Neuron-derived orphan receptor 1 transduces survival signals in neuronal cells in response to hypoxia-induced apoptotic insults

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OBJECTIVE Hypoxia can induce cell death or trigger adaptive mechanisms to guarantee cell survival. Neuron-derived orphan receptor 1 (NOR-1) works as an early-response protein in response to a variety of environmental stresses. In this study, the authors evaluated the roles of NOR-1 in hypoxia-induced neuronal insults.

METHODS Neuro-2a cells were exposed to oxygen/glucose deprivation (OGD). Cell viability, cell morphology, caspase-3 activity, DNA fragmentation, and cell apoptosis were assayed to determine the mechanisms of OGD-induced neuronal insults. RNA and protein analyses were carried out to evaluate the effects of OGD on expressions of *NOR-1*, *cAMP* response element-binding (*CREB*), and cellular inhibitor of apoptosis protein 2 (*clAP2*) genes. Translations of these gene expressions were knocked down using RNA interference. Mice subjected to traumatic brain injury (TBI) and NOR-1 was immunodetected.

RESULTS Exposure of neuro-2a cells to OGD decreased cell viability in a time-dependent manner. Additionally, OGD led to cell shrinkage, DNA fragmentation, and cell apoptosis. In parallel, treatment of neuro-2a cells with OGD time dependently increased cellular NOR-1 mRNA and protein expressions. Interestingly, administration of TBI also augmented NOR-1 levels in the impacted regions of mice. As to the mechanism, exposure to OGD increased nuclear levels of the transcription factor CREB protein. Downregulating CREB expression using RNA interference simultaneously inhibited OGD-induced NOR-1 mRNA expression. Also, levels of *clAP2* mRNA and protein in neuro-2a cells were augmented by OGD. After reducing *clAP2* translation, OGD-induced cell death was reduced. Sequentially, application of NOR-1 small interfering RNA to neuro-2a cells significantly inhibited OGD-induced *clAP2* mRNA expression and concurrently alleviated hypoxia-induced alterations in cell viability, caspase-3 activation, DNA damage, and cell apoptosis.

CONCLUSIONS This study shows that NOR-1 can transduce survival signals in neuronal cells responsible for hypoxia-induced apoptotic insults through activation of a CREB/*clAP2*-dependent mechanism.

KEY WORDS NOR-1; neuronal cell; hypoxia; survival signals; CREB/*clAP2*; traumatic brain injury

CEREBRAL hypoxia is commonly caused by traumatic brain injury (TBI) and brain tumors. A prospective study reported that almost half of patients with a severe head injury had epidemiologically experienced a period of hypoxia.⁶ This low-oxygen condition can subsequently lead to brain edema and neurodegeneration.³⁰ To neurologically critically ill patients, cerebral hypoxia

is further recognized as a major potential cause of secondary injury.²² Clinically, hypoxia is highly associated with a significant increase in morbidity and mortality from severe head injuries.^{22,30} Because brain ischemia and hypoxia are vital causes of brain damage, preservation of sufficient tissue oxygenation is a primary objective in the field of neurocritical care.²⁶ Additionally, hypoxia has

ABBREVIATIONS *clAP2* = cellular inhibitor of apoptosis protein 2; CREB = *cAMP* response element-binding; DMEM = Dulbecco's modified Eagle's medium; DMSO = dimethyl sulfoxide; ELISA = enzyme-linked immunosorbent assay; GBM = glioblastoma multiforme; mAb = monoclonal antibody; NOR-1 = neuron-derived orphan receptor 1; OGD = oxygen/glucose-deprivation; RT-PCR = reverse transcription polymerase chain reaction; pAb = polyclonal antibody; PAGE = polyacrylamide gel electrophoresis; PBS = phosphate-buffered saline; PCNA = proliferating cell nuclear antigen; PI = propidium iodide; PMSF = phenylmethylsulfonyl fluoride; RNAi = RNA interference; SDS = sodium dodecyl sulfate; siRNA = small interfering RNA; TBI = traumatic brain injury.

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been implicated in many characteristics of tumor development, angiogenesis, and growth.⁶ A low-oxygen condition can enhance tumor vascularization, mutation rate, and metastatic spread in tumor development.³⁰ Under hypoxic stress, brain tumor cells either survive by augmenting the glycolysis rate or undergo cell death.^{7,12} For the highly aggressive glioblastoma multiforme (GBM), intratumoral hypoxia has been reported to be associated with resistance to radiotherapy and chemotherapy.¹⁵ Therefore, brain hypoxia plays multiple physiological and pathophysiological roles in TBIs and brain tumors.

