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Oxidative Stress-Induced Attenuation of Thrombospondin-1 Expression in Primary Rat Astrocytes

Jen-Kun Chen,[1] Yan-Jie Zhan,[2] Chung-Shi Yang,[1*] and Shun-Fen Tzeng[2**]

[1] *Center for Nanomedicine Research, National Health Research Institutes, Zhunan, Miaoli County, Taiwan*

[2] *Department of Life Sciences, National Cheng Kung University, Tainan, Taiwan*

ABSTRACT

Astrocytes, the major glial population in the central nervous system (CNS), can secrete thrombospondin (TSP)-1 that plays the role in synaptogenesis and axonal sprouting during CNS development and tissue repair. However, little is known about the regulation of TSP-1 expression in astrocytes under oxidative stress condition. Here, a hypoxic mimetic reagent, cobalt chloride ($CoCl_2$), was used to initiate hypoxia-induced oxidative stress in primary rat astrocytes. $CoCl_2$ at the concentration range of 0.1–0.5 mM was found to cause no significant cell death in primary rat astrocytes. However, $CoCl_2$ at 0.2–0.5 mM increased intracellular reactive oxygen species (ROS) levels and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene expression that is known as a hallmark for oxidative damage. We further found that TSP-1 mRNA expression in astrocytes was inhibited dose- and time-dependently by $CoCl_2$. TSP-1 mRNA levels were increased in $CoCl_2$-exposed astrocytes in the presence of the inhibitors (U0126 and PD98059) of mitogen-activated protein kinase/extracellular signal-regulated kinases (MAPK/ERK), when compared to that detected in the culture only exposed to $CoCl_2$. Moreover, the inhibition in TSP-1 mRNA expression by $CoCl_2$ was blocked by the addition of the potent antioxidant, N-acetylcysteine (NAC). Thus, we conclude that $CoCl_2$ inhibits TSP-1 mRNA expression in astrocytes via a ROS mechanism possibly involving MAPK/ERK. This inhibition may occur after CNS injury and impair the supportive function of astrocytes on neurite growth in the injured CNS tissues. J. Cell. Biochem. 112: 59–70, 2011.

KEY WORDS: ASTROCYTES; THROMBOSPONDIN-1; OXIDATIVE STRESS; *N*-ACETYLCYSTEINE; HYPOXIA; GLIA

Glia play an important role in neuronal survival and transmission; therefore, they are functionally considered to be neuronal support cells. Astrocytes, the predominant glial population in the central nervous system (CNS), regulate neuronal maturation, neuronal metabolic/neurotransmitter homeostasis, and synaptic plasticity in developing and adult CNS [Voutsinos-Porche et al., 2003; Solecki et al., 2004; Wadiche and Jahr, 2005]. In addition, astrocytes are the first cells of the brain parenchyma to encounter foreign molecules crossing the blood–brain barrier [Tiffany-Castiglioni et al., 1989b]. The soluble factors derived from astrocytes regulate synapse formation and neuronal survival in developing CNS and support neuronal survival and tissue repair after CNS injury [Ransom et al., 2003].

Thrombospondins (TSPs) belong to the multimeric extracellular-matrix glycoprotein family, which was initially identified as the regulator for cell motility, growth, and differentiation in diverse cell types [Adams, 2001; Adams and Lawler, 2004]. Among identified TSP members, the role of TSP-1 and TSP-2 in promoting neurite outgrowth and synaptogenesis has been characterized [Christopherson et al., 2005]. The two molecules are known to be produced by astrocytes [Asch et al., 1985; Christopherson et al., 2005]. The increase in TSP-1 expression that occurred during the early period after cerebral ischemia is thought to be critical for stabilizing neurons and their synapses surrounding the injured site [Lin et al., 2003; Tran and Neary, 2006]. In vitro study has shown that TSP-1 was secreted from reactive astrocytes induced by mechanic strain [Tran and Neary, 2006]. The findings indicate that the effect of astrocyte-secreting TSP-1 on neuronal dendritic growth, axonal sprouting, and synaptogenesis is important for CNS development and repair.

Divalent cobalt ion, Co^{2+}, is an essential component of vitamin B12 (hydroxocobalamin) and serves as a cofactor for a few proteins,

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*Correspondence to: Chung-Shi Yang, Center for Nanomedicine Research, National Health Research Institutes, Zhunan, Miaoli County, Taiwan. E-mail: cyang@nhri.org.tw

**Correspondence to: Shun-Fen Tzeng, Department of Life Sciences, National Cheng Kung University, #1 Ta-Hsueh Road, Tainan 70101, Taiwan. E-mail: stzeng@mail.ncku.edu.tw

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such as methionine aminopeptidase 2 and nitrile hydratase [Domingo, 1989; Kobayashi and Shimizu, 1999]. Exposure to Co^{2+} results in the release of intracellular Ca^{2+} and cellular hypoxic injury [Karovic et al., 2007], and inhibits neurotransmission at the synapse [Gerber and Gahwiler, 1991]. The cellular uptake of Co^{2+} is through the relatively unspecific uptake systems, such as divalent metal transporter 1/2 [Forbes and Gros, 2003]. Moreover, neurons are able to uptake Co^{2+} through non-NMDA ionotropic glutamate receptors, such as kainate receptors [Pruss et al., 1991] or AMPA receptors [Malva et al., 2003]. These transport systems are also found in glial cells [Pruss et al., 1991; Lis et al., 2004]. Co^{2+} induces the stabilization of hypoxia inducible factor-1α (HIF-1α) and increases the expression of HIFα-targeted genes, which are important for cells to survive under hypoxic insult [Sharp and Bernaudin, 2004; Karovic et al., 2007]. Based on this characteristic effect, thus, Co^{2+} is used as a chemical agent for in vivo ischemic or in vitro hypoxic study [Jones and Bergeron, 2001; Grasselli et al., 2005].

The aim of this study was to examine whether oxidative stress induced by in vitro chemical hypoxic agent $CoCl_2$mediated the expression of TSP-1 mRNA and proteins in rat cortical astrocytes. To verify $CoCl_2$-induced oxidative stress in astrocytes, we firstly evaluated the generation of ROS and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in astrocytes exposed to $CoCl_2$. Furthermore, we found that TSP-1 production was substantially reduced through extracellular signal-regulated kinase (ERK/MAPK). Finally, evidence provided here indicates that an increased in ROS by Co^{2+}-induced oxidative stress caused a decline in TSP-1 mRNA expression in astrocytes. This may decrease the stimulatory effect of astrocytes on neurite growth in the injured CNS.

MATERIALS AND METHODS

MATERIALS

Media and antibiotics were purchased from Invitrogen (Carlsbad, CA). Fetal bovine serum (FBS) and goat serum were from Hyclone (Logan, UT). Cell cultureware and Petri dishes were from BD Biosciences (San Jose, CA). *N*-Acetylcysteine (NAC), resveratrol, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), protease inhibitor cocktail, cobalt chloride ($CoCl_2$), PD98059 (inhibitor of MAPK/ERK kinase [MEK]), and other chemicals were purchased from Sigma–Aldrich (St. Louis, MO). NE-PER kit used for nuclear protein extraction was from Pierce (Rockford, IL). U0126 (MEK inhibitor), T4 polynucleotide kinase, and consensus DNA sequences specific for the binding of ATF-1/cAMP-response element binding protein (CREB) were obtained from Promega (Madison, WI). The primers used for examination of GAPDH, TSP-1, and cyclophilin A (CyPA) mRNA expression were synthesized by MWG Biotech AG (Ebersberg, Germany). Nitrocellulose membrane and enhanced chemiluminescence (ECL) solution were the products of Millipore (Bedford, MA) and Perkin Elmer (Boston, MA), respectively. Antibodies used in this study were listed as follows: mouse anti-TSP-1 antibody (Thermo Scientific, CA), rabbit anti-activating transcription factor 1 (ATF-1) antibody (Santa Cruz Biotechnology, USA), rabbit neurofilament-200 kDa (NF-200) antibody (Sigma–Aldrich), mouse anti-GAPDH antibody (Chemicon, Temecula, CA), biotinylated secondary antibodies (Vector Laboratories, Burlingame, CA), fluorescein-avidin D (Vector Laboratories), and horseradish peroxidase (HRP)-conjugated secondary antibodies (Jackson Immunoresearch, West Grove, PA). Nitric acid (ultrapure reagent grade) was from J.T. Baker (Canada).

