Cobalt chloride treatment induces autophagic apoptosis in human glioma cells via a p53-dependent pathway

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Abstract. Malignant glioma is the most aggressive brain tumor. Hypoxic condition has been explored for killing cancer stem cells or drug-resistant tumor cells. This study investigated the effects of hypoxia on autophagic death and the possible mechanisms. Exposure of human malignant glioma U87-MG cells to cobalt chloride ($CoCl_2$) increased cellular hypoxia-inducible factor-1α levels and concurrently decreased cell viability concentration- and time-dependently. In parallel, treatment with $CoCl_2$ suppressed proliferation of human U87-MG cells. Autophagic cells and levels of LC3-II were concentration- and time-dependently induced in human U87-MG cells after exposure to $CoCl_2$. However, pretreatment with 3-mehyladenine (3-MA) and chloroquine, inhibitors of cell autophagy, caused significant alleviations in $CoCl_2$-induced cell autophagy. In contrast, exposure to rapamycin, an inducer of cell autophagy, synergistically induced hypoxia-induced autophagy of U87-MG cells. Administration of human U87-MG cells with $CoCl_2$ triggered caspase-3 activation and cell apoptosis. Interestingly, pretreatment with 3-MA and chloroquine remarkably suppressed $CoCl_2$-induced caspase-3 activation and cell apoptosis. Application of p53 small interference (si)RNA into human U87-MG cells downregulated levels of this protein and simultaneously lowered hypoxia- and 3-MA-induced alterations in cell autophagy, apoptosis, and death. The hypoxia-induced autophagy and apoptosis of DBTRG-05MG cells were significantly lowered by 3-MA pretreatment and p53 knockdown. Therefore, the present study shows that $CoCl_2$ treatment can induce autophagy of human glioma cells and subsequent autophagic apoptosis via a p53-dependent pathway. Hypoxia-induced autophagic apoptosis may be applied as a therapeutic strategy for treatment of glioma patients.

Introduction

Gliomas are primary solid tumors of the brain and may arise from transformations of three major types of glial cells, including astrocytes, oligodendrocytes, and ependymal cells (1). According to the World Health Organization grading system, gliomas are classed into low-grade tumors (grades I and II) and high-grade tumors (grades III and IV). Glioblastoma multiforme (GBM), classified as a malignant grade IV glioma, is the most aggressive brain tumor (2). The location of tumor occurrence in the brain and the high variability in genetic abnormalities are two major risk factors existing in malignant gliomas (3). In addition, malignant gliomas present unique features of rapid growth and high migration that consequently result in poor prognoses and high mortality rates in GBM patients (4). In the clinic, the median overall survival time and the 5-year survival rate of GBM patients are ~10.2-14.6 months and 5%, respectively (5). Therefore, discovering more-effective therapeutic strategies for glioma patients remains a challenge today.

Hypoxia is a condition in which a tissue is not satisfactorily oxygenated. Under hypoxic stimulation, tissues/cells can be driven either to survive by expanding the glycolysis rate or to death via a programmed pathway (6). In contrast, hypoxic microenvironments inside cancers are able to promote tumor growth, angiogenesis, and invasion (7). Brain hypoxia frequently occurs in brain neoplasms and traumatic brain injuries and is furthermore recognized as a major potential cause of secondary injury in neurologically critically ill patients (8). In hypoxic conditions, hypoxia-inducible factor (HIF)-1α,

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a transcription factor, is highly induced and essentially participates in regulating cell survival or death (9,10). HIF-1α functions by binding to specific recognition sequences in the genome to stimulate transcriptions of certain genes involved in various metabolic pathways that are necessary for cells in response to an oxygen-poor environment (11,12). Joseph *et al*, reported that an HIF-1α-mediated signaling axis contributes to a hypoxia-induced mesenchymal shift, migration, and invasion in malignant gliomas (13). Nonetheless, a previous study showed the positive roles of HIF-1α in hypoxia-induced glioblastoma cell death (14). Therefore, hypoxia has bidirectional roles in controlling glioma growth and death.

Autophagy is a preserved progression inside cells that uses double-membrane vesicles, called autophagosomes, to deliver cytoplasmic contents to lysosomes for degradation of long-lived and misfolded proteins, damaged and dysfunctional organelles, and foreign particles (15,16). Microtubule-associated protein light chain 3 (LC3), a consistent marker of autophagy, is important for the progression of autophagy because it is essential for the formation of autophagosomes (17). Autophagy is critical for maintaining homeostasis in certain diseases, including neurodegeneration, cancers and aging (15,18). Recently, induction of autophagy by drugs was considered a novel therapeutic strategy for treating GBM cancer stem cells and temozolomide (TMZ)-resistant glioma cells (19). Dolma *et al* showed that inhibition of the D4 dopamine receptor delays autophagic flux, proliferation, and survival of glioblastoma stem cells (20). In our lab, we proved the effects of honokiol, a polyphenol, on initiating autophagy and subsequent cell death in human malignant gliomas *in vitro* and *in vivo* (21). Recently, induction of hypoxia was investigated as an effective treatment strategy (19,22). However, autophagic cells can survive or undergo apoptosis (23,24). Our previous studies showed honokiol-induced autophagic death in neuroblastomas and gliomas (21,25). p53, a tumor suppressor protein, is involved in regulating cell survival, autophagy and apoptosis (26). In this study, we investigated the effects of hypoxia on autophagy and apoptosis of human malignant glioma cells and the possible mechanisms.

Materials and methods

Cell culture and drug treatment. Human glioma U87-MG cells and DBTRG-05MG cells purchased from American Type Culture Collection (Manassas, VA, USA) were cultured in minimum essential medium (MEM; Gibco-BRL Life Technologies, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 IU/ml penicillin, 100 mg/ml streptomycin, 1 mM sodium pyruvate, and 1 mM non-essential amino acids at 37°C in a humidified atmosphere of 5% CO_2. Cells were grown to confluence before drug treatment. Cobalt chloride ($CoCl_2$) purchased from Sigma (St. Louis, MO, USA) was freshly dissolved in in 1X phosphate-buffered saline (PBS; 0.14 M NaCl, 2.6 mM KCl, 8 mM Na_2HPO_4 and 1.5 mM KH_2PO_4). Human U87-MG cells were exposed to different concentrations of $CoCl_2$ and/or other agents indicated in the text for various intervals. Control cells received PBS only. 3-Methyladenine (3-MA) and chloroquine, inhibitors of cell autophagy, and rapamycin (Rapa), an inducer of cell autophagy, were purchased from Sigma. To determine the effects of 3-MA, chloroquine, and Rapa on

$CoCl_2$-induced autophagy, caspase-3 activation, cell apoptosis, and cell death, the glioma cells were pretreated with 1 mM 3-MA, 20 μM chloroquine, or 0.5 μM Rapa for 1 h and then exposed to $CoCl_2$.

Assays of cell morphology and cell viability. The toxicity of hypoxia to human glioma U87-MG cells was determined by analyses of cell morphology and viability as described previously (12). Briefly, U87-MG cells (10^4 cells/well) were seeded in 96-well tissue culture plates overnight. After drug treatment, U87-MG cells were cultured in new medium containing 0.5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide for a further 3 h. The blue formazan products in U87-MG cells were dissolved in dimethyl sulfoxide and spectrophotometrically measured at a wavelength of 550 nm. Cell morphologies were observed and photographed using a light microscope (Nikon, Tokyo, Japan).