Apoptosis, the process of programmed cell death, can occur in developing and adult animal tissues.¹³ A variety of intrinsic or extrinsic molecular events can trigger the progression of cell apoptosis. When cells undergo apoptosis, specific apoptotic characteristics may appear, including a changed morphology, caspase cascade activation, DNA fragmentation, and cell cycle arrest at the sub-G₁ phase.^{21,35} In neuronal development, because apoptosis can precisely regulate the programmed natural death of neurons, it is thought to play important roles in neurogenesis.^{14,29} However, premature or irregular apoptotic regulation can lead to the pathogenesis of neurodegeneration, subsequently leading to various acute or chronic brain diseases. A deviation in the supply and consumption of oxygen in brain tissues initiates hypoxic stress that causes a complex cycle of biochemical and molecular events and accordingly results in neuronal death.^{7,36} Our previous study showed that treatment of neural cells with oxygen/glucose deprivation (OGD) can mimic hypoxic conditions and induce cell shrinkage and cell cycle arrest at the sub-G₁ phase.⁷ Fatemi et al. reported that hypoxia-induced apoptotic insults to neonatal brains are a crucial cause of cerebral palsy, mental retardation, and epilepsy.¹¹ To overcome hypoxia-induced adverse situations, brain neurons should adopt some cellular mechanisms such as induction of various transcription factors.

In addition to inducing cell apoptosis, hypoxia can also trigger adaptive mechanisms to guarantee cell survival. A large array of hypoxia-associated proteins is involved in hypoxic stress-induced neural damage and survival.³¹ Neuron-derived orphan receptor 1 (NOR-1), a member of the nuclear receptor 4A subfamily, functions as a ligand-independent transcription factor.³⁹ NOR-1 has been reported to be an early-response protein accountable for a pleiotropy of environmental cues.²⁵ Kim et al. have shown that after transient global ischemia for 3 hours, NOR-1 is rapidly induced in the dentate gyrus of the hippocampal formation and piriform cortex.¹⁷ In addition, exposure to hyperbaric air increases transcription of the *NOR-1* gene in rat vascular tissues.¹⁰ As a transcription factor, NOR-1 can regulate certain survival-related gene expressions.³⁸ Cellular inhibitor of apoptosis protein 2 (cIAP2), a ubiquitin ligase, has a RING domain in the carboxyl terminal that specifically binds to caspases or nuclear factor (NF)- κ B and mediates cell survival signals.^{34,37} In endothelial cells, knocking down NOR-1 expression concurrently downregulated cellular cIAP2 levels and enhanced hypoxia-induced cell damage.²⁴ Thus, cIAP2 could be a downstream target of NOR-1 in response to hypoxic stress. In this study, we evaluated the roles of NOR-1 in

hypoxia-induced insults to neurons and the possible molecular mechanisms.

Methods

Cell Culture and Hypoxia Treatment

Mouse neuroblastoma neuro-2a cells purchased from American Type Culture Collection were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL) supplemented with 10% fetal calf serum, L-glutamine, penicillin (100 IU/ml), and streptomycin (100 μ g/ml) in 75-cm² flasks at 37°C in a humidified atmosphere of 5% CO₂. Cells were grown to confluence prior to drug treatment.

Neuro-2a cells were treated with OGD to induce hypoxia as described previously.⁷ Glucose-free DMEM (OGD medium) and 1 $\times$ phosphate-buffered saline (PBS) (OGD buffer), containing 0.14 M NaCl, 2.6 mM KCl, 8 mM Na₂HPO₄, and 1.5 mM KH₂PO₄, were prepared by bubbling with 100% N₂ for 30 minutes. Neuro-2a cells were washed twice with OGD buffer and then seeded in OGD medium for various time intervals.

Assay of Cell Viability

Cell morphology and cell viability were assayed to evaluate the toxic effects of hypoxia to neuro-2a cells as described previously.¹⁹ Briefly, neuro-2a cells (10⁴ cells/well) were seeded in 96-well tissue culture plates overnight. After OGD treatment, cells were cultured with new medium containing 0.5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide for a further 3 hours. The blue formazan products in neuro-2a cells were dissolved in dimethyl sulfoxide (DMSO) and spectrophotometrically measured at a wavelength of 550 nm. After OGD treatment, the cell morphology was observed and photographed using a reverse-phase microscope (Nikon).

Quantification of DNA Fragmentation

DNA fragmentation was quantified using a cellular DNA fragmentation enzyme-linked immunosorbent assay (ELISA) kit (Boehringer Mannheim) as described previously.²⁰ Briefly, neuro-2a cells (2 $\times$ 10⁵ cells) were subcultured in 24-well tissue culture plates and labeled with BrdU overnight. Cells were harvested and suspended in culture medium. One hundred microliters of a cell suspension was added to each well of 96-well tissue culture plates. Neuro-2a cells were treated with OGD for different time intervals at 37°C in a humidified atmosphere of 5% CO₂. Amounts of BrdU-labeled DNA in the cytoplasm were quantified using a microplate photometer (Anthos Labtec Instruments).