CELL CULTURE

Primary rat astrocytic culture was prepared as described below. Cerebral cortices from neonatal Sprague–Dawley rat brains (P1–P2) were removed and carefully dissected. Animal care was provided in accordance with the *Laboratory Animal Welfare Act, Guide for the Care and Use of Laboratory Animals,* Animal Center of National Cheng Kung University. The tissue was dissociated in DMEM containing 0.0025% trypsin/EDTA and passed through a 70 μm pore nylon mesh. After centrifugation, the cell pellet was resuspended in DMEM/F12 medium containing 10% heat-inactivated FBS, 50 U/ml penicillin, and 50 mg/ml streptomycin (P/S). The cells (10^7 cells/flask) were then plated onto PDL-coated 75T tissue culture flasks. The medium was renewed every 2–3 days. Eight days later, microglia were dislodged using the shake-off method [McCarthy and de Vellis, 1980]. There were approximately 90–92% astrocytes on the flask after shake-off procedure, which was verified using immunostaining for the specific astrocytic marker GFAP. Less than 5–10% of cells were B4 isolectin$^+$ microglia in astrocytic culture. Astrocytes were then trypsinized and replated on the culture plate at the density of 5×10^4 cells/cm^2. Four days after replating, astrocytes at approximately 90% confluency were exposed to $CoCl_2$ at the different concentrations for the distinct time points.

A mouse neuroblastoma cell line Neuro2A (ATCC, CCL-131) was maintained as monolayer cultures in DMEM medium containing 10% FBS and P/S at 37°C in 5% CO_2 humidified air. For coculture experiments, Neuro2A cells (1×10^4 cells/well) were plated onto 24-well culture plates. Astrocytes (5×10^4 cells/insert) were plated on tissue culture inserts with 3-μm pores and then pretreated with 0.5 mM of $CoCl_2$ for 6 h. The culture inserts were washed with DMEM at least three times to remove the residual $CoCl_2$ in the culture, and then placed onto 24-well plates containing Neuro2A cells. The medium volume of the dual-well system was maintained at 1 ml of serum-free DMEM medium without $CoCl_2$ for 2 days. Neuro2A cells were fixed in 4% paraformaldehyde for 10 min and subjected to immunostaining for neurofilament-200 kDa (NF-200).

CELL VIABILITY ASSAY

MTT is converted to a formazan product by mitochondrial dehydrogenase in viable cells. Accordingly, the levels of the formazan product by a colorimetric method are correlated with the relative number of cells. To study the effect of $CoCl_2$ on the cell viability of astrocytes, cells were treated with $CoCl_2$ (0–0.5 mM) in serum-free DMEM medium (DMEM) for distinct time courses, followed by MTT assay as previously described [Yang et al., 2008]. The assay was performed by the addition of MTT solution (5 mg/ml) to each well; 4 h later, SDS (10% in 0.01 N HCl) was added to each well; then the culture was incubated overnight at 37°C. MTT absorbance was measured using an ELISA reader at 595 nm. To confirm the results from MTT assay, cells were harvested using 0.00125% trypsin/EDTA solution, and then collected by centrifugation at 1,500 rpm for 5 min. The cell pellets were re-suspended in

PBS containing 0.4% trypan blue dye. Blue dye-incorporating cells were considered as dying/dead cells. The cell counting was performed using a hemacytometer under a light microscope. The number of live cells was determined and expressed as a percentage of the total of 250–300 cells from randomly chosen fields. Data are presented as the percentage versus control, which is expressed as 100% cell viability.

QUANTITATIVE REAL-TIME RT-PCR (Q-PCR)

Total RNA isolation and Q-PCR were performed as described previously [Liu et al., 2008] with minor modification. Polymerase chain reaction amplification of GAPDH or TSP-1 was performed for 10 min at 95°C, followed by 50 cycles at 95°C for 10 s, annealing at 60°C for 10 s, and extension at 72°C for 20 s. Results (Cycle at threshold, Ct) were normalized to those of the housekeeping gene cyclophilin A (CyPA) as ΔCt ($Ct_{TSP\text{-}1\ or\ GAPDH} - Ct_{CyPA}$). Values are expressed as the relative mRNA levels by comparison of $\Delta Ct_{treatment}$ to $\Delta Ct_{control}$ ($2^{\Delta Ct_{treatment} - \Delta Ct_{control}}$). Polymerase chain reactants were also analyzed on 1% agarose gels to confirm primer specificity by observing the purity of single PCR products during amplification. The specific primer sequences for rat GAPDH (GenBank accession number NM_017008) and TSP-1 (GenBank accession number NM_001013062) are as follows: GAPDH, 5′-TCTACCCACGGCAA-GTTC-3′(forward), 5′GATGTTAGCGGGATCTCG-3′ (reverse); TSP-1, 5′-CCAGTTCAACCAACGTCCTT-3′ (forward), 5′TTGCGAATGCTGT-CCTGTAG3′(reverse).

CELLULAR ROS MEASUREMENT

ROS production was analyzed with CM-H_2 DCFDA [Li et al., 2005], which was converted by endogenous esterases to carboxy-H_2 DCF and then oxidized to the fluorescent probe (CM-DCF) after exposure to ROS. Astrocytes were pre-incubated with 10 μM CM-H_2 DCFDA at 37°C for 45 min, followed by treatment with $CoCl_2$ at the different concentrations for the distinct time points. The cells were harvested and processed for the flow cytometrical analysis to quantify the fluorescence intensity of the end-product of CM-DCF.

WESTERN BLOTTING

After treatment, cells were harvested and gently homogenized on ice in phosphate-buffered saline (PBS) containing 1% SDS, 1 mM phenylmethylsulphonyl fluoride (PMSF), 1 mM EDTA, 1 mM sodium orthovanadate, and proteinase inhibitor cocktail. Protein concentration was determined with a Bio-Rad DC kit. Subsequently, 100 μg of total protein was loaded onto 10% SDS polyacrylamide electrophoresis gels and transferred to nitrocellulose membrane. Proteins were identified by incubating the membrane overnight at 4°C with primary antibodies against TSP-1, GAPDH, or MAPK/ERK1/2 at the appropriate dilution. The membrane was then incubated in HRP-conjugated secondary antibodies for 1 h, and followed by ECL solution for 1 min.

MEASUREMENT OF COBALT

After treatment, the cultures (5×10^5 cells/culture) were washed twice with PBS to remove residual culture media. The cell pellets were lysed with 0.5 ml of double deionized water. Thereafter, nitric acid (0.08 ml) was added to the cell lysate; and after 10 min the reactant was diluted to 4 ml using double deionized water. Furthermore, the cell lysate was subjected to ICP-MS (Agilent 7500cx, Agilent Technologies, Inc., Tokyo, Japan) for determination of intracellular cobalt levels. The detection limit for cobalt in samples was 0.01 ng/mL.