Cell proliferation assay. The effect of hypoxia on cell proliferation was analyzed by measuring the incorporation of bromodeoxyuridine (BrdU) into genomic DNA as described previously (27). Human glioma U87-MG cells were seeded at $3x10^3$ cells/well in 96-well microtiter plates. After drug treatment, 10 mM BrdU was added to the culture medium for incorporation into the DNA of replicating cells. After 2 h of incubation, cells were fixed in 4% paraformaldehyde. BrdU incorporation was determined by a cell proliferation enzyme-linked immunosorbent assay (ELISA) BrdU kit (Roche, Mannheim, Germany).

Assay of cell autophagy. Cell autophagy was assayed by quantifying acidic vesicular organelles using flow cytometry as described previously (25). After drug treatment, human glioma U87-MG and DBTRG-05MG cells (10^5) were treated with 1 μg/ml of acridine orange (AO) for 20 min. Then, the cells were collected in phenol red-free MEM. The green and red fluorescence of AO in cells were measured with a flow cytometer (Becton-Dickinson, San Jose, CA, USA). Fluorescent intensities were quantified with the aid of CellQuest software (Becton-Dickinson). A fluorescent microscope (Nikon) was used to observe and photograph cells with fluorescent signals.

Assay of caspase-3 activity. Caspase-3 activity was determined using a fluorometric substrate assay kit as described previously (28). Briefly, after drug administration, human glioma U87-MG cells were lysed using a buffer containing 1% Nonidet P-40, 200 mM NaCl, 20 mM Tris/HCl (pH 7.4), 10 mg/ml leupeptin, 0.27 U/ml aprotinin, and 100 mm PMSF. Cell extracts (25 mg of total protein) were incubated with 50 mM of a specific fluorogenic peptide substrate in 200 ml of a cell-free system buffer composed of 10 mM HEPES (pH 7.4), 220 mM mannitol, 68 mM sucrose, 2 mM NaCl, 2.5 mM KH_2PO_4, 0.5 mM EGTA, 2 mM $MgCl_2$, 5 mM pyruvate, 0.1 mM PMSF, and 1 mM dithiothreitol. The peptide substrate for the caspase-3 enzyme assay was DEVD. The peptide was conjugated to 7-amino-4-trifluoromethyl coumarin for fluorescence detection. Intensities of the fluorescent products were measured with a spectrometer.

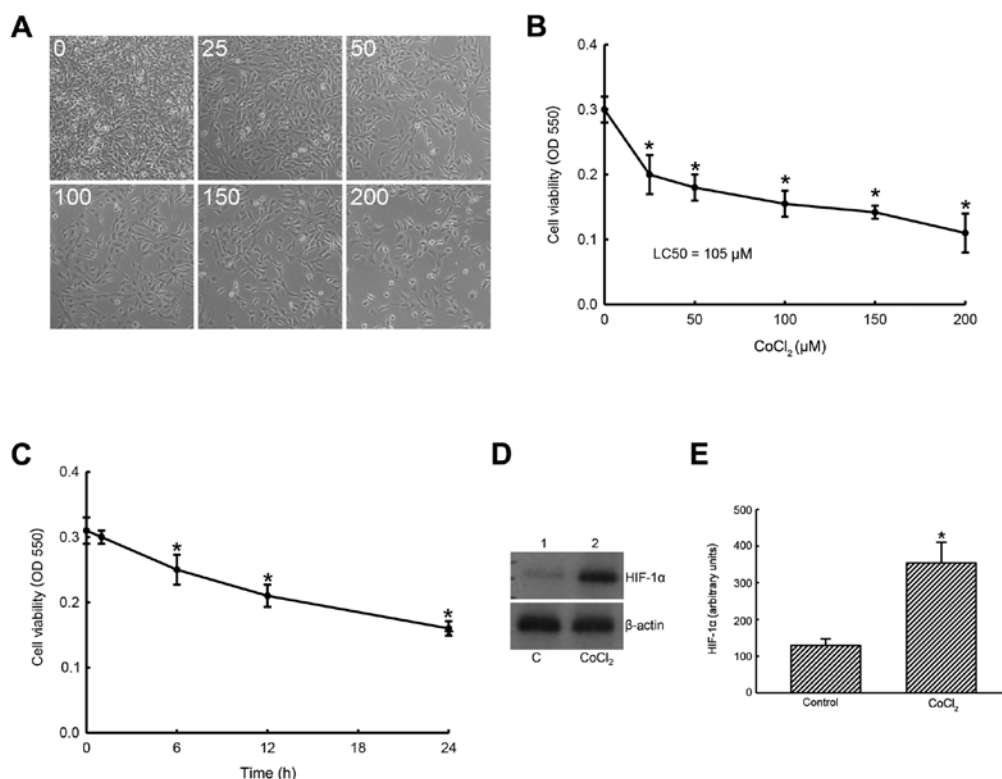*Quantification of apoptotic cells.* Apoptosis of human glioma U87-MG and DBTRG-05MG cells was determined using

Figure 1. Effects of cobalt chloride (CoCl₂) on cell morphology and cell viability. Human glioma U87-MG cells were treated with 25, 50, 100, 150 and 200 μM CoCl₂ for 24 h (A and B) or with 100 μM CoCl₂ for 1, 6, 12 and 24 h (C). Cell morphology was observed and photographed using a light microscope (A). Cell viability was assayed using a colorimetric method (B and C). Human U87-MG cells were exposed to 100 μM CoCl₂ for 24 h, and cellular proteins were prepared. Levels of hypoxia-inducible factor (HIF)-1α were immunodetected (D, left-top panel). β-actin was analyzed as the internal control (left-bottom panel). These protein bands were quantified and statistically analyzed (D, right panel). Each value represents the mean ± SEM for n=6. *p<0.05, value significantly differed from the control group.

propidium iodide (PI) to detect DNA injury in nuclei according to a previously described method (29). Briefly, after drug administration, human glioma U87-MG cells were harvested and fixed in cold 80% ethanol. Following centrifugation and washing, fixed cells were stained with PI and analyzed using a FACScan flow cytometer (Becton-Dickinson).