Quantification of Apoptotic Cells

Cell apoptosis was quantified using propidium iodide (PI) according to a previously described method.⁸ After OGD treatment, neuro-2a cells were harvested and fixed in cold 80% ethanol. Following centrifugation and washing, fixed cells were stained with PI and analyzed by a flow cytometer (EPICS XL, Beckman Coulter).

Fluorogenic Substrate Assay for Caspase-3 Activity

Caspase-3 activity was determined using a fluorometric substrate assay kit.⁵ Briefly, after OGD administration, neuro-2a 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 phenylmethylsulfonyl fluoride (PMSF). Cell extracts (25 mg 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 ethylene glycol tetraacetic acid, 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 using a spectrometer.

Immunoblotting Analyses of NOR-1, cIAP2, and β -Actin

Protein analyses were carried out according to a previously described method.³⁸ After OGD treatment, cell lysates were prepared in ice-cold radioimmunoprecipitation assay buffer (25 mM Tris-HCl [pH 7.2], 0.1% sodium dodecyl sulfate [SDS], 1% Triton X-100, 1% sodium deoxycholate, 0.15 M NaCl, and 1 mM EDTA). To avoid degradation of the cytosolic proteins by proteinases, a mixture of 1 mM phenyl methyl sulfonyl fluoride, 1 mM sodium orthovanadate, and 5 $\mu\text{g}/\text{ml}$ leupeptin was added to the radioimmunoprecipitation assay buffer. Protein concentrations were quantified using a bicinchoninic acid protein assay kit (Pierce, Rockford, IL, USA). Proteins (50 $\mu\text{g}/\text{well}$) were subjected to SDS-polyacrylamide gel electrophoresis (PAGE), and transferred to nitrocellulose membranes. NOR-1 and cIAP2 were immunodetected using a rabbit polyclonal antibody (pAb) (Santa Cruz Biotechnology). The cellular β -actin protein was immunodetected using a mouse monoclonal antibody (mAb) against mouse β -actin (Sigma) as the internal standard. These protein bands were quantified using a digital imaging system (UVtec).

Extraction of Nuclear Proteins and Immunodetection

The amounts of nuclear *cAMP* response element-binding (CREB) protein were quantified following a previously described method.⁴ Briefly, after drug treatment, nuclear extracts of neuro-2a cells were prepared. Protein concentrations were quantified by a bicinchoninic acid protein assay kit (Pierce). Nuclear proteins (50 $\mu\text{g}/\text{well}$) were subjected to SDS-PAGE and transferred to nitrocellulose membranes. After blocking, nuclear CREB was immunodetected using a rabbit pAb against mouse CREB (Santa Cruz Biotechnology). Cellular proliferating cell nuclear antigen (PCNA) was immunodetected using a mouse mAb against mouse PCNA (Sigma) as the internal control. Intensities of the immunoreactive bands were determined using a digital imaging system (UVtec).

Reverse Transcription and Quantitative Polymerase Chain Reaction Assays

Messenger (m)RNA from neuro-2a cells exposed to OGD was prepared for reverse transcription polymerase

chain reaction (RT-PCR) and quantitative PCR analyses of NOR-1, cIAP2, and β -actin mRNA. Oligonucleotides for the PCR analyses of NOR-1, cIAP2, and β -actin were designed and synthesized by Clontech Laboratories. The oligonucleotide sequences of the upstream and downstream primers for these mRNA analyses were respectively 5'-CTGCAATGGACTTGTCTGC-3' and 5'-GAGCTTGTCGATGACAGAAG-3' for NOR-1,¹⁶ 5'-TAGAGGGACCATCAAGGGCA-3' and 5'-CATCTGTGCTCCCGGTTCTT-3' for cIAP2, and 5'-GTGGGCCGCTCTAGGCACCAA-3' and 5'-CTCTTTGATGTACGCACGATTTC-3' for β -actin.⁷ The PCR products were loaded onto a 1.8% agarose gel containing 0.1 $\mu\text{g}/\text{ml}$ ethidium bromide and were electrophoretically separated. DNA bands were visualized and photographed under ultraviolet-light exposure. The intensities of the DNA bands in the agarose gel were quantified using a digital imaging system (UVtec). A quantitative PCR analysis was carried out using iQSYBR Green Supermix (Bio-Rad) and the MyiQ Single-Color Real-Time PCR Detection System (Bio-Rad) as described previously.⁴