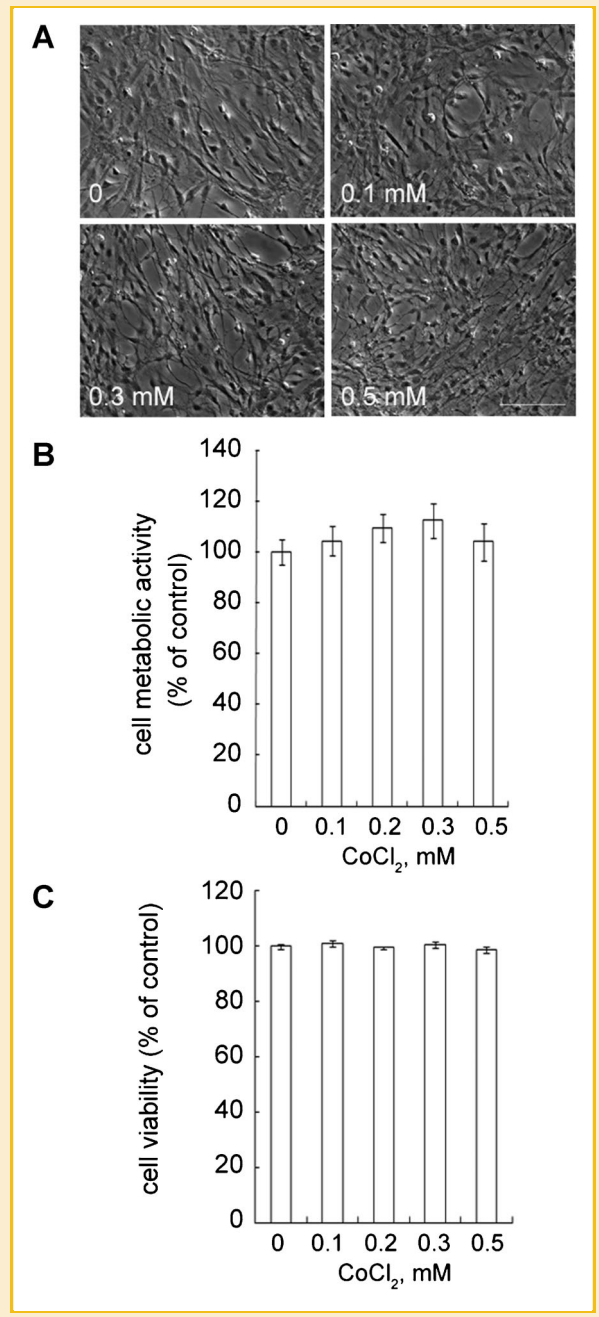

Fig. 1. Effect of $CoCl_2$ on the cell viability of primary rat astrocytes. Primary rat astrocytes were exposed for 24 h to $CoCl_2$ at the different concentrations indicated above. Morphological examinations (A), MTT assay for cell metabolic activity (B), and trypan blue exclusion assay (C) were performed. Data are means ± SEM of at least three independent experiments.

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The cultures were fixed in PBS containing 4% paraformaldehyde for 10 min, and then incubated with PBS containing 0.1% Triton X-100 and 5% normal goat serum for 20 min. Subsequently, cells were incubated at room temperature with anti-ATF-1 antibody at the dilution of 1:200 overnight at 4°C, followed by biotinylated secondary antibody (1:200) for 1 h. FITC-avidin (1:200) was added to the cultures for 45 min. The slides were mounted, and the results were observed under a fluorescence microscope.

QUANTIFICATION OF NEURITE LENGTH

Neuro2A cells were fixed by 4% paraformaldehyde for 10 min, incubated with anti-NF-200 at the dilution of 1:200 overnight at 4°C, and followed by biotinylated secondary antibody (1:200) for 1 h. Subsequently, FITC-avidin (1:200) was added to the cultures for 45 min. The culture was subjected to nuclei counterstaining in PBS containing 1 μg/ml DAPI for 2 min. The resulting images were captured at 20× magnifications under a fluorescence microscope equipped with a cooling CCD system. Five images from each culture were randomly selected and subjected for measurement of neurite length using a ImageJ plugin for automated quantification of neurite outgrowth so called as NeuroTracer [Pool et al., 2008]. The results were expressed as the average neurite length (in μm) per cell.

ELECTROPHORETIC MOBILITY SHIFT ASSAY (EMSA)

Nuclear proteins were isolated using the NE-PER kit according to the manufacturer's directions. EMSA was performed as described

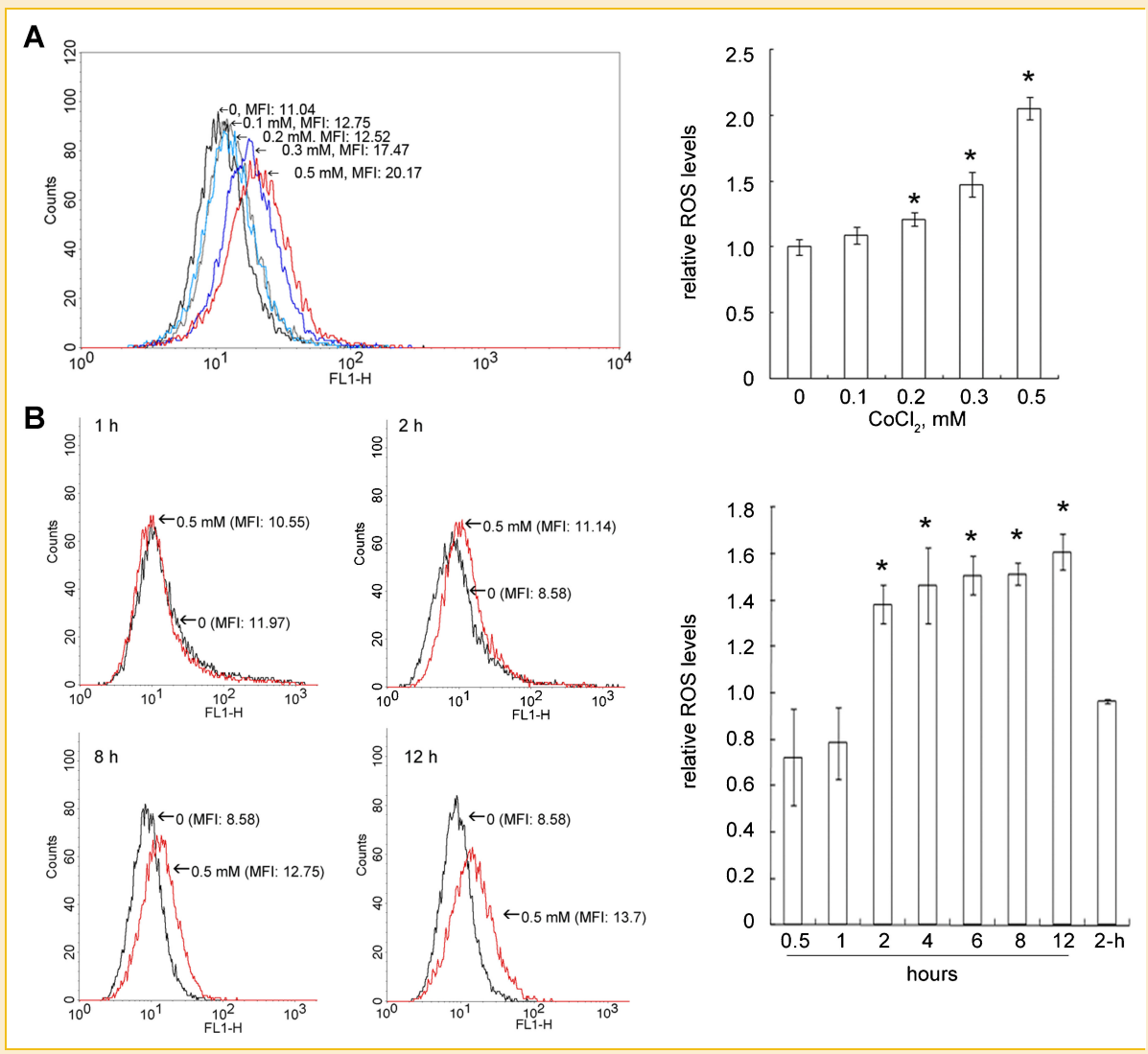

Fig. 2. An increase in ROS generation induced by $CoCl_2$. Primary rat astrocytes were exposed to $CoCl_2$ at the different concentrations (A), or treated with $CoCl_2$ (0.5 mM) for the distinct time periods (B). Alternatively, the cultures were exposed to $CoCl_2$ (0.5 mM) for 2 h (2-h), and then were refilled with fresh medium without $CoCl_2$ for another 22 h (B). After treatment, the cultures were subjected to Flow cytometric analysis for the measurement of intracellular ROS levels in astrocytes. The pictures (left panels in A and B) shown are representative for three independent experiments performed. Data shown in the graph of the quantification (right panels in A and B) are means ± SEM of three independent experiments. $^*P < 0.05$ versus control culture (A), or versus 0.5-h exposure to $CoCl_2$. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

