH-DA staining followed by photofluorography to observe the intracellular ROS in PC12 cells. (A) Control group. (B) PC12 cells exposed to 500 μmol/l NAC for 60 min followed by a 6-h culture. (C) PC12 cells were treated with 20 μmol/l SB203580, an inhibitor of p38MAPK, for 60 min followed by a 6-h culture. (D) PC12 cells subjected to 600 μmol/l $CoCl_2$ treatment for 6 h. (E) PC12 cells were pretreated with 500 μmol/l NAC for 60 min prior to exposure to $CoCl_2$. (F) PC12 cells were preconditioned with 20 μmol/l SB203580 for 60 min followed by $CoCl_2$ treatment. (G) Quantitative analysis of the mean fluorecence intensity (MFI) of DCF with ImageJ 1.41o software. #P<0.01 vs. the control group; *P<0.01 vs. the 600 μmol/l $CoCl_2$ group.

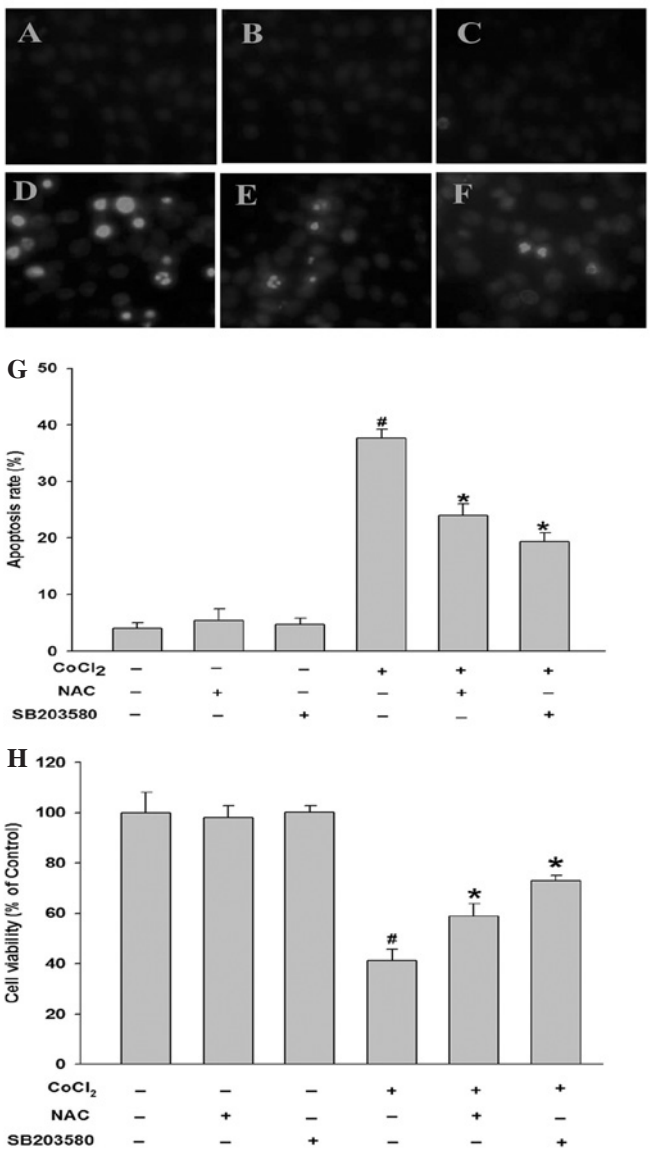

Figure 2. Protection of NAC and SB203580 against $CoCl_2$-induced apoptosis and cytotoxicity in PC12 cells. (A-F) Hoechst 33258 nuclear staining followed by photofluorography to observe cellular apoptosis. (A) Control group. (B) PC12 cells were treated with 500 μmol/l NAC for 60 min followed by a 48-h culture. (C) PC12 cells were exposed to 20 μmol/l SB203580 for 60 min followed by culture for 48 h. (D) PC12 cells were treated with 600 μmol/l $CoCl_2$ for 48 h. (E) PC12 cells were pretreated with 500 μmol/l NAC for 60 min prior to exposure to $CoCl_2$ for 48 h. (F) PC12 cells were pretreated with 20 μmol/l SB203580 for 60 min prior to $CoCl_2$ treatment. (G) The apoptotic rate was analyzed with a cell counter from the ImageJ 1.41o software. #P<0.01 vs. the control group; *P<0.01 vs. the 600 μmol/l $CoCl_2$ group. (H) PC12 cells were treated with 600 μmol/l $CoCl_2$ for 24 h in the presence or absence of pretreatment with NAC or pretreatment with SB203580. The CCK-8 assay was performed to measure cell viability. #P<0.01 vs. the control group. *P<0.01 vs. the 600 μmol/l $CoCl_2$ group.