CREB, cIAP2, and NOR-1 Knockdown

Translation of CREB and NOR-1 mRNA in neuro-2a cells was knocked down using an RNA interference (RNAi) method as described previously.⁹ CREB, cIAP2, and NOR-1 small interfering (si)RNAs were purchased from Santa Cruz Biotechnology, which is a pool of three target-specific 20- to 25-nucleotide siRNAs designed to respectively knock down CREB, cIAP2, and NOR-1 expressions. CREB, cIAP2, and NOR-1 siRNAs were transfected into neuro-2a cells according to an siRNA transfection protocol provided by Santa Cruz Biotechnology. Briefly, after culturing neuro-2a cells in antibiotic-free DMEM at 37°C in a humidified atmosphere of 5% CO_2 for 24 hours, the siRNA duplex solution, which was diluted in siRNA transfection medium (Santa Cruz Biotechnology), was added to neuro-2a cells. After transfection for 24 hours, the medium was replaced with normal RPMI medium, and neuro-2a cells were treated with drugs. Scrambled siRNA, purchased from Santa Cruz Biotechnology, was applied to neuro-2a cells as a negative standard.

Animal Model of TBI

Male ICR mice (weighting 20–25 g) were purchased from the Animal Center of the College of Medicine, National Taiwan University. All procedures were performed following the National Institutes of Health Guidelines for the Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee of Taipei Medical University, Taipei, Taiwan. Before the experiments began, the mice were allowed to acclimatize for 1 week in their animal quarters with air conditioning and an automatically controlled photoperiod of 12 hours of light daily. The animal was anesthetized and placed in a stereotactic frame, and its head was positioned in the horizontal plane with the nose bar set at zero. Following a midline incision exposing the skull, a 4-mm craniotomy was made lateral to the sagittal suture. The impactor tip diameter was 3 mm. The impact velocity and the depth of cortical defor-

mation were set at 3.5 m/sec and 1.0 mm, respectively. After injury, the skin was sutured and the animal was placed in an incubator until consciousness was regained.

Immunohistochemical and Immunoblotting Analyses of NOR-1

After TBI administration, the mice were killed and the brains were removed for immunohistological and immunoblotting assays.⁴ For immunohistological analyses, the brain tissues were fixed with a fixing reagent (acetone:methanol, 1:1). Following slicing and rehydration, the brain specimens were incubated with 0.2% Triton X-100. A rabbit pAb against mouse NOR-1 (Santa Cruz Biotechnology) was used for immunohistological detection of NOR-1. For immunoblotting analyses, the brains were homogenized with lysis buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1 mM ethylene glycol tetraacetic acid, 1% NP-40 supplemented with 1 mM PMSF, 1 μ M aprotinin, 1 μ M leupeptin, 1 mM Na₂VO₄, and 1 mM NaF). Protein concentrations were quantified using a bicinchoninic acid protein assay kit (Pierce). Proteins (50 μ g/well) were subjected to SDS-PAGE and transferred to nitrocellulose membranes. NOR-1 protein was immunodetected using a pAb (Santa Cruz Biotechnology). β -Actin was detected using a mouse mAb against mouse β -actin (Sigma) as an internal control. Intensities of these protein bands were determined using a digital imaging system (UVtec).

Statistical Analysis

Statistical differences between the control and OGD-treated groups were considered significant when the *p* value of a one-way ANOVA with the Bonferroni multiple-comparison test was < 0.05.

Results

Hypoxia Induces Apoptotic Insults to Neuro-2a Cells

To evaluate the effects of hypoxia on neuronal insults and the possible death mechanism, cell morphology, cell viability, DNA fragmentation, and apoptotic cells were analyzed (Fig. 1). Exposure of neuro-2a cells to OGD for 1 hour decreased cell number and induced a change in morphology (Fig. 1A). At 3, 6, 12, and 24 hours after OGD administration, the insults to neuro-2a cells became more aggressive. Analysis of cell viability further showed that treatment of neuro-2a cells with OGD for 1, 3, 6, 12, and 24 hours caused significant decreases in cell survival by 12%, 31%, 45%, 69%, and 88%, respectively (Fig. 1B). As to the mechanism, exposure of neuro-2a cells to OGD for 1 hour induced DNA fragmentation by 33% (Fig. 1C). Administration of OGD for 3, 6, 12, and 24 hours led to 80%, 173%, 233%, and 227% induction of DNA fragmentation in neuro-2a cells. In parallel, OGD treatment for 1 hour caused 12% of neuro-2a cells to undergo apoptosis (Fig. 1D). After exposure to OGD for 3, 6, 12, and 24 hours, the percentages of apoptotic cells were significantly raised by 29%, 45%, 81%, and 89%, respectively.