Immunoblot analyses. Protein analyses were performed according to a previously described assay (30). Briefly, after drug treatment, cell lysates were prepared in iced radioim-munoprecipitate test buffer (25 mM Tris HCl, 0.1% sodium dodecylsulfate (SDS). 1% Triton X-100, 1% sodium deoxycho-late, 0.15 M NaCl, and 1 mM EDTA). Protein concentrations were measured using a bicinchonic acid protein assay kit (Pierce, Rockford, IL, USA). The protein (50 μg/well) was subjected to SDS polyacrylamide gel electrophoresis (PAGE), and transferred to nitrocellulose membranes. After blocking, LC3-I, LC3-II, HIF-1α, and p53 were immunodetected using rabbit polyclonal antibodies or mouse monoclonal antibodies against related proteins (Cell Signaling, Danvers, MA, USA). Cellular β-actin protein was immunodetected using a mouse monoclonal antibody against mouse β-actin (Sigma) as the internal control. These protein bands were quantified using a digital imaging system (UVtec, Cambridge, UK).

p53 knockdown. Translation of p53 messenger (m)RNA in human glioma U87-MG and DBTRG-05MG cells was knocked-down using an RNA interference (RNAi) method

following a small interfering (si)RNA transfection protocol provided by Santa Cruz Biotechnology (Santa Cruz, CA, USA) as described previously (31). p53 siRNA was purchased from Santa Cruz Biotechnology, and is a pool of three target-specific 20-25-nt siRNAs designed to suppress p53 expression. Scrambled siRNA, purchased from Santa Cruz Biotechnology, contained non-targeting 20-25-nt siRNA and was applied to control cells as a negative standard. Briefly, after culturing U87-MG cells in antibiotic-free MEM at 37°C in a humidified atmosphere of 5% CO₂ for 24 h, the siRNA duplex solution, which was diluted in an siRNA transfection medium (Santa Cruz Biotechnology), was added to human glioma cells. After transfection with scrambled or p53 siRNA for 24 h, the medium was replaced with normal MEM, and the cells were treated with CoCl₂.

Statistical analysis. Statistical differences between the control and drug-treated groups were considered significant when the p-value of Duncan's multiple-range test was <0.05. Statistical analysis between drug-treated groups was carried out using a two-way analysis of variance (ANOVA).

Results

Cell morphology, cell viability, and cellular HIF-1α levels were analyzed to determine the effects of CoCl₂ on inducing of hypoxia and cytotoxicity in human glioma U87-MG cells (Fig. 1). Exposure of human U87-MG cells to 25 μM CoCl₂
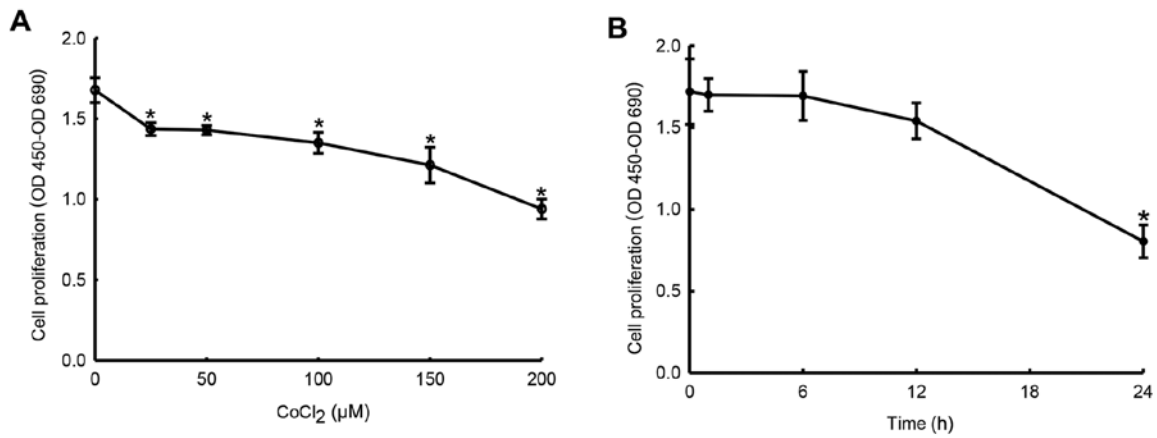
Figure 2. Effects of cobalt chloride (CoCl$_2$) on cell proliferation. Human glioma U87-MG cells were treated with 25, 50, 100, 150 and 200 μM CoCl$_2$ for 24 h (A) or with 100 μM CoCl$_2$ for 1, 6, 12 and 24 h (B). Cell proliferation was analyzed using a thymidine incorporation assay. Each value represents the mean ± SEM for n=6. *p<0.05, value significantly differed from the control group.
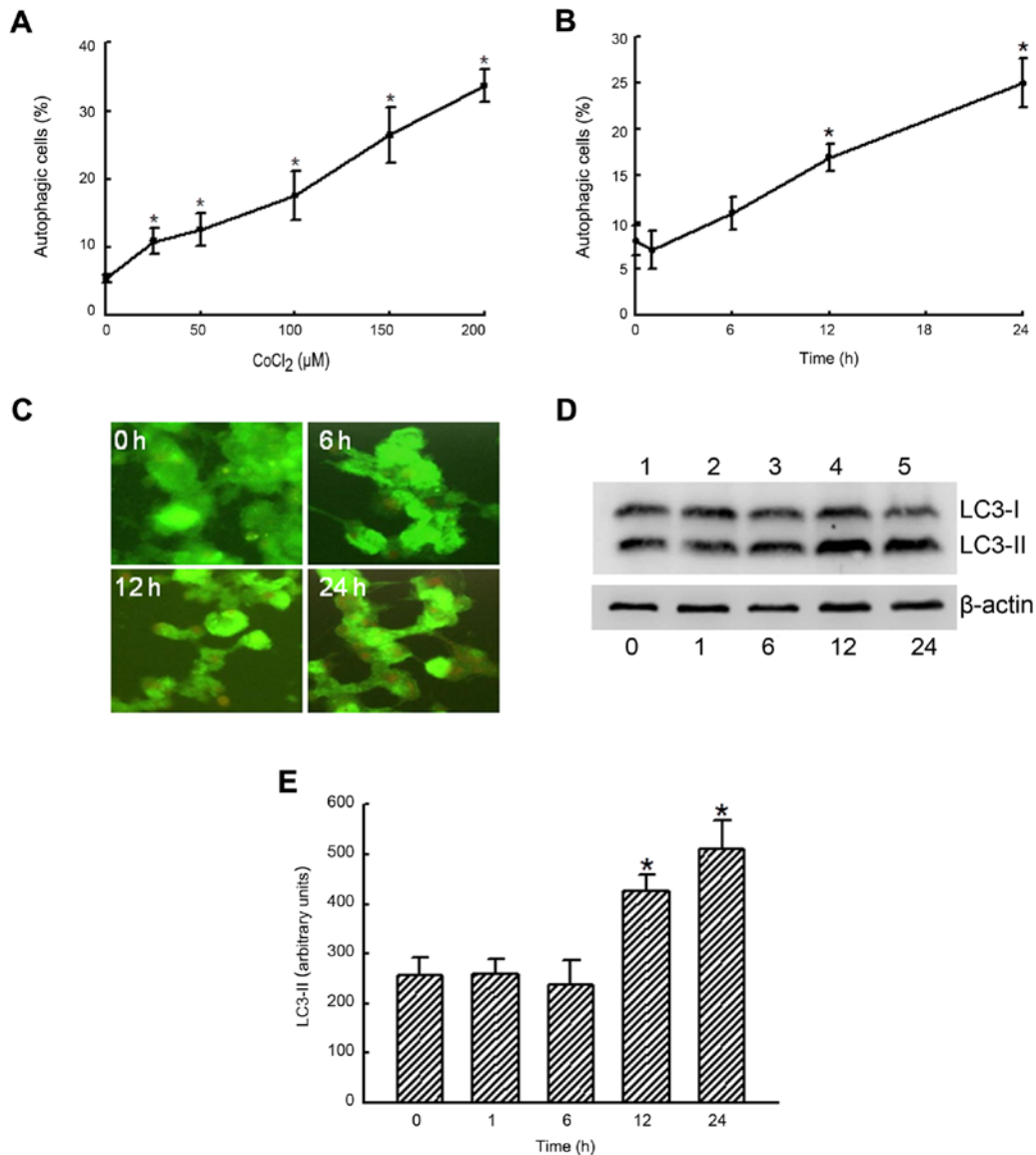