by one-way ANOVA with SPSS 13.0 (SPSS Inc.). P<0.05 was considered to be statistically significant.

Results

NAC attenuates $CoCl_2$-induced ROS and neuronal injuries. Exposure of PC12 cells to 600 μmol/l $CoCl_2$ for 6 h led to a significant increase in DCF-derived fluorescence, manifesting an accumulation of intracellular ROS (Fig. 1D and G). Further study showed that $CoCl_2$-induced ROS production was markedly decreased by pretreatment with 500 μmol/l NAC, a ROS scavenger, for 60 min (Fig. 1E and G). Moreover, our findings showed that treatment of cells with 600 μmol/l $CoCl_2$ for 24 h markedly reduced cell viability and that NAC at 500 μmol/l significantly suppressed this inhibitory effect of $CoCl_2$ (Fig. 2H). In addition, exposure of PC12 cells to 600 μmol/l $CoCl_2$ for 48 h caused a marked increase in the percentage of apoptotic cells (Fig. 2D and G). $CoCl_2$-induced apoptosis was considerably depressed by pretreatment with 500 μmol/l NAC for 60 min (Fig. 2E and G). Furthermore, the results of the Rh123 staining assay indicated that after PC12 cells were subjected to 600 μmol/l $CoCl_2$ for 24 h, mitochondria were dramatically damaged, resulting in a decrease in the uptake of Rh123, indicating dissipation of MMP (Fig. 3D and G). The loss of MMP was ameliorated by pretreatment with 500 μmol/l NAC for 60 min (Fig. 3E and G). NAC at 500 μmol/l alone did not alter ROS production, cell viability, apoptosis or MMP, respectively (Figs. 1-3).