Hypoxia and TBI Induce NOR-1 Gene Expression

Protein and RNA analyses were conducted to determine

the effects of hypoxia on *NOR-1* gene expression (Fig. 2). Treatment of neuro-2a cells with OGD for 1 hour increased cellular NOR-1 levels (Fig. 2A Lane 2). After being exposed to OGD for 3 and 6 hours, amounts of NOR-1 were more augmented (Fig. 2A, Lanes 3 and 4). β -Actin was immunodetected as the internal standard (Fig. 2A). These immunorelated protein bands were quantified and statistically analyzed (Fig. 2B). Exposure of neuro-2a cells for 1, 3, and 6 hours caused significant 59%, 96%, and 134% augmentations of cellular NOR-1 amounts, respectively. RT-PCR analyses revealed that administration of OGD for 1 hour induced NOR-1 mRNA expression (Fig. 2C Lane 2). At 3 and 6 hours after exposure to OGD, NOR-1 mRNA in neuro-2a cells was induced (Lanes 3 and 4). β -Actin mRNA was assayed as the internal standard (bottom panel). These DNA bands were quantified and statistically analyzed (Fig. 2D). Treatment of neuro-2a cells with OGD for 1, 3, and 6 hours induced NOR-1 expression by 103%, 132%, and 92%, respectively. The effects of OGD on NOR-1 mRNA expression were further confirmed using quantitative PCR (Fig. 2E). Exposure of neuro-2a cells to OGD for 1, 3, and 6 hours led to 32%, 95%, and 110% enhancements in levels of NOR-1 mRNA, respectively.

Mice received a TBI and NOR-1 was immunodetected (Fig. 2F). Compared with sham groups, treatment of mice with TBI obviously increased levels of NOR-1 in the impacted regions. Results of an immunoblotting analysis also revealed that TBI enhanced NOR-1 production in mouse brains (Fig. 2F). β -Actin was measured as an internal control. These protein bands were quantified and statistically analyzed (Fig. 2F). Administration of TBI caused a 2.2-fold increase in NOR-1 protein in the impacted regions compared with sham groups.

CREB Participates in Regulation of Hypoxia-Induced NOR-1 Gene Expression

Roles of transcription factor CREB in regulation of hypoxia-induced *NOR-1* gene expression were assessed (Fig. 3). Exposure of neuro-2a cells to OGD for 1, 3, and 6 hours obviously enhanced nuclear CREB levels (Fig. 3A, Lanes 2–4). β -Actin was determined as the internal standard. These protein bands were quantified and statistically analyzed (Fig. 3B). Treatment of neuro-2a cells with OGD for 1, 3, and 6 hours significantly increased amounts of nuclear CREB by 91%, 109%, and 101%, respectively. Application of CREB siRNA to neuro-2a cells suppressed translation of cellular CREB (Fig. 3C, Lane 2). Levels of β -actin were analyzed as the internal standard. These protein bands were quantified and statistically analyzed. When applying CREB siRNA to neuro-2a cells, translation of this transcription factor decreased by 79%. The effects of CREB knockdown on OGD-induced NOR-1 mRNA expression were further evaluated (Fig. 3D). OGD led to 2.2-fold increase of NOR-1 mRNA in neuro-2a cells. CREB siRNA did not influence basal levels of NOR-1 mRNA. However, treatment of neuro-2a cells with CREB siRNA caused a significant 83% decrease in OGD-induced NOR-1 mRNA expression (Fig. 3D).

clAP2 Is Induced by Hypoxia and Adjusts Cell Survival

RNA and protein analyses were carried out to deter-

FIG. 1. Effect of hypoxia on neuronal insults. **A:** Neuro-2a cells were exposed to OGD for 1, 3, 6, 12, and 24 hours. Cell morphology was analyzed using a light microscope. Original magnification $\times 100$. **B:** Cell viability was determined by a colorimetric method. **C:** DNA fragmentation was quantified using ELISA. **D:** Apoptotic cells were quantified using a flow cytometer. Each value represents the mean $\pm$ SEM for $n = 6$. *Values significantly differed from those of the respective control ($p < 0.05$). OD = optical density.

mine the effects of hypoxia on regulation of *cIAP2* gene expression (Fig. 4). Exposure of neuro-2a cells to OGD for 1 hour slightly increased levels of cellular *cIAP2* (Fig. 4A, Lane 2). In comparison, after OGD administration for 3 and 6 hours, amounts of *cIAP2* in neuro-2a cells were increased (Lanes 3 and 4). β -Actin was detected as the internal control (bottom panel). These protein bands were quantified and statistically analyzed (Fig. 4B). Treatment of neuro-2a cells with OGD for 3 and 6 hours caused significant increases—93% and 89%—in cellular *cIAP2* protein levels. Quantitative PCR analyses further demonstrated that exposure of neuro-2a cells to OGD for 1 hour did not affect *cIAP2* mRNA expression (Fig. 4C). However, 3 and 6 hours after treatment with OGD, *cIAP2* mRNA syntheses were increased by 68% and 94%, respectively. Knocking down *cIAP2* expression caused a significant in-

crease (by 75%) in OGD-induced death of neuro-2a cells (Fig. 4D).