Figure 3. Effects of cobalt chloride (CoCl$_2$) on cell autophagy and levels of light chain 3-I (LC3-I) and LC3-II. Human glioma U87-MG cells were treated with 25, 50, 100, 150 and 200 μM CoCl$_2$ for 24 h (A) or with 100 μM CoCl$_2$ for 1, 6, 12 and 24 h (B-D). Cell autophagy was analyzed using a flow cytometer (A and B). Cells with fluorescent signals were observed and photographed with a fluorescent microscope at x200 (C). Levels of LC3-I and LC3-II were immunodetected (D, top panel). β-actin was analyzed as the internal control (bottom panel). These protein bands were quantified and statistically analyzed (E). Each value represents the mean ± SEM for n=6. *p<0.05, value significantly differed from the control group.
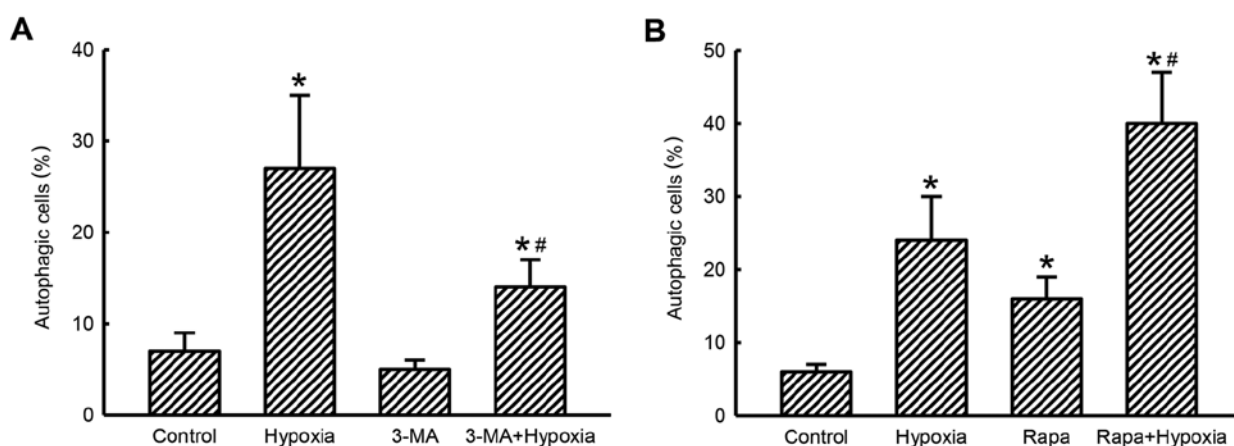
Figure 4. Effects of 3-methyladenine (3-MA) and rapamycin (Rapa) on cobalt chloride (CoCl₂)-induced cell autophagy. Human glioma U87-MG cells were pretreated with 1 mM 3-MA, an inhibitor of cell autophagy (A), or 0.5 μM Rapa, an inducer of cell autophagy (B), for 1 h and then exposed to 100 μM CoCl₂ for another 24 h (hypoxia). Cell autophagy was analyzed using flow cytometry. Each value represents the mean ± SEM for n=6. $^{*,\#}$p<0.05, values significantly differed from the control and hypoxia-treated groups, respectively.

for 24 h decreased cell numbers (Fig. 1A). After exposure to 50, 100, 150 and 200 μM CoCl₂ for 24 h, cell numbers time-dependently decreased. Treatment of human U87-MG cells with 25 μM CoCl₂ for 24 h caused a 31% reduction in cell viability (Fig. 1B). When the administered concentrations of CoCl₂ reached 50, 100, 150 and 200 μM, cell viabilities were diminished by 36, 45, 52 and 63%, respectively. Exposure of U87-MG cells to 100 μM CoCl₂ for 1 h did not influence cell viability (Fig. 1C). In contrast, viabilities of U87-MG cells were lowered by 19, 30 and 47% after CoCl₂ treatment for 6, 12 and 24 h, respectively. Low levels of HIF-1α were detected in untreated U87-MG cells (Fig. 1D, top panel, lane 1). However, exposure to 100 μM CoCl₂ for 24 h obviously enhanced amounts of cellular HIF-1α (lane 2). β-actin was analyzed as the internal control (bottom panel). These protein bands were quantified and statistically analyzed (Fig. 1E). Treatment with 100 μM CoCl₂ for 24 h significantly increased levels of HIF-1α in human U87-MG cells by 2.7-fold.

To decide the effects of CoCl₂ treatment on cell proliferation, a BrdU incorporation assay was conducted (Fig. 2). Exposure of human glioma U87-MG cells to 25 μM CoCl₂ for 24 h caused a 15% diminution in cell proliferation (Fig. 2A). In comparison, CoCl₂ at 50, 100, 150 and 200 μM suppressed proliferation of human U87-MG cells by 17, 20, 28 and 42%, respectively. Exposure of human U87-MG cells to 100 μM CoCl₂ for 1, 6, and 12 h did not affect cell proliferation (Fig. 2B). Nonetheless, treatment with CoCl₂ for 24 h caused a significant 49% decline in the proliferation of human U87-MG cells.

Autophagic cells and cellular LC3-II levels were examined in order to determine the effects of CoCl₂ treatment on autophagy of human U87-MG cells (Fig. 3). Treatment with 25 μM CoCl₂ for 24 h induced 16% of human U87-MG cells to undergo autophagy (Fig. 3A). Fractions of autophagic cells in human U87-MG cells were significantly augmented to 19, 28, 41 and 52% following respective exposure to 50, 28, 41 and 52% following respective exposure to 50, 100, 150 and 200 μM CoCl₂ for 24 h. Treatment of human U87-MG cells with 100 μM CoCl₂ for 1 and 6 h did not change cell autophagy (Fig. 3B). In contrast, after exposure for 12 and

24 h, CoCl₂ administration induced cell autophagy by 17 and 25%, respectively. Staining with AO revealed that exposure of human U87-MG cells to CoCl₂ for 6 h did not change the proportion of cells with acidic vesicular organelles (Fig. 3C). In comparison, after exposure to CoCl₂ for 12 or 24 h, proportions of human U87-MG cells stained with AO were augmented. Exposure of human U87-MG cells to 100 μM CoCl₂ for 1 or 6 h did not affect levels of the LC3-II protein (Fig. 3D, top panel, lanes 2 and 3). However, after treatment with CoCl₂ for 12 or 24 h, amounts of LC3-II in U87-MG cells were obviously augmented (lanes 4 and 5). β-actin was analyzed as the internal control (bottom panel). These protein bands were quantified and statistically analyzed (Fig. 3E). Exposure of human U87-MG cells to 100 μM CoCl₂ for 12 or 24 h led to 72 and 98% increases in amounts of the LC3-II protein.