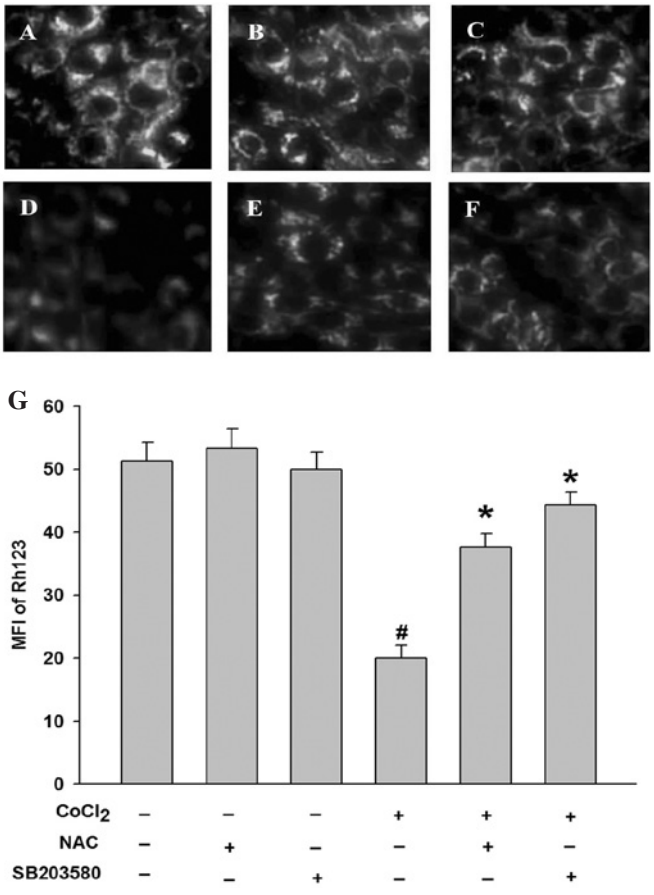

Figure 3. Protection of NAC and SB203580 against $CoCl_2$-induced loss of MMP in PC12 cells. (A-F) Rh123 staining following by photofluorography to measure mitochondrial membrane potential (MMP). (A) Control group. (B) PC12 cells were treated with 500 μmol/l NAC for 60 min followed by a 24-h culture. (C) PC12 cells were treated with 20 μmol/l SB203580 for 60 min followed by a 24-h culture. (D) PC12 cells were treated with 600 μmol/l $CoCl_2$ for 24 h. (E) PC12 cells were pretreated with 500 μmol/l NAC for 60 min prior to $CoCl_2$ treatment. (F) PC12 cells were pretreated with SB203580 for 60 min prior to $CoCl_2$ treatment. (H) Quantitative analysis of MFI of Rh123 in A-F with the ImageJ 1.41o software. $^{\#}$P<0.01 vs. the control group; *P<0.01 vs. the 600 μmol/l $CoCl_2$ group.

p38MAPK is involved in $CoCl_2$-induced neuronal injuries. As shown in Fig. 4A and B, after PC12 cells were exposed to 600 μmol/l $CoCl_2$ for 2 h, the expression of p-p38MAPK was increased 4.7-fold compared with the control group (P<0.01). However, the expression of total p38MAPK was not altered. Further study revealed that pretreatment of cells with 20 μmol/l SB203580 (an inhibitor of p38MAPK) for 60 min prior to exposure to $CoCl_2$ significantly attenuated $CoCl_2$-induced cytotoxicity, leading to an increase in cell viability (72.16±2.3%) (P<0.01), whereas SB203580 alone had no effect on cell viability in PC12 cells (Fig. 2H). Additionally, pretreatment with SB203580 also blocked $CoCl_2$-induced apoptosis (Fig. 2F and G) and dissipation of MMP (Fig. 3F and G). These results suggest that $CoCl_2$ is capable of activating the p38MAPK pathway, which mediates neuronal injuries induced by $CoCl_2$.

$CoCl_2$-induced ROS activates the p38MAPK pathway in PC12 cells. Since $CoCl_2$-induced neuronal injuries are attributed to its induction of ROS and p38MAPK activation (Figs. 2 and 3), we further investigated whether $CoCl_2$-induced activation of p38MAPK is due to its induction of ROS. As shown in Fig. 4C and D, pretreatment of cells with 500 μmol/l NAC for 60 min before exposure to $CoCl_2$ at 600 μmol/l for 2 h markedly inhibited $CoCl_2$-induced phosphorylation of p38MAPK, whereas pretreatment with NAC had no effect on the expression of total p38MAPK (Fig. 4C and D).

$CoCl_2$-induced activation of p38MAPK promotes ROS production in PC12 cells. To determine the role of the p38MAPK pathway in $CoCl_2$-induced ROS production, PC12 cells were pretreated with 20 μmol/l SB203580 for 60 min prior to exposure to 600 μmol/l $CoCl_2$ for 6 h, followed by DCFH-DA staining and photofluorography to observe the intracellular ROS. As shown in Fig. 1, pretreatment with SB203580 led to a decrease in DCF-derived fluorescence, exhibiting a decrease in intracellular ROS (Fig. 1F and G). These findings suggest that the p38MAPK pathway is involved in $CoCl_2$-induced ROS production.