NOR-1 Transcriptionally Regulates *cIAP2* Gene Expression

Translation of *NOR-1* gene expression was knocked down to determine the role of this survival protein in regulation of *cIAP2* gene expression (Fig. 5). Application of NOR-1 siRNA into neuro-2a cells obviously reduced levels of cellular NOR-1 protein (Fig. 5A Lane 2). β -Actin was quantified as the internal standard. These protein bands were quantified and statistically analyzed (Fig. 5B). Treatment of neuro-2a cells with NOR-1 siRNA led to a 92% reduction in translation of this survival protein. Exposure to OGD increased *cIAP2* mRNA expression by 2.1-fold (Fig. 5C). NOR-1 siRNA did not change basal lev-

FIG. 2. Effect of hypoxia and TBI on NOR-1 expression. Neuro-2a cells were exposed to OGD for 1, 3, and 6 hours. Amounts of NOR-1 were immunodetected (**A**, upper panel). β -Actin was detected as the internal standard (lower panel). These protein bands were quantified and statistically analyzed (**B**). Levels of NOR-1 mRNA were analyzed using RT-PCR (**C**, upper panel). β -Actin mRNA was measured as the internal standard (lower panel). These DNA bands were quantified and statistically analyzed (**D**). NOR-1 mRNA was further quantified using a quantitative PCR analysis (**E**). Mice received a TBI, and NOR-1 was analyzed using immunohistological and immunoblotting assays (**F**). Nuclei were stained with hematoxylin. Original magnification $\times 100$. Each value represents the mean $\pm$ SEM ($n = 6$). *Values significantly differed from those of the respective control ($p < 0.05$). Figure is available in color online only.

FIG. 3. Roles of CREB in hypoxia-induced NOR-1 mRNA expression. Neuro-2a cells were exposed to OGD for 1, 3, and 6 hours. Levels of nuclear CREB were immunodetected (A, upper panel). Amounts of PCNA were measured as the internal standard (lower panel). These protein bands were quantified and statistically analyzed (B). Neuro-2a cells were exposed to CREB siRNA (siRNA) for 48 hours. Control cells received scrambled siRNA. Levels of nuclear CREB were immunodetected (C, upper panel). PCNA was measured as the internal standard. These protein bands were quantified and statistically analyzed (lower panel). Neuro-2a cells were treated with OGD, CREB siRNA (siRNA), and a combination of siRNA and OGD for 6 hours. Levels of NOR-1 mRNA were analyzed using a quantitative PCR (D). Each value in the bar graph represents the mean $\pm$ SEM ($n = 6$). *Values significantly ($p < 0.05$) differed from those of the control and OGD-treated groups, respectively. #Values significantly ($p < 0.05$) differed from those of the control and OGD-treated groups, respectively.

els of cIAP2 mRNA in neuro-2a cells. However, application of it lowered OGD-induced cIAP2 mRNA expression by 70% (Fig. 5C).

NOR-1 Transduces Survival Signals in Hypoxia-Induced Apoptotic Insults

Roles of NOR-1 in mediating survival signals were also determined (Fig. 6). Exposure of neuro-2a cells to OGD decreased 46% of cell viability (Fig. 6A). NOR-1 siRNA did not affect cell viability. However, application of NOR-1 siRNA to neuro-2a cells increased OGD-induced cell death by 71% (Fig. 6A). As to the mechanism, administration of OGD to neuro-2a cells caused significant increases in caspase-3 activity and DNA fragmentation by 180% and 96%, respectively (Fig. 6B and C). NOR-1 siRNA alone neither changed caspase-3 activity nor induced DNA frag-

mentation. In comparison, application of NOR-1 siRNA caused significant increases in OGD-induced alterations in caspase-3 activation and DNA damage by 52% and 49%, respectively (Fig. 6B and C). Consequently, the OGD-induced apoptosis of neuro-2a cells was augmented by NOR-1 siRNA by 68% (Fig. 6D).