To confirm CoCl₂-induced autophagy, 3-MA, an inhibitor of autophagy, and Rapa, an inducer of autophagy, were applied to human U87-MG cells (Fig. 4). Treatment of human U87-MG cells with 100 μM CoCl₂ for 24 h induced 28% of glioma cells to undergo autophagy (Fig. 4A). Pretreatment with 3-MA alone did not influence cell autophagy but significantly decreased hypoxia-induced cell autophagy by 68%. In contrast, pretreatment with Rapa alone triggered 16% of human U87-MG cells to undergo autophagy (Fig. 4B). Interestingly, pretreatment with Rapa synergistically induced hypoxia-induced cell autophagy by 40%.

Caspase-3 activity and apoptotic cells were analyzed to determine the effects of CoCl₂-induced autophagy on cell apoptosis (Fig. 5). Treatment of human U87-MG cells with 100 μM CoCl₂ for 1 and 6 h did not influence caspase-3 activity (Fig. 5A). When the treatment time intervals reached 12 and 24 h, caspase-3 activities were significantly augmented in human U87-MG cells by 2.5- and 3.8-fold, respectively. Administration of 100 μM CoCl₂ to human U87-MG cells for 1 and 6 h did not affect cell apoptosis (Fig. 5B). Nevertheless, after exposure to CoCl₂ for 12 or 24 h, the fractions of human U87-MG cells that underwent apoptosis were significantly increased to 24 and 37%. Pretreatment of human U87-MG cells
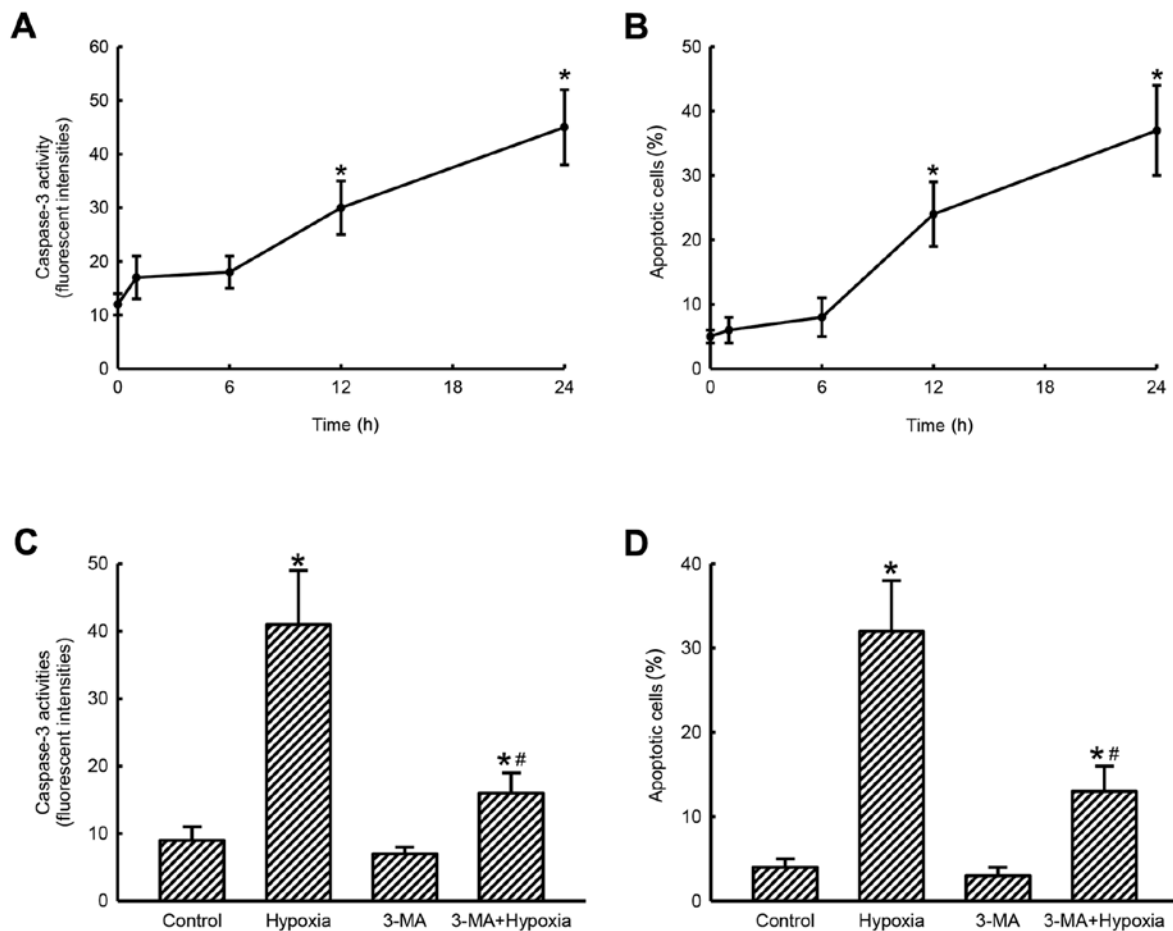
Figure 5. Effects of cobalt chloride (CoCl$_2$) and 3-methyladenine (3-MA) on caspase-3 activity and cell apoptosis. Human glioma U87-MG cells were treated with 100 μM CoCl$_2$ for 1, 6, 12 and 24 h. Caspase-3 activity was analyzed using a fluorometric substrate assay (A). Cell apoptosis was assayed using a flow cytometer (B). Human glioma U87-MG cells were pretreated with 1 mM 3-MA and exposed to 100 μM CoCl$_2$ for another 24 h (hypoxia). Caspase-3 activity (C) and cell apoptosis (D) were analyzed. Each value represents the mean $\pm$ SEM for n=6. [*,#]p<0.05, values significantly differed from the control and hypoxia-treated groups, respectively.

Table I. Effects of chloroquine (CLQ) on cobalt chloride (CoCl$_2$)-induced insults to human glioma cells.

	Cell viability (OD$_{550}$)	Cell autophagy (%)	Caspase-3 activity (FI)	Apoptotic cells (%)
Control	0.412±0.089	6±1	11±3	5±1
CoCl$_2$	0.133±0.048[a]	29±7[a]	41±8[a]	39±7[a]
CLQ	0.402±0.102	5±1	9±2	6±2
CLQ+hypoxia	0.268±0.069[a,b]	14±3[a,b]	16±4[a,b]	19±4[a,b]

Human glioma U87-MG cells were pretreated with 20 μM CLQ, an inhibitor of cell autophagy, for 1 h and then exposed to 100 μM CoCl$_2$ for another 24 h (hypoxia). Cell viability was assayed using a colorimetric method. Autophagic and apoptotic cells were quantified using flow cytometry. Caspase-3 activity was analyzed with a fluorometric substrate assay. Each value represents the mean $\pm$ SEM for n=6. [a,b]p<0.05, Values significantly differed from the control and CoCl$_2$-treated groups, respectively. FI, fluorescent intensities.

with 3-MA alone did not change caspase-3 activity (Fig. 5C) or cell apoptosis (Fig. 5D). In contrast, pretreatment with 3-MA caused significant 61 and 58% alterations in hypoxia-induced caspase-3 activation and cell apoptosis, respectively (Fig. 5C and D).