Discussion

It is widely accepted that oxidative stress is associated with hypoxia/ischemia-linked neuronal cell death, which is involved in a number of neurogenerative disorders, such as Parkinson's disease, Alzheimer's disease and amyotrophic lateral sclerosis (26-29). Under oxidative stress, ROS, including free radicals such as superoxide (O_2^-), hydroxyl radical (HO^-) and H_2O_2, are generated at high levels (12,30). Excessive amounts of ROS induced by oxidative stress may modify protein, lipids and DNA and alter their functions, resulting in apoptosis of neuronal cells (19,31). Our previous study and other studies (6) have demonstrated that $CoCl_2$ elicits oxidative stress, which contributes to neuronal apoptosis. In agreement with the previous studies (6), our results showed that $CoCl_2$ induced ROS generation, which mediated $CoCl_2$-induced neuronal injuries, including cytotoxicity, mitochondrial damage and apoptosis. This is supported by the following results: i) $CoCl_2$-induced cytotoxicity was prevented by NAC, a ROS scavenger; ii) NAC blocked a loss of MMP induced by $CoCl_2$; iii) NAC attenuated $CoCl_2$-induced apoptosis.

p38MAPK is a distinct member of the MAPK family. In neuronal cells, the p38 signaling pathway is preferentially activated by inflammatory cytokines and environmental stress, such as osmotic stress, ultraviolet light, heat shock and hypoxia. It can also be activated by ROS (12,18-21). Previous studies have demonstrated that p38MAPK activation is implicated in neuronal damage associated with ischemia and neurodegenerative disease (32), and it is one of the apoptotic markers during PC12 cell death induced by a variety of stimuli (15-18). In the present study, we found that exposure of PC12 cells to $CoCl_2$ significantly upregulated expression of p-p38MAPK and that pretreatment of cells with SB203580, an inhibitor of p38MAPK, markedly blocked $CoCl_2$-induced neuronal injuries, indicating that activation of p38MAPK mediates neuronal damage induced by $CoCl_2$. Our results are consistent with the results reported by Zou *et al* (4) and are comparable with the evidence that H_2O_2-induced apoptosis of neuronal cells is associated with activation of p38MAPK (18).