Discussion

This study showed that NOR-1 can transduce survival signals in neuronal cells responsible for hypoxia-induced insults. Exposure of neuro-2a cells to OGD led to cell death. In parallel, NOR-1 was rapidly induced in neuro-2a cells responsible for OGD stimulation. Application of NOR-1 siRNA to neuro-2a cells decreased the production of this emergent protein and concurrently alleviated OGD-induced neuronal death. Previous functional studies have

FIG. 4. Roles of cIAP2 in hypoxia-induced neuronal insults. Neuro-2a cells were exposed to OGD for 1, 3, and 6 hours. Levels of cIAP2 were immunodetected (**A**, upper panel). Amounts of β -actin were detected as the internal standard (lower panel). These protein bands were quantified and statistically analyzed (**B**). Levels of cIAP2 mRNA were analyzed using quantitative PCR analyses (**C**). Neuro-2a cells were exposed to cIAP2 siRNA (siRNA) for 48 hours and then treated with OGD, and a combination of OGD and siRNA for 6 hours. Scrambled siRNA was administered to control cells as the negative control. Cell viability was determined by a colorimetric method (**D**). Each value represents the mean $\pm$ SEM ($n = 6$). Each value represents the mean $\pm$ SEM ($n = 6$). *Values significantly ($p < 0.05$) differed from those of the control and OGD-treated groups, respectively.

shown that NOR-1 participates in regulating cell proliferation, apoptosis, and differentiation.^{23,39} In particular, NOR-1 can be rapidly induced by a pleiotropy of stimuli such as inflammatory cytokines and growth factors.²⁴ The present results revealed that TBI could increase NOR-1 production in the impacted regions of mouse brain tissues. Cerebral hypoxia is a typical stress that commonly occurs in cases of TBI and brain tumors. In severe head injuries, hypoxia has been shown to be highly associated with significant increases in morbidity and mortality rates.^{6,30} In addition, hypoxic conditions can enhance tumor vascularization, the mutation rate, and metastatic spread during development of brain tumors.¹ As a result, preserving sufficient tissue oxygenation is a primary objective in the field of neurocritical care.²⁶ Hypoxia can induce cell apoptosis and also trigger adaptive mechanisms to guarantee cell survival. The present study demonstrated that *NOR-1* is an early response gene in neuronal cells under low-oxygen conditions. Clinically, NOR-1 can be applied as an immediate indicator of the prognosis and diagnosis of brain diseases such as TBIs and brain tumors.

NOR-1 transduces neural survival via an antiapoptotic mechanism. Exposure of neuro-2a cells to OGD induced cell shrinkage, DNA fragmentation, and cell cycle arrest at the sub-G₁ phase. A change in morphology, DNA damage, and cell cycle arrest are characteristic features of cells undergoing apoptosis.^{13,21,35} In addition, OGD activated caspase-3 in neuro-2a cells. Caspase-3 plays a key role in triggering intrinsic and extrinsic cell apoptosis.¹³ Thus, OGD can trigger low-oxygen conditions and subsequently induce the death of neuro-2a cells via an apoptotic pathway. On the other hand, when knocking down NOR-1 expression using RNAi, OGD-induced modifications of these apoptotic features were simultaneously attenuated. A previous study showed that induction of *NOR-1* gene expression was associated with cadmium-induced apoptosis of human peripheral blood mononuclear cells.³³ Nomiya et al. reported that in smooth muscle cells a NOR-1 deficiency decreased cell proliferation and elevated serum deprivation-induced apoptotic insults.²⁸ In contrast, overexpression of NOR-1 can lessen low-oxygen condition-induced apoptosis of endothelial cells.²⁴ In this study, we

FIG. 5. Participation of NOR-1 in regulation of cIAP2 mRNA expression. NOR-1 siRNA was administered to neuro-2a cells for 48 hours. Control cells received scrambled siRNA as the negative control. Levels of NOR-1 were immunodetected (A, upper panel). β -Actin was quantified as the internal standard (lower panel). These protein bands were quantified and statistically analyzed (B). Neuro-2a cells were exposed to OGD, NOR-1 siRNA (siRNA), and a combination of OGD and siRNA for 6 hours. NOR-1 mRNA was analyzed using quantitative PCR (C). Each value represents the mean $\pm$ SEM ($n = 6$). *#Values significantly ($p < 0.05$) differed from those of the control and OGD-treated groups, respectively.

further showed that NOR-1 can transduce survival signals in hypoxia-induced neuronal insults through an antiapoptotic mechanism.

CREB participates in regulating *NOR-1* gene expression. The present study demonstrated that, after exposure to OGD, levels of nuclear CREB in neuro-2a cells were augmented in a time-dependent manner. Being a transcription factor, phosphorylated CREB in nuclei is capable of specifically binding to the cAMP response elements that exist in the 5'-promoter regions of certain genes and, afterward, of transcriptionally adapting these gene expressions in response to intracellular and extracellular stimuli.²⁷ CREB has been shown to play an important role in regulating neuronal activities.² In the ischemic brain, CREB activation consequently induces antiapoptotic *Bcl-2* gene

expression that contributes to neuron survival and protection.¹⁸ In the present study, we demonstrated that knocking down CREB translation decreased production of this transcription factor and concomitantly inhibited NOR-1 mRNA expression. Rius et al. reported that in vascular smooth muscle cells, treatment with a dominant-negative of CREB or initiation of a specific mutation in the cAMP response element present in the NOR-1 promoter abolished low-density lipoprotein-induced NOR-1 promoter activity.³² As a result, this study showed that CREB can function as a transcription factor to regulate *NOR-1* gene expression in neuronal cells responsible for hypoxic stress.