Chloroquine, another inhibitor of cell autophagy, was further applied to human U87-MG cells to confirm CoCl$_2$-induced cell insults (Table I). Pretreatment of human U87-MG cells with chloroquine did not affect cell viability, cell autophagy, caspase-3 activity, or cell apoptosis. In contrast, chloroquine pretreatment caused significant 51, 52, 61 and 51% decreases in CoCl$_2$-induced alterations in cell viability, autophagic cells, caspase-3 activation, and apoptotic cells, respectively (Table I).
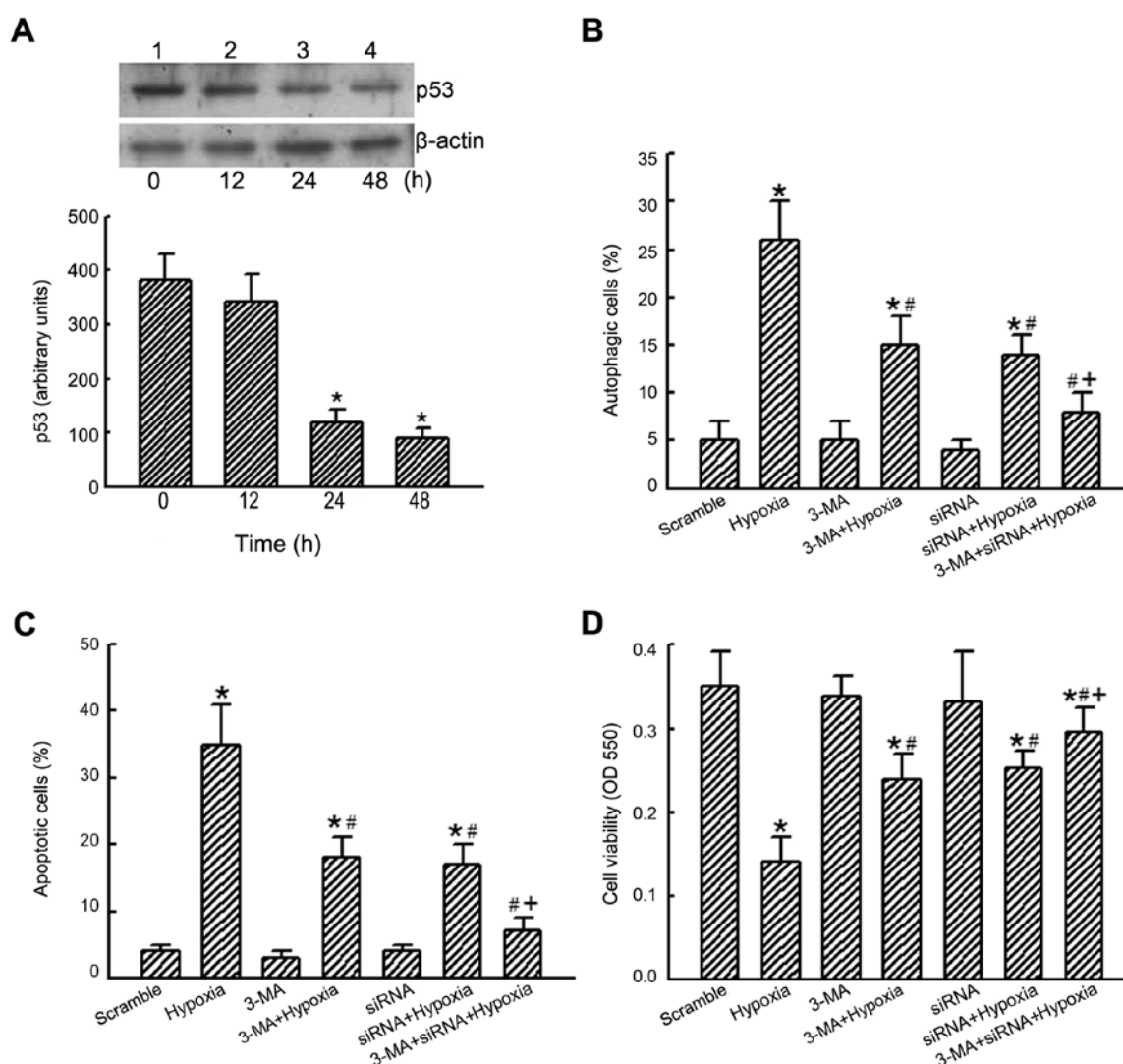
Figure 6. Roles of p53 in cobalt chloride (CoCl$_2$)-induced cell autophagy, apoptosis, and viability. Human glioma U87-MG cells were exposed to p53 siRNA (siRNA) for 6, 12 and 24 h. Scrambled siRNA was applied into control cells as the negative control (scramble). Levels of p53 were immunodetected, and β-actin was analyzed as the internal control (A, top panel). These protein bands were quantified and statistically analyzed (bottom panel). U87-MG cells were pretreated with p53 siRNA (siRNA) and 3-methyladenine (3-MA) and then exposed to 100 μM CoCl$_2$ for another 24 h (hypoxia). Cell autophagy (B) and cell apoptosis (C) were determined using flow cytometry. Cell viability was assayed using a colorimetric method (D). Each value represents the mean ± SEM for n=6. *,#,+p<0.05, values significantly differed from the control, hypoxia-, and p53 siRNA-treated groups, respectively.

RNAi was applied to human U87-MG cells to determine the roles of p53 in CoCl$_2$-induced autophagic death (Fig. 6). Application of p53 siRNA to human U87-MG cells for 12 h did not affect levels of p53 (Fig. 6A, top panel, lane 2). However, after exposure to p53 siRNA for 24 or 48 h, amounts of p53 in human U87-MG cells were significantly decreased (lanes 3 and 4). β-actin was analyzed as the internal control (top panel). These protein bands were quantified and statistically analyzed (bottom panel). Application of p53 siRNA to human U87-MG cells for 24 or 48 h caused significant 69 and 77% reductions in levels of p53, respectively. Exposure to hypoxia induced autophagy of 28% of human U87-MG cells (Fig. 6B). Pretreatment with 3-MA and p53 siRNA did not affect cell autophagy but significantly alleviated hypoxia-induced autophagy of human U87-MG cells. Co-treatment with 3-MA and p53 siRNA synergistically lowered hypoxia-induced cell autophagy (Fig. 6B). At the same time, pretreatment of human U87-MG cells with a combination of 3-MA and p53 siRNA

caused noteworthy alleviations of hypoxia-induced cell apoptosis and cell death (Fig. 6C and D).