previously [Hsiao et al., 2006]. The double-stranded consensus DNA sequences specific for the binding of ATF-1/CREB were end-labeled with [γ-^{32}P] ATP using T4 polynucleotide kinase at 37°C for 10 min and purified by passage through a G25 spin column. Twenty micrograms of nuclear protein extracts were incubated at RT for 20 min in the final volume of 20 μl binding buffer (10 mM Tris-HCl, pH 7.5, 250 mM NaCl, 5 mM $MgCl_2$, 2.5 mM EDTA, 2.5 mM dithiothreitol, and 5% glycerol), which contained 1 μg poly-dIdC and 5 ng end-labeled oligomer. Alternatively, prior to DNA binding reaction, 20 μg of nuclear extracts were incubated with the specific antibody against ATF-1 for 60 min at RT. The reaction mixture was fractionated on a 6% polyacrylamide gel for 2 h at 180 V. The gel was dried and exposed to film at −70°C for 2–3 h.

STATISTICAL ANALYSIS

Data are expressed as mean ± SEM. Each experiment was repeated at least three times. Statistical significance of differences between the two groups of data (P-value <0.05) was analyzed using one-way analysis of variance (ANOVA) followed by Tukey's pairwise comparison (Minitab, State College, PA).

RESULTS

INDUCTION OF OXIDATIVE STRESS IN PRIMARY RAT ASTROCYTES BY $CoCl_2$

Morphological examination showed that exposure to $CoCl_2$ at the concentrations of 0.1–0.5 mM did not induce significant change in astrocytic morphology. Moreover, MTT assay for cell metabolic activity (Fig. 1B) and trypan blue exclusion analysis were performed for examination of cell viability. The results indicated that exposure to $CoCl_2$ for 24 h at the used concentrations did not induce cell death in astrocytic culture (Fig. 1C).

The results from further experiments indicated that exposure to $CoCl_2$ at the concentrations of 0.2, 0.3 and 0.5 mM for 24 h significantly increased ROS levels in astrocytes (Fig. 2A). Time course experiments indicated that ROS increase in primary rat astrocytes was initially detected at 2 h after exposure to 0.5 mM of $CoCl_2$, and sustained over the entire time period analyzed (Fig. 2B). We also noticed that the amount of ROS in the rat astrocytes returned to basal levels when the cultures were incubated in $CoCl_2$-free medium for 22 h after 2-h exposure to 0.5 mM of $CoCl_2$ (Fig. 2B). The results reveal that cobalt-induced rise of ROS in astrocytes is reversible.

On the other hand, GAPDH gene is expressed at steady state under normoxic condition, but its expression is increased in response to hypoxic and oxidative stress [Ito et al., 1996; Graven et al., 1998]. Accordingly, we examined GAPDH expression levels to further confirm the characteristic effect of cobalt on cellular oxidative stress. As shown in Figure 3A, a 6- to 10-fold increase in GAPDH mRNA expression was detected when astrocytes were treated for 24 h with $CoCl_2$ at the concentration range of 0.2–0.5 mM. Moreover, GAPDH mRNA levels were time dependently increased (Fig. 3B). The findings further demonstrate that sustained exposure to $CoCl_2$ caused hypoxia-induced oxidative stress in astrocytes.

REDUCTION IN TSP-1 mRNA AND PROTEIN LEVELS BY $CoCl_2$

An increase in TSP-1 was observed at the early phase after tissue ischemia or trauma [Lin et al., 2003; Thakar et al., 2005; Tran and Neary, 2006]. In vitro study also indicated that TSP-1 levels were

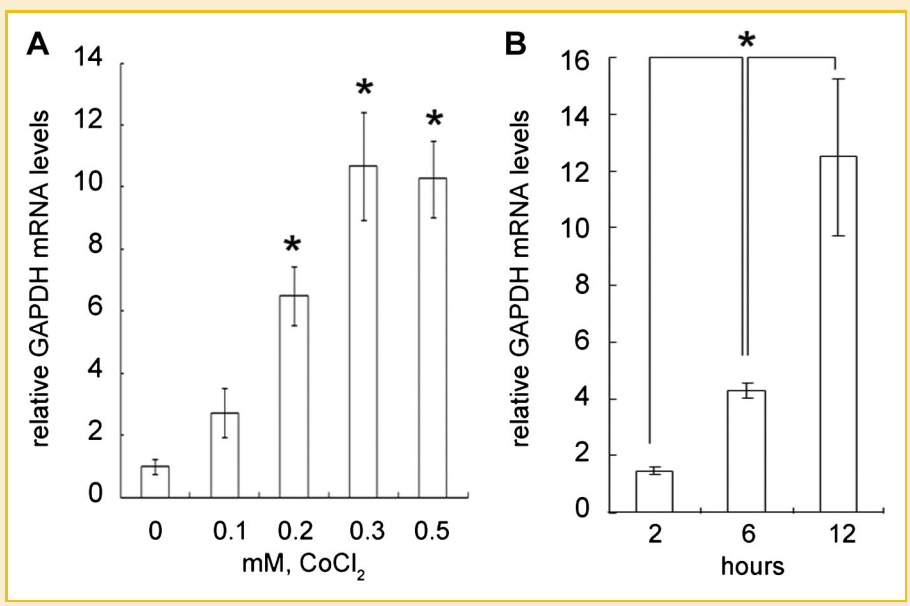

Fig. 3. Upregulation of GAPDH mRNA expression induced by $CoCl_2$. Astrocytes were treated with $CoCl_2$ for 24 h at concentrations of 0.1–0.5 mM (A). In addition, time-course experiments for examination of GAPDH mRNA expression levels were performed after astrocytes were treated with $CoCl_2$ (0.5 mM) for 2, 6, and 12 h (B). After treatment, the cultures were subjected to Q-PCR analysis for the examination of GAPDH mRNA expression. Data shown in the graph of the quantification are means ± SEM of three independent experiments. *$P<0.05$ versus control culture.

increased in astocytes under mechanical strain-induced stress [Tran and Neary, 2006]. Therefore, we examined whether TSP-1 gene expression was altered in astrocytes treated with $CoCl_2$. Treatment with $CoCl_2$ for 24 h at 0.2 mM induced approximately 50% inhibition in TSP-1 mRNA expression ($P = 0.0002$), and 0.5 mM of $CoCl_2$ reduced TSP-1 mRNA levels by 80% ($P = 0.0004$), when compared to that observed in the control culture (Fig. 4A). The results from time-course experiments showed that the downregulation of TSP-1 mRNA expression was observed as early as 12 h after exposure to 0.5 mM of $CoCl_2$ ($P = 0.03$ vs. control at 12 h). In addition, as shown in Figure 4C, approximately 50–70% decrease in TSP-1 proteins levels in astrocytes treated for 24 h with $CoCl_2$ ($P = 0.02$, 0.3 mM vs. control; $P = 0.01$, 0.5 mM vs. control). In parallel experiments, TSP-1 protein levels in the cultured medium of $CoCl_2$-treated astrocytes were also reduced (Fig. 4C).