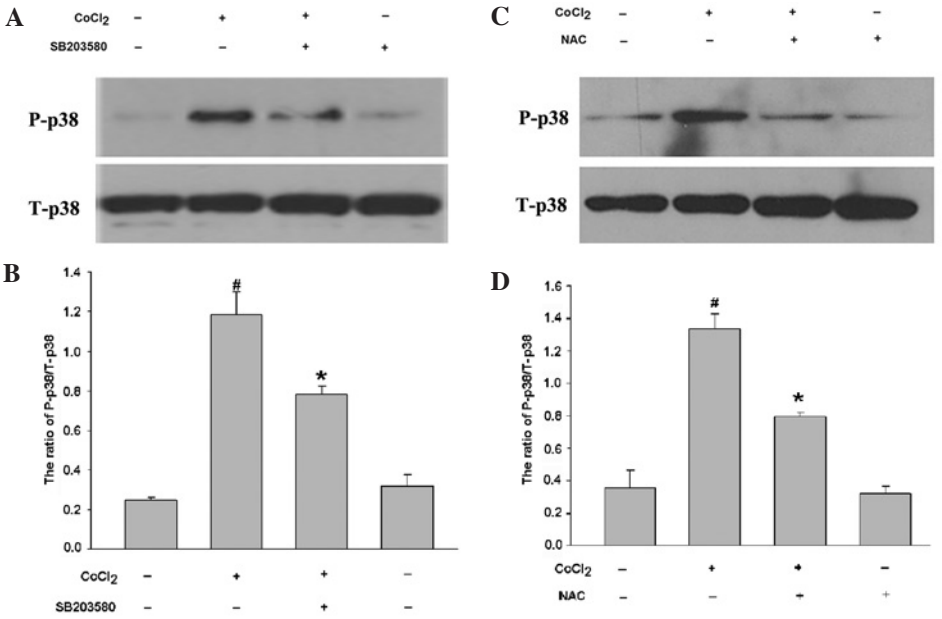

Figure 4. Inhibitory effects of SB203580 and NAC on the phosphorylation of p38MAPK induced by $CoCl_2$ in PC12 cells. (A and B) PC12 cells were treated with 600 μmol/l $CoCl_2$ for 2 h in the presence or absence of pretreatment with 20 μmol/l SB203580, an inhibitor of p38MAPK, for 60 min. (C and D) PC12 cells were treated with 600 μmol/l $CoCl_2$ for 2 h in the presence or absence of pretreatment with 500 μmol/l NAC for 60 min. (A and C) Expression of p38MAPK was detected by Western blot analysis and (B and D) quantified by densitometric analysis with ImageJ 1.41 software. #P<0.01 vs. the control group; *P<0.01 vs. the 600 μmol/l $CoCl_2$ group.

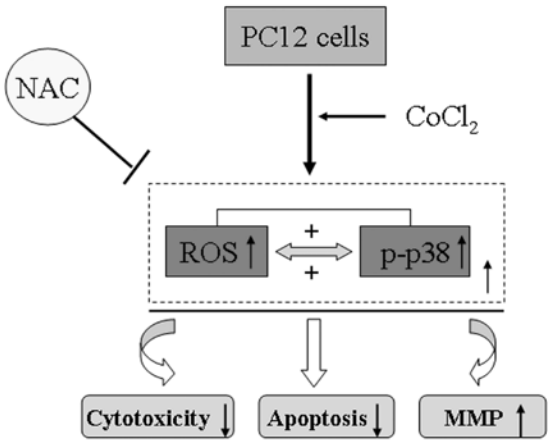

Figure 5. Proposed positive interaction between $CoCl_2$ induction of ROS and activation of p38MAPK during $CoCl_2$-induced injuries in PC12 cells. +, stimulation.

Notably, our studies further demonstrated that NAC, a ROS scavenger, attenuated not only $CoCl_2$-induced ROS, but also activation of p38MAPK by $CoCl_2$, suggesting that $CoCl_2$ induction of ROS activates the p38MAPK pathway. Our results are supported by a recent study, which showed that H_2O_2 induction of ROS contributes to p38MAPK activation (18). Collectively, the findings from our group and others (18-20) support the notion that ROS activation of the p38MAPK pathway may be a common mechanism by which oxidative stress or hypoxia/ischemia induces neuronal cell death or neurodegenerative disorders.

Importantly, here, for the first time, we showed that $CoCl_2$-induced activation of p38MAPK is capable of regulating ROS overproduction induced by $CoCl_2$ in PC12 cells. After PC12 cells were pretreated with SB203580 prior to exposure to $CoCl_2$, $CoCl_2$-induced ROS production was markedly blocked, suggesting that p38MAPK may be a positive regulator for $CoCl_2$-induced ROS production in PC12 cells. Previous studies reported that ROS appeared to be upstream of p38MAPK (33). However, based on our results, ROS and p38MAPK may locate at a parallel level, and there is interaction between ROS and p38MAPK during $CoCl_2$-induced neuronal injuries.

In conclusion, the present study provided novel evidence that $CoCl_2$ induction of ROS activates the p38MAPK pathway which is involved in $CoCl_2$-induced neuronal injuries and that $CoCl_2$-induced activation of p38MAPK also promotes ROS production induced by $CoCl_2$. The positive interaction between $CoCl_2$ induction of ROS and activation of p38MAPK may play a significant role in $CoCl_2$-induced neuronal injuries (Fig. 5). However, further studies are required to elucidate the molecular mechanisms responsible for the interaction between ROS induction and p38MAPK activation during $CoCl_2$-induced injuries in PC12 cells.

Acknowledgements

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