To confirm the roles of p53 in CoCl$_2$-induced autophagic death of glioma cells, human glioma DBTRG-05MG cells were further used as our experimental model (Fig. 7). Exposure of DBTRG-05MG cells to hypoxia led to a 3.6-fold induction of cell autophagy (Fig. 7A). Pretreatment with 3-MA did not affect autophagy of DBTRG-05MG cells but attenuated hypoxia-induced cell autophagy by 56%. Administration of hypoxia induced apoptosis of DBTRG-05MG cells by 20% (Fig. 7B). In contrast, pretreatment of DBTRG-05MG cells with 3-MA did not affect cell apoptosis but caused a significant 60% reduction in hypoxia-induced apoptosis. Application of p53 siRNA into DBTRG-05MG cells for 24 h did not influence autophagy and apoptosis (Fig. 7C and D). However, when knocking down p53 translation in DBTRG-05MG cells, the hypoxia-induced autophagy and apoptosis were simultaneously reduced by 100 and 75%, respectively (Fig. 7C and D).
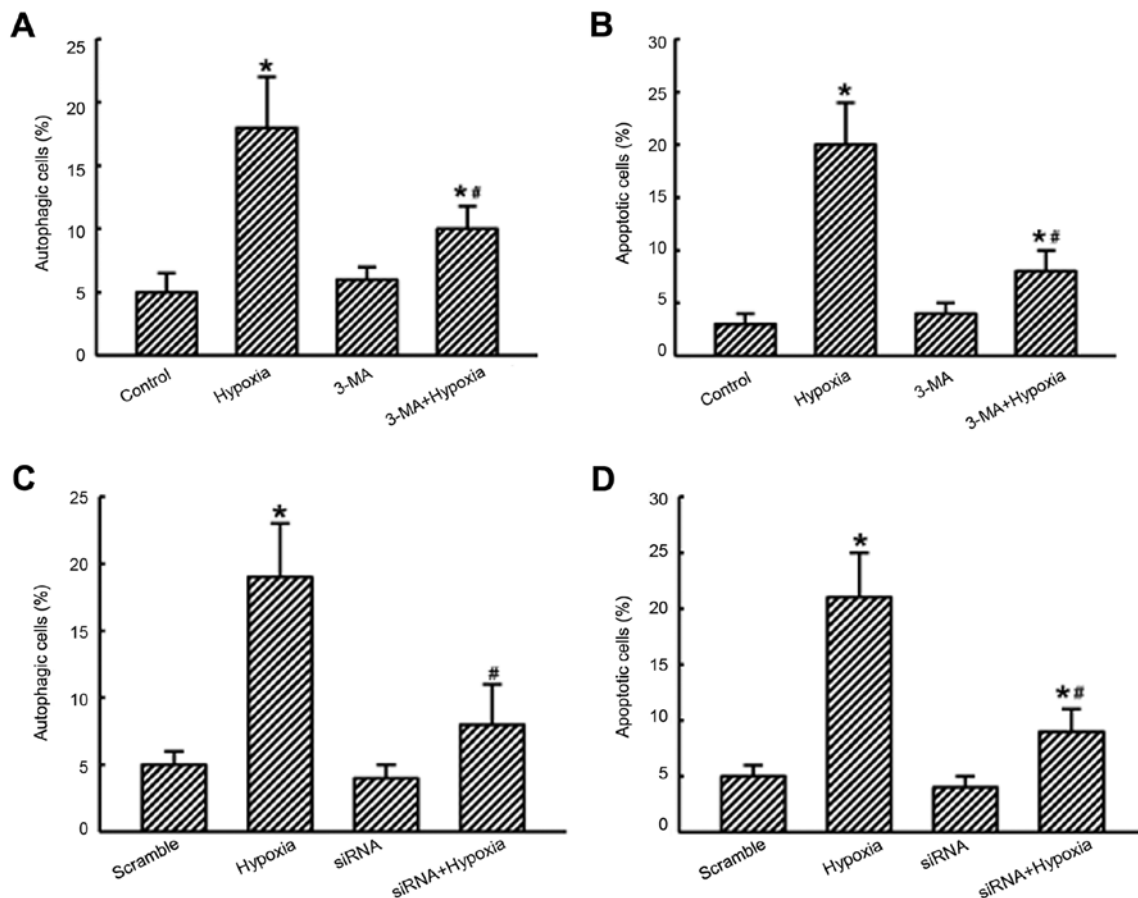
Figure 7. Participation of p53 in cobalt chloride (CoCl$_2$)-induced autophagy and apoptosis in human glioma DBTRG-05MG cells. DBTRG-05MG cells were pretreated with 3-methyladenine (3-MA) for 1 h and then exposed to 100 μM CoCl$_2$ for another 24 h (hypoxia) (A and B). Or, DBTRG-05MG cells were exposed to p53 siRNA (siRNA) for 24 h and then to 100 μM CoCl$_2$ for another 24 h (hypoxia) (C and D). Scrambled siRNA was applied into control cells as the negative control (scramble). Cell autophagy (A and C) and cell apoptosis (B and D) were determined using flow cytometry. Each value represents the mean ± SEM for n=6. *,#p<0.05, values significantly differed from the control, hypoxia-, and p53 siRNA-treated groups, respectively.

Discussion

The present study showed that CoCl$_2$ treatment can induce autophagy and subsequent death of human malignant glioma cells. Malignant gliomas are the most prevalent and aggressive brain tumors (2). In the clinic, patients suffering from malignant gliomas usually have poor prognoses, and their median overall survival rates are less than 1 year. Induction of hypoxia has recently been explored as a novel strategy for treating aggressive tumors such as cancer stem cells and chemotherapy-resistant tumor cells (19,22). This study showed that administration of CoCl$_2$ induced hypoxic stress in human glioma cells. Separately, exposure of human U87-MG cells to hypoxia triggered cell autophagy, apoptosis, and death. Interestingly, suppressing autophagy using the inhibitor, 3-MA, concurrently lessened hypoxia-induced apoptosis and death in human glioma U87-MG and DBTRG-05MG cells. Hence, CoCl$_2$ treatment can induce autophagic death in human glioma cells. Brain hypoxia is thought to have bidirectional roles in controlling cell survival and death in human malignant glioma cells (13,14). Hu *et al* reported that hypoxia-induced autophagy promotes glioblastoma cell survival (23). Nevertheless, our study provides *in vitro* data which demonstrate that delayed hypoxia can induce autophagy

and autophagic death in human glioma cells. TMZ is an oral chemotherapeutic drug that is used to treat brain tumors (32). A previous study further showed that bortezomib induces cell death in GBM stem cells and TMZ-resistant glioma cells via a hypoxia-dependent mechanism (22). As a result, discovering a novel scheme to induce hypoxia may be beneficial for therapy of malignant gliomas and TMZ-resistant GBM through an autophagic apoptosis pathway.

Exposure to CoCl$_2$ induces hypoxic insults to human glioma cells. In a hypoxic microenvironment, HIF-1α is highly produced and functions as a transcription factor to regulate a large array of gene expressions in response to hypoxic conditions (9). Characteristically, HIF-1α is recognized as a typical marker indicating that cells are experiencing hypoxia. Our present data revealed that CoCl$_2$ treatment augmented amounts of HIF-1α in human U87-MG cells. As to the mechanism, CoCl$_2$ can diminish HIF-1α degradation by suppressing prolyl-4-hydroxylase activity (33). Consequently, treatment of human glioma cells with CoCl$_2$ can stimulate cellular hypoxic stress by means of enriching HIF-1α expression. At the same time, exposure to hypoxia decreased the viability of human U87-MG cells. Brain hypoxia often occurs in brain neoplasms and traumatic brain injury and is recognized as a major cause of secondary injury to neurologically critically

ill patients (8). With glioblastomas, hypoxia was reported to promote tumor survival and angiogenesis (23). In neuroblastoma cells, our previous study demonstrated that hypoxia can induce cell death via an apoptotic mechanism due to targeting antiapoptotic *bcl-x$_L$* gene expression (12). The present study additionally proved the effects of hypoxia on triggering insults to human glioma cells.