In response to hypoxia-induced apoptotic insults, NOR-1 can act as a transcriptional factor to regulate *cIAP2* gene expression in neuronal cells. Our results indicate that exposure of neuro-2a cells to OGD time-dependently induced expression of cIAP2 mRNA and protein. In addition, when RNAi techniques were used to reduce amounts of cIAP2 in neuro-2a cells, the OGD-induced decrease in cell viability was simultaneously attenuated. Previous studies have shown that cIAP2 binds to apoptosis-related proteins such as nuclear factor- κ B, then suppresses caspase activity, and accordingly protects against cell death.^{34,37} Thus, cIAP2 may be a survival protein that mediates survival signals during the processing of hypoxia-induced insults to neuronal cells. Interestingly, our present data show that application of NOR-1 siRNA to neuro-2a cells significantly decreased levels of this transcription factor, while simultaneously inhibiting cIAP2 mRNA expression. Martorell et al. have reported that attenuating NOR-1 levels in endothelial cells caused a noteworthy downregulation of cIAP2 mRNA production.²⁴ NOR-1 comprises an evolutionarily ancient and highly conserved group of transcription factors that can regulate certain survival-related gene expressions.²³ In this study, we provide in vitro data to demonstrate that NOR-1 can protect neuronal cells against hypoxia-induced apoptotic insults by transcriptionally regulating cIAP-2 gene expression.

Conclusions

This study shows that exposure of neuro-2a cells to OGD decreased cell viability and induced DNA fragmentation and cell apoptosis in a time-dependent manner. In parallel, OGD time-dependently triggered NOR-1 mRNA and protein expression. Additionally, TBI increased amounts of NOR-1 in the impacted regions. As to the mechanism, the levels of nuclear CREB were augmented in neuro-2a cells, and application of CREB siRNA decreased amounts of this transcription factor and concurrently inhibited OGD-induced NOR-1 mRNA expression. Thus, CREB works as a transcription factor to regulate *NOR-1* gene expression in OGD-treated neuronal cells. In addition, exposure of neuro-2a cells induced cIAP2 mRNA and protein expression. Knocking down cIAP2 expression augmented OGD-induced death of neuro-2a cells. Interestingly, attenuation of NOR-1 translation using RNAi concomitantly decreased levels of cIAP2 mRNA. Consequently, when knocking down NOR-1 translation, the OGD-induced reduction of cell viability was furthered, and caspase-3 activation, DNA fragmentation, and cell apoptosis were simultaneously increased. Therefore, this study demonstrated that

FIG. 6. Roles of NOR-1 in OGD-induced apoptotic insults to neuronal cells. Neuro-2a cells were exposed to OGD, NOR-1 siRNA, and a combination of OGD and siRNA for 6 hours. Control cells received Scrambled siRNA as the negative control. Cell viability was assayed with a colorimetric method (A). Caspase-3 activity was analyzed using a fluorometric substrate assay (B). DNA fragmentation was quantified using an ELISA method (C). Apoptotic cells were quantified using a flow cytometer (D). Each value represents the mean $\pm$ SEM (n = 6). *Values significantly (p < 0.05) differed from those of the control and OGD-treated groups, respectively.

OGD-induced CREB can regulate *NOR-1* gene expression and that augmented NOR-1 can function as an early-response protein to transduce survival signals in neuronal cells by transcriptionally regulating *cIAP2* gene expression in response to hypoxic stress. Clinically, NOR-1 has potential as an effective biomarker for diagnosis and prognosis of hypoxia-related brain diseases such as TBI and brain tumors.

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Disclosures

The authors report no conflict of interest concerning the materials or methods used in this study or the findings specified in this paper.

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Conception and design: RM Chen, Chio, Wei. Acquisition of data: RM Chen, Chio, TG Chen, Shieh. Analysis and interpretation of data: RM Chen, Chio, Wei, Lin, Shieh, Yeh. Drafting the article: RM Chen, Chio. Critically revising the article: RM Chen. Reviewed submitted version of manuscript: all authors. Approved the final version of the manuscript on behalf of all authors: RM Chen. Statistical analysis: RM Chen, Wei, TG Chen, Lin.

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