INCREASED GAPDH mRNA EXPRESSION AND REDUCED TSP-1 GENE EXPRESSION AFTER $CoCl_2$ Removal

When astrocytes were pretreated with 0.5 mM of $CoCl_2$ for 2 and 6 h and then maintained in $CoCl_2$-free culture medium (Fig. 5A), a decrease in TSP-1 mRNA levels in astrocytes remained observed (Fig. 5B). Moreover, an increase in GAPDH gene expression was still detected after $CoCl_2$ was removed from the culture by supplying fresh medium (Fig. 5B). However, ROS generation was decreased to the basal levels when astrocytes were cultured in $CoCl_2$-free medium after a 2-h exposure to 0.5 mM of $CoCl_2$ (Fig. 2B). These findings implicated that cobalt entered into the cells may play a regulatory role in the expression of astrocytic TSP-1 and GAPDH. To examine whether cobalt was up-taken by astrocytes, the amounts of intracellular cobalt were analyzed, revealing that intracellular cobalt levels were indeed increased in a dose- and time-dependent manner (Fig. 5C). Based on our observations, we suggest that cobalt

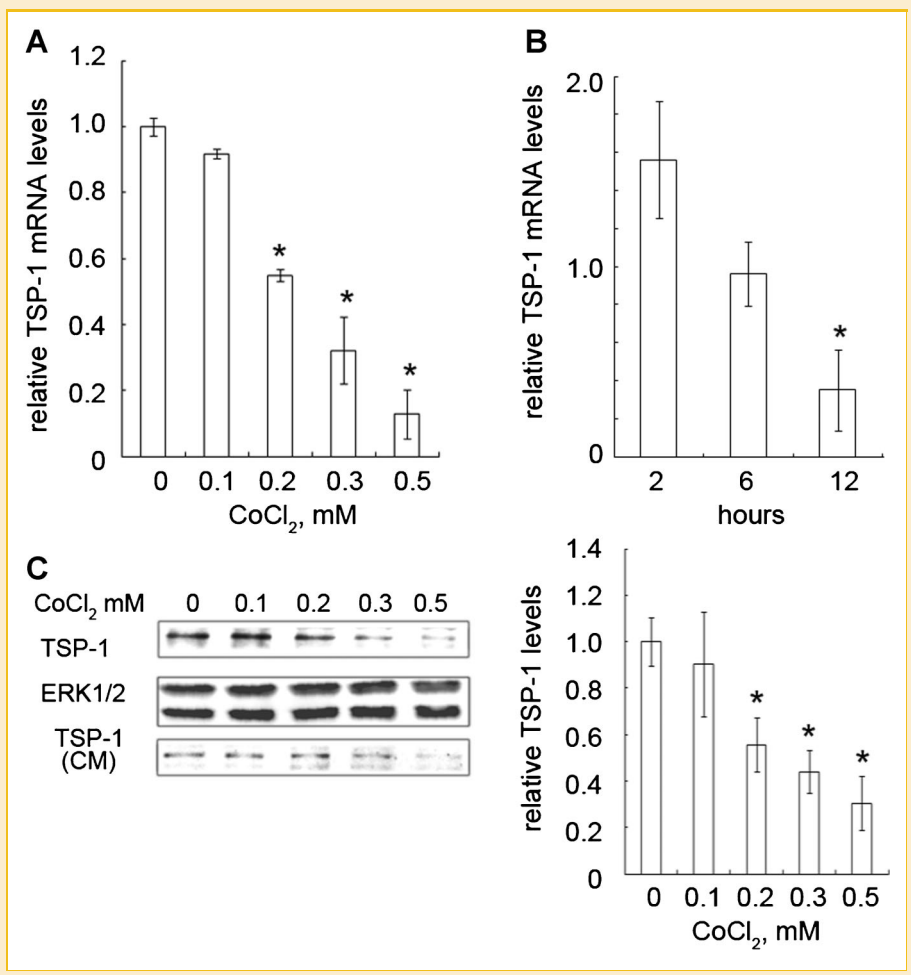

Fig. 4. $CoCl_2$-induced decrease in TSP-1 production in primary rat astrocytes. Astrocytes were treated with $CoCl_2$ at the different concentrations (A), or exposed to $CoCl_2$ (0.5 mM) for the distinct time periods indicated as above (B). Subsequently, the cultures were subjected to Q-PCR for examination of TSP-1 mRNA expression. For TSP-1 protein production, astrocytes were exposed to $CoCl_2$ for 24 h at the different concentrations indicated above (C). After harvest, the cells and culture medium were subjected to Western blotting. Immunoblot images (left panel in C) are representative of three independent experiments performed. Relative intensity of the bands corresponding to TSP-1 in the left panel was quantified by densitometry. The level of ERK1/2 protein was used as a loading control (right panel in C). TSP-1 intensity was normalized by ERK1 (44-kDa) intensity. Values are expressed as relative levels by normalized TSP-1 intensity from the treated cultures over that from the control. Data are means ± SEM of three independent experiments. $^*P < 0.05$ versus control culture.

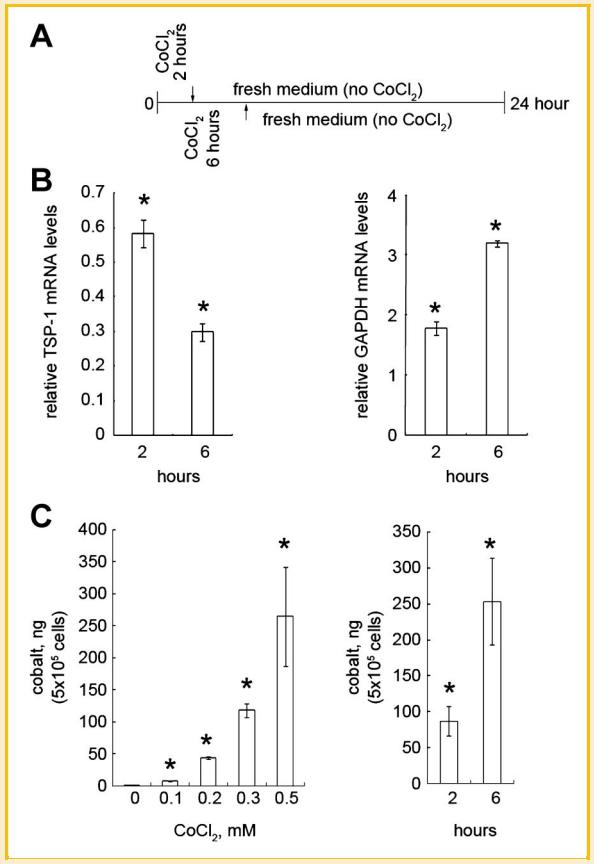

Fig. 5. Expression of TSP-1 and GAPDH mRNA in primary rat astrocytes after $CoCl_2$ removal. A: The diagram indicates that astrocytes were exposed to $CoCl_2$ (0.5 mM) for 2 or 6 h; then the cultures were maintained in $CoCl_2$-free medium over 18–24 h. B: After treatment, total RNA was isolated and then subjected for the expression of TSP-1 or GAPDH mRNA using Q-PCR analysis. C: ICP-MS analysis was performed to measure intracellular cobalt levels after astrocytes were treated for 24 h with $CoCl_2$ at the different concentrations, or exposed to $CoCl_2$ (0.5 mM) for 2 or 6 h. Data are means ± SEM of three independent experiments. $^*P < 0.05$ versus control cultures.

entered into the cells may also play a role in the inhibition of astrocytic TSP-1 expression.

Next, astrocyte-Neuro2A coculture was performed to examine if the supporting function of astrocytes for the neurite extension was impaired after pre-exposure to $CoCl_2$. Astrocytes were pretreated with 0.5 mM of $CoCl_2$ for 6 h, and then indirectly cocultured with Neuro2A cells in $CoCl_2$-free medium for 2 days. DAPI nuclear staining showed that there was no significant cell death in Neuro2A culture when these cells were cocultured indirectly either with control astrocytes or with $CoCl_2$-pretreated astrocytes (Fig. 6A). When Neuro2A cells were cultured in serum-free medium for 2 days, approximately 80% of Neuro2A cells extended neurite-like cell processes (Fig. 6B). Furthermore, coculture of Neuro2A cells indirectly with astrocytes significantly enhanced neurite extension in Neuro2A cells as compared to Neuro2A cells without astrocyte coculture (Fig. 6C). As expected, $CoCl_2$-pretreated astrocytes significantly reduced the neurite length of Neuro2A cells when compared to that observed in the group with astrocyte coculture (Fig. 6C).