CoCl$_2$ treatment lessens the proliferation of human glioma U87-MG cells. Hypoxia broadly exists in diverse types of tumor microenvironments (7,9). During tumorigenesis, low levels of oxygen may develop to promote tumor cell proliferation, angiogenesis, and metastasis through an HIF-1α-involved multifaceted network (34). Our present results showed that under extended hypoxic stress, incorporation of BrdU in human glioma U87-MG cells was repressed in concentration- and time-dependently. BrdU incorporation in cells can be measured to explain cell cycle processing in order to monitor the status of cell proliferation (35). Thus, this study demonstrated the suppressive effects of extended hypoxia on the proliferation of human glioma cells. In the clinic, rapid tumor growth because of speedy cell proliferation is one of major features and reasons illuminating the casual recurrence and poor prognoses of human malignant gliomas (4). Li *et al* reported that hypoxic conditions can activate a self-protective mechanism against glioma proliferation (36). As a result, CoCl$_2$ treatment is able to prevent tumor cell proliferation and then suppress tumor growth and angiogenesis in human malignant gliomas.

CoCl$_2$ treatment can induce autophagic insults to human glioma cells. Autophagy is a self-degradative process inside cells (15,16). Autophagosomes, acidic vesicular organelles, and elevation in LC3-II levels are considered as consistent markers of cell autophagy (37,38). Our preliminary study has shown that treatment of human U87-MG cells with CoCl$_2$ could induce autophagosomes, acidic vesicular organelles, and LC3-II amounts. Herein, we demonstrated the concentration- and time-dependent effects of CoCl$_2$ treatment on raising the proportions of human glioma cells with acidic vesicular organelles and the levels of LC3-II protein. Moreover, 3-MA and chloroquine are typically used as inhibitors of cell autophagy because it can block the formation of autophagosomes (39). Our current data showed the repressive effects of pretreatment with 3-MA and chloroquine on CoCl$_2$-induced autophagy of human glioma cells. Separately, pretreatment with Rapa, an inducer of autophagy, synergistically amplified hypoxia-induced cell autophagy. Therefore, we provide several lines of evidence to show that delayed hypoxia can induce autophagy in human glioma cells. Nevertheless, autophagic cells can reverse course and survive or proceed to death (23,24). This study showed that hypoxia decreased the viability of human U87-MG cells. Pretreatment with 3-MA and chloroquine caused concurrent defenses against CoCl$_2$-induced cell death. Consequently, CoCl$_2$ treatment can induce autophagy and autophagic death of human glioma cells. Bevacizumab is reported to kill high-grade gliomas via a hypoxia-induced autophagic pathway (19). Thus, induction of prolonged hypoxia has the potential to be a new therapeutic strategy for human gliomas.

Autophagy-induced apoptosis is involved in CoCl$_2$ treatment-induced damage to human glioma cells. Exposure of human glioma U87-MG cells to CoCl$_2$ induced caspase-3 activation and sequential cell cycle arrest at the sub-G$_1$ phase. Caspase-3 activation and cell cycle arrest at the sub-G$_1$ phase are two typical characteristics indicating that cells are undergoing apoptosis (40,41). Hence, CoCl$_2$ treatment can induce apoptosis of human glioma U87-MG cells. Autophagy is reported to closely cross-talk with apoptosis (42). In general, autophagy can block apoptosis, and certain apoptosis-associated proteins disrupt the autophagic process. In some cases, autophagy may induce cell apoptosis or necrosis, known as autophagic cell death (42). This study demonstrated that pretreatment with 3-MA alone did not influence apoptosis of human U87-MG cells but caused significant alleviation of CoCl$_2$-induced apoptotic injury. Thus, CoCl$_2$ treatment can induce autophagic apoptosis in human glioma cells. The key principles of cancer therapy focus on inducing cell death and inhibiting cell proliferation (42). Our previous studies showed that honokiol, a polyphenol, induced autophagic apoptosis in neuroblastomas and gliomas (21,25). The present study additionally supports hypoxia being applied to kill glioma cells via inducing cell autophagy and consequent autophagic apoptosis.

p53 participates in CoCl$_2$-induced autophagic apoptosis in human glioma cells. p53, a tumor suppressor protein, can transcriptionally regulate expression of certain genes and then control cell survival or death (26). Also, p53 is reported to control cell autophagy by adjusting downstream damage-regulated autophagy modulator (DRAM) expression, a lysosomal protein (10,40). This study showed that knocking down p53 expression using RNAi instantaneously attenuated CoCl$_2$-induced autophagy and subsequent apoptosis. Our recent study proved that activation of p53 sequentially results in suppression of mammalian target of rapamycin (mTOR) activity and then induction of autophagy of glioma cells (10). Hence, p53 participates in CoCl$_2$-induced cell autophagy in human glioma cells. Feng *et al* reported that p53 can upregulate unc-51-like kinase 1/2, which are necessary for sustained autophagy in response to camptothecin-induced DNA damage (44). Furthermore, a previous study demonstrated the contribution of p53 to triggering cell autophagy and death by inducing DRAM gene expression (45). The present study indicated that co-treatment with 3-MA and p53 siRNA synergistically protected human glioma cells from CoCl$_2$ treatment-induced caspase-3 activation, cell apoptosis, and cell death. As a result, p53 plays a crucial role in mediating hypoxia-induced cell autophagy and subsequent cell apoptosis in human gliomas.

In conclusion, this study verified the effects of CoCl$_2$ in inducing hypoxic stress in human glioma U87-MG cells. In parallel, exposure to CoCl$_2$ led to significant reductions in the viability and proliferation of human glioma cells. As to the mechanism, treatment of human U87-MG cells with CoCl$_2$ meaningfully augmented levels of acidic vesicular autophagosomes and the cellular LC3-II protein. The gain- and loss-of-function strategies further showed induction of autophagy in human glioma cells by CoCl$_2$. Pretreatment with 3-MA and chloroquine decreased the proportion of human U87-MG cells undergoing autophagy and concurrently attenuated CoCl$_2$-triggered caspase-3 activation and cell apoptosis. Consecutively, knocking down p53 synthesis by RNAi could weaken hypoxia-induced cell autophagy, apoptosis, and death. In contrast, co-treatment with 3-MA and p53 siRNA synergistically protected human glioma cells against CoCl$_2$-

induced autophagic death. The CoCl$_2$-induced autophagic apoptosis were supplementarily confirmed in human glioma DBTRG-05MG cells. Taken together, CoCl$_2$ treatment can induce autophagic apoptosis of human glioma cells via a p53-dependent pathway. Induction of delayed hypoxia may have the potential to be clinically applied for treating human malignant gliomas. An *in vivo* intracranial model is being investigated to confirm our *in vitro* findings on the suppressive effects of hypoxia on glioma growth through an autophagic apoptosis pathway.

Acknowledgements

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