INVOLVEMENT OF MAPK/ERK AND ATF-1 IN COBALT-INDUCED INHIBITION OF TSP-1 mRNA EXPRESSION

It has been reported that $CoCl_2$ activated MAPK to induce cell apoptosis in C6 glioma and pheochromocytoma PC12 cells [Zou et al., 2002; Yang et al., 2004]. To determine the involvement of MAPK in cobalt-induced inhibition of TSP-1, we used the two specific inhibitors (U0126 and PD98059) of MAPK kinase (MEK) that acts as the upstream activator of MAPK/ERK. Although U0126 itself was found to reduce TSP-1 mRNA levels in astrocytes, this reduction was not biostatistically different ($P = 0.133$) as compared to the control. However, pretreatment with U0126 caused an increase in TSP-1 mRNA expression by 40% in $CoCl_2$-treated astrocytes as compared to the cultures with $CoCl_2$ exposure, but without U0126 pretreatment ($P = 0.014$; Fig. 7A). Although PD98059 alone suppressed TSP-1 mRNA expression in astrocytes, TSP-1 mRNA levels were increased in $CoCl_2$-treated astrocytes by pretreatment with PD98059, when compared to that observed in the group by treatment only with $CoCl_2$ (Fig. 7A). The results suggest that MAPK/ERK was involved in the action of $CoCl_2$-induced oxidative stress on the inhibition of TSP-1 mRNA expression.

The activation of activating transcription factor-1 (ATF-1) is regulated by MAPK/ERK signaling pathway [Gupta and Prywes, 2002]. ATF-1 has also known to act as a negative regulator of TSP-1 expression in nickel-treated 3T3 cells and papillary carcinoma of the thyroid triggered by hepatocyte growth factor [Salnikow et al., 1997; Ghoneim et al., 2007]. To determine whether ATF-1 is involved in $CoCl_2$-mediated TSP-1 expression, we conducted a nuclear staining for ATF-1 in astrocytes treated with 0.3 and 0.5 mM of $CoCl_2$ for 24 h (Fig. 7B). Moreover, the results from EMSA indicated that a time-dependent increase in the binding activity of nuclear extracts to ATF/CREB-responsive elements was induced by 0.5 mM of $CoCl_2$ (Fig. 7C). The binding specificity was confirmed by the observation that the binding to ATF/CREB-responsive elements was decreased by preincubation of the nuclear extracts with anti-ATF-1 antibody (Fig. 7C). These results reveal that cobalt may suppress TSP-1 mRNA expression by the activation of the signaling pathway associated with MEK/MAPK and ATF-1.

EFFICIENCY OF *N*-ACETYL-L-CYSTEINE (NAC) ON THE BLOCKAGE OF COBALT INHIBITION IN TSP-1 mRNA EXPRESSION

To determine whether cobalt-induced rise in ROS levels is a critical player in suppression of astrocytic TSP-1 mRNA expression, the potent ROS scavenger NAC was used. As shown in Figure 8A, pretreatment with NAC effectively reduced ROS production in $CoCl_2$-treated astrocytes when compared to that detected in astrocytes treated only with $CoCl_2$ ($P = 0.0009$). In addition, pretreatment with NAC blocked the inhibitory effect of $CoCl_2$ on TSP-1 mRNA expression in astrocytes (Fig. 8B). However, resveratrol, a naturally occurring antioxidant, did not attenuated ROS generation in $CoCl_2$-treated astrocytes (Fig. 8C). As shown in Figure 8D, resveratrol failed to rescue $CoCl_2$-induced inhibition in TSP-1 mRNA expression. Moreover, resveratrol alone significantly reduced TSP-1 mRNA levels in astrocytes. The results indicate that

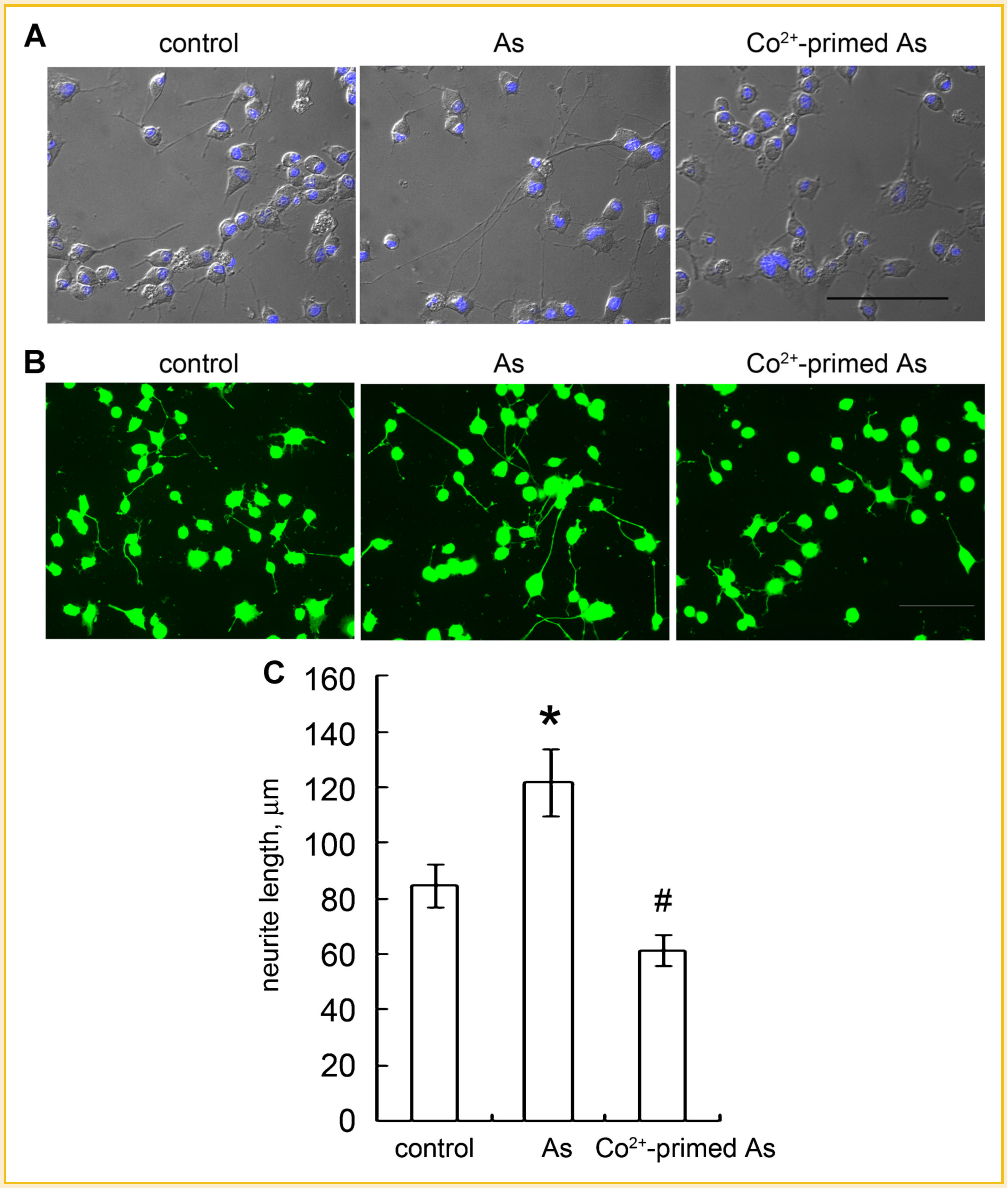

Fig. 6. Inhibition of neurite extension of Neuro2A cells by $CoCl_2$-treated astrocytes. Astrocytes cultured in cell inserts were treated without (As) or with $CoCl_2$ (0.5 mM) for 6 h (Co^{2+}-primed As). The cell inserts (As or Co^{2+}-primed As) were washed, and then placed onto Neuro2A cells-seeded culture plates. The cocultures were maintained in serum-free medium without $CoCl_2$ (0.5 mM) for 2 days. Neuro2A cells without astrocyte coculture were referred as the control group. The cultures were subjected to DAPI nuclear staining (A), or immunostaining for NF-200 for morphological examination (B). Neurite length (C) in the differentiated Neuro2A was quantified as described in the Materials and Methods Section. $^{*}P < 0.05$, As group versus control group; * or $^{\#}P < 0.01$; however, * indicates As versus control; # indicates Co^{2+}-primed As to As. Scale bar, 100 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

cobalt-induced oxidative stress may be involved in the inhibition of TSP-1 mRNA expression in astrocytes.

DISCUSSION

Cobalt is known to disrupt the conversion of citrate to α-ketoglutarate in Krebs cycle, and subsequently interfere with the process of cellular respiration [Wiberg, 1968]. The inhibition further causes ATP depletion and reduces mitochondrial membrane potential, leading to mitochondrial impairment and cell death [Gupta, 2001; Joza et al., 2001]. Among neural cell populations in the CNS, astrocytes are more resistant to heavy metal-induced oxidative stress [Tiffany-Castiglioni et al., 1989a; Yang et al., 2008; Wang et al., 2009]. It could be due to the fact that astrocytes can produce higher levels of antioxidants (e.g., glutathione) and antioxidative enzymes (e.g., superoxide dismutase, glutathione peroxidase) when compared to that detected in neurons [Khanna and Nehru, 2007]. A previous study has reported that treatment of primary mouse astrocytes with $CoCl_2$ at the concentration range of 0.2–0.8 mM caused ATP depletion and cell death by apoptosis and necrosis [Karovic et al., 2007]. In this study, primary rat astrocytes

that were exposed to $CoCl_2$ for 24 h at the concentrations of 0.1–0.5 mM displayed no significant reduction in cell viability. The discrepancy could be due to the distinct plating cell density, since the culture with the lower seeding density was susceptible to cobalt-induced cytotoxicity (unpublished observation of Zhan and Tzeng).

In consistence with the findings from other laboratories [Chandel et al., 1998; Grasselli et al., 2005; Kotake-Nara et al., 2005], we have shown that exposure to cobalt increased intracellular levels of ROS in astrocytes. Interestingly, cobalt-induced production of ROS in mouse astrocytes has been reported to be suppressed by heme oxygenase 1 (HO-1) that is the target gene of HIF-1α and a substance known to be increased by cobalt exposure [Karovic et al., 2007]. The findings may explain our results that only 1.5- to 2-fold increase in ROS levels was observed in $CoCl_2$-treated astrocytes compared to the control cultures. GAPDH, another indicator for hypoxic stress, has been known to not simply only serve as glycolytic enzyme, but also have roles in several cellular functions, such as endocytosis, nuclear RNA export, DNA replication and DNA repair [Sirover, 1999, 2005]. GAPDH transcription has been reported to be remarkably upegulated under hypoxia [Ito et al., 1996; Graven et al., 1998; Zhong and Simons, 1999], since its promoter gene region contains hypoxic regulatory elements (HREs) for HIF-1 proteins [Graven et al., 1999; Lu et al., 2002]. We did observe that GAPDH gene expression was profoundly increased in astrocytes after exposure to $CoCl_2$, although GAPDH proteins levels were marginally increased by $CoCl_2$ (unpublished observation of Zhan and Tzeng). Overall, our results, in conjunction with the findings from others, point to cobalt inducing hypoxia and oxidative stress in astrocytes.

Further evidence indicates that exposure to $CoCl_2$ significantly suppressed the expression of TSP-1 mRNA and proteins in astrocytes. It has been reported that oxidative stress is not a major factor for $CoCl_2$ cytotoxicity in primary mouse astrocytes [Karovic et al., 2007]. However, our results reveal that efficient reduction in $CoCl_2$-induced ROS production by NAC, but not resveratrol, prevents the inhibition of TSP-1 mRNA expression in $CoCl_2$-treated astrocytes. Thus, oxidative stress is involved in $CoCl_2$-induced inhibition of TSP-1 mRNA expression in astrocytes. On the other hand, our data show that cobalt-induced increase in intracellular ROS was blocked after the removal of $CoCl_2$ from the culture medium. Yet, the decrease in TSP-1 mRNA expression was still detected after astrocytes were recovered from the incubation in $CoCl_2$-containing medium. Possibly, cobalt that has entered astrocytes may associate with its intracellular targets to mediate TSP-1 mRNA expression in astrocytes. Based on our observations, cobalt-induced hypoxic stress is considered as a critical factor to inhibit TSP-1 mRNA expression in $CoCl_2$-treated astrocytes.

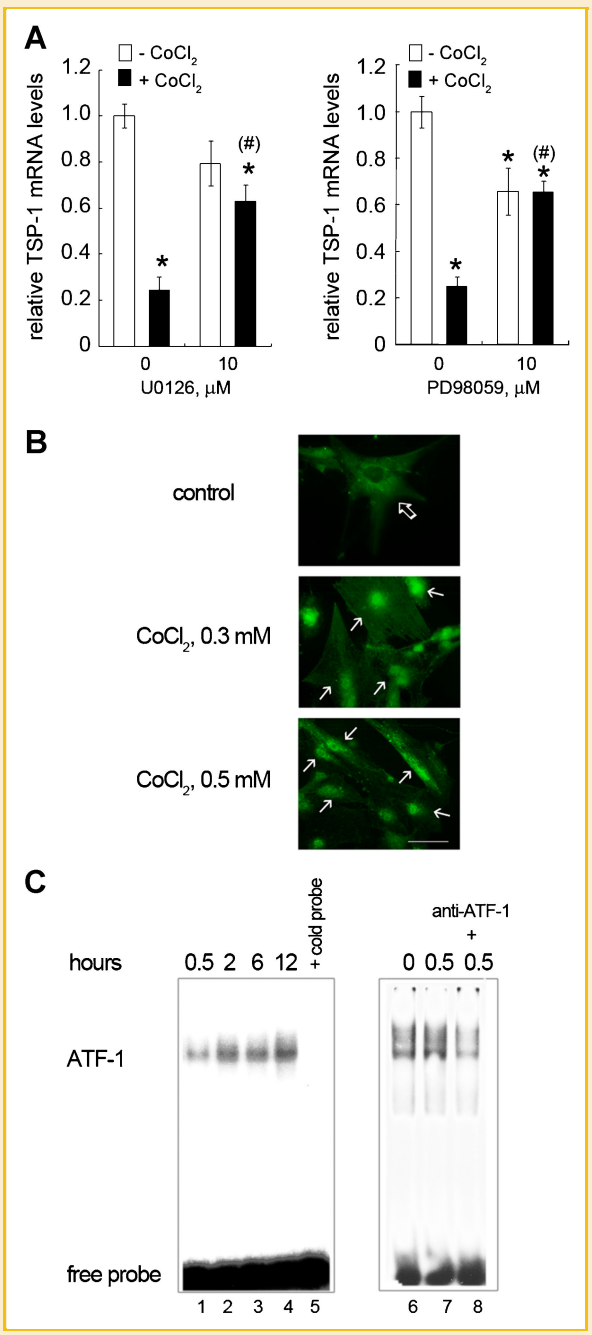

Fig. 7. Involvement of MAPK/ERK and ATF-1 in $CoCl_2$-induced reduction of TSP-1 mRNA expression. A: Astrocytes were pretreated with 10 μM of U0126 (MEK inhibitor) and 10 μM of PD98059 (MEK inhibitor) for 10 min, and then exposed to $CoCl_2$ (0.5 mM) for 24 h. The cells were subjected to Q-PCR for measurement of TSP-1 mRNA levels. Data are means ± SEM of three independent experiments. $^{*}P < 0.05$ versus control cultures. $^{\#}P < 0.05$ versus $CoCl_2$-treated cultures. B: Astrocytes were exposed to $CoCl_2$ (0.3 and 0.5 mM) for 24 h. Cellular localization of ATF-1 was observed by immmunofluorescence. The subcellular location of ATF-1 was indicated by open arrow (cytoplasmic ATF-1) or arrows (nuclear ATF-1), respectively. Scale bar, 50 μm. C: Astrocytes were exposed to $CoCl_2$ (0.5 mM) for the distinct time periods indicated above. The binding of nuclear extracts isolated from $CoCl_2$-treated astrocytes was analyzed by EMSA which was performed with labeled oligonucleotides containing the consensus sequences of ATF-1/CREB. The binding specificity was confirmed in the presence of unlabeled oligonucleotides (100-fold molar excess). DNA/ATF-1 complexes were confirmed by examining the inhibition of the DNA binding when nuclear extracts were incubated with anti-ATF-1 antibody in the presence of labeled probes. The experiments were repeated twice with similar observations. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

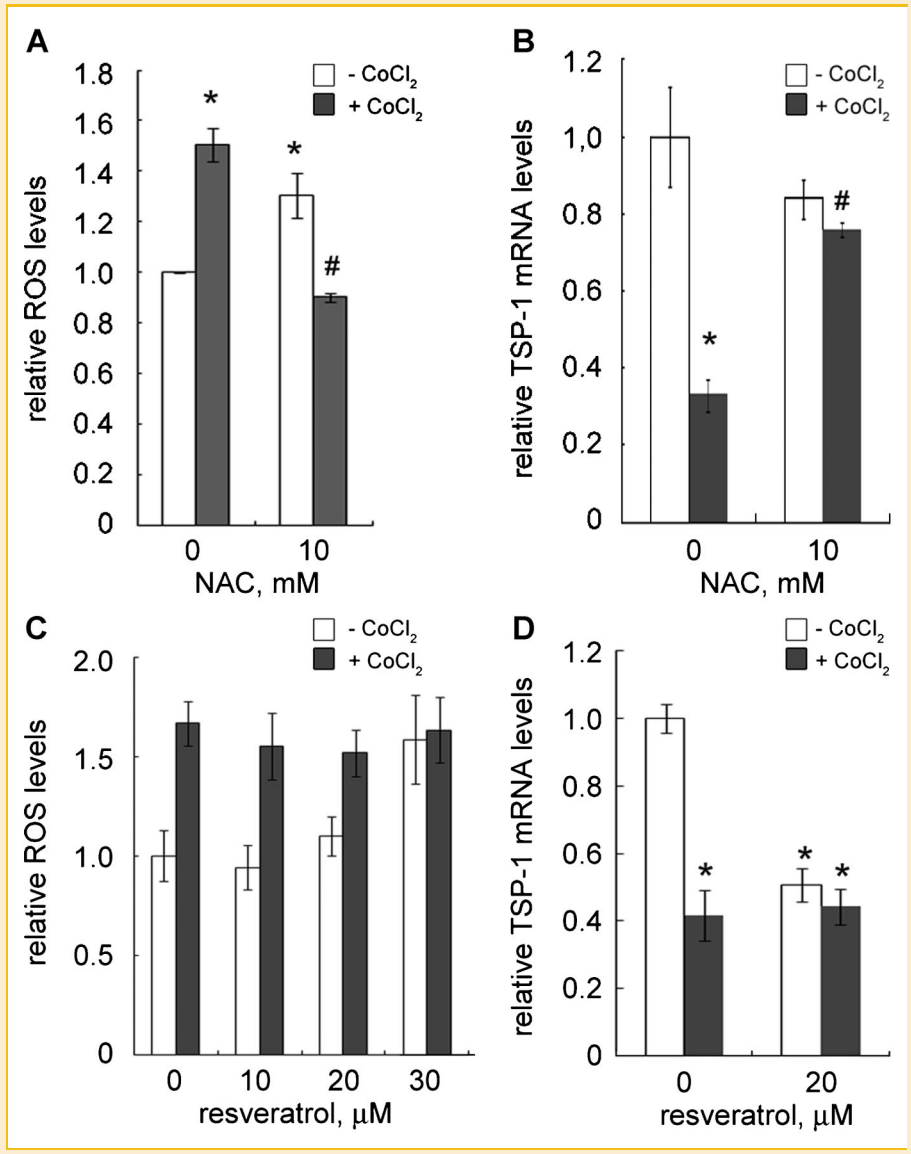

Fig. 8. Blockade of $CoCl_2$-induced inhibition on TSP-1 mRNA expression by N-acetylcysteine (NAC). A: Astrocytes were preloaded with the ROS indicator CM-H_2DCFDA for 45 min, and then treated with NAC (10 mM) for 1 h. The cultures were subsequently exposed to $CoCl_2$ (0.5 mM) for 6 h, and then measured intracellular ROS levels analyzed by Flow Cytometry. B: Astrocytes were pretreated with NAC (10 mM) for 1 h and exposed to $CoCl_2$ (0.5 mM) for 24 h. Total RNA was isolated and subjected to Q-PCR analysis for measurement of TSP-1 mRNA levels in astrocytes. C: Astrocytes were pretreated for 30 min with resveratrol at the different concentrations as indicated above, and then exposed to $CoCl_2$ (0.5 mM) for 24 h. The cultures were subsequently subjected to ROS measurement. D: Astrocytes were pretreated for 30 min with resveratrol (20 μM), and exposed to $CoCl_2$ (0.5 mM) for 24 h. The cultures were subjected to Q-PCR analysis for measurement of TSP-1 mRNA expression in astrocytes. Data are means ± SEM of three independent experiments. $^{*}P < 0.05$ versus control group. $^{\#}P < 0.05$ in A and B versus $CoCl_2$-treated group.

Given that stress-activated protein kinase JNK is activated under hypoxia [Comerford et al., 2004], we examined the role of JNK in the regulation of TSP-1 mRNA expression in $CoCl_2$-treated astrocytes. However, our unpublished observation showed that treatment with SP600125 (JNK inhibitor) alone reduced TSP-1 mRNA levels by 80% in astrocytes, indicating that JNK is essential for the maintenance of TSP-1 mRNA expression in astrocytes. In contrast, our data show that $CoCl_2$ failed to reduce TSP-1 mRNA expression in astrocytes pretreated with the inhibitors for MAPK/ERK, raising the possibility that MAPK/ERK signaling is involved in the effect of cobalt-induced oxidative stress on the reduction of TSP-1 mRNA expression. ATF-1 has been identified as a negative regulator for TSP-1 mRNA expression via interaction with the CRE site on the promoter of TSP-1 [Salnikow et al., 1997; Ghoneim et al., 2007]. It is also known that ATF-1 can be activated by MAPK/ERK signaling [Gupta and Prywes, 2002]. Thus, our further study that exposure to $CoCl_2$ induces nuclear localization of ATF-1 in astrocytes and increases the binding of astrocytic nuclear extract to ATF-1/CREB, suggests that MAPK/ERK may activate ATF-1 to regulate TSP-1 mRNA expression in astrocytes.

In summary, we demonstrate cobalt-induced oxidative stress inhibits TSP-1 expression in primary rat astrocytes through MAPK/

ATF-1 action. Inhibited TSP-1 expression in astrocytes by exposure to cobalt may lessen neurite extension. Suppression of ROS generation by treatment with NAC can abolish cobalt-induced inhibition in astrocytic TSP-1 mRNA expression. Our study suggests that hypoxia-induced oxidative stress reduces TSP-1 production by astrocytes and hinders the promotion of neurite growth, which may be prevented by the addition of the antioxidant NAC.

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