Upregulation of HIF-1α protects neuroblastoma cells from hypoxia-induced apoptosis in a RhoA-dependent manner

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Abstract. Hypoxic conditions regulate several metabolic enzymes and transcription factors that are involved in cancer, ischemia and pulmonary diseases. The Ras homolog (Rho) family, including Rho member A (RhoA), is involved in reorganization of the actin cytoskeleton, cell migration and in the regulation of apoptosis and gene transcription. The aim of the present study was to investigate the expression of hypoxia-inducible factor (HIF)-α and the activity of RhoA in PC12 neuroblastoma cells under hypoxic conditions. The upregulation of HIF-α and RhoA by hypoxia was determined using reverse transcription-quantitative polymerase chain reaction and western blot assays, cell apoptosis was analyzed using flow cytometry, and the activity of caspase 3 was examined using a western blot assay and caspase 3 activity assay kit. The PC12 cells were induced to apoptosis following exposure to hypoxia, and exhibited increased expression of HIF-α and increased mRNA and protein expression levels of RhoA. The overexpression of HIF-α attenuated the hypoxia-induced apoptosis of the PC12 cells. In addition, RhoA knockdown using small interfering RNA abrogated the antagonism of HIF-1α towards hypoxia-induced apoptosis. The results of the present study confirmed the protective role of HIF-1α and RhoA in hypoxia-induced PC12 cell apoptosis, and that the upregulation of HIF-1α by hypoxia is RhoA-dependent.

Introduction

Accumulating evidence has demonstrated that nerve terminals are vulnerable to various stimuli, including traumatic, toxic and disease-associated neurodegenerative factors (1). As one of these pathological stimuli, ischemia/hypoxia has been extensively investigated in the central nervous system (CNS), including stroke-induced brain damage (2). However, few studies have focused on the stimulation of ischemia/hypoxia on the peripheral nervous system (PNS), although the PNS can be highly susceptible to reductions in blood supply and oxygen levels during surgical and pathological conditions (3,4) and can cause neurological complications, ranging from mild functional loss to complete limb paralysis and permanent functional deficits (5). Hypoxia is considered to be the key pathological stimulus in CNS ischemia (6) and also induces functional changes at the neuromuscular junction (7,8). The mechanisms underlying nerve terminal vulnerability to hypoxia-reperfusion injury in situations, including trauma, remain to be fully elucidated (9) and require further investigation. The majority of current in vivo animal models of hypoxia-reperfusion rely on the experimental application of high-pressure tourniquets, which may induce considerable mechanical stress and potential crush injury to the underlying nerves, making it difficult to conclusively distinguish the effects of ischemia-reperfusion injury from the effects of mechanical trauma (10).

During ischemia/hypoxia-induced damage to the CNS/PNS or other organs, there are two major processes, necrosis and apoptosis, leading to neural cell death. In the core ischemic area, the severe restriction of blood flow leads to necrotic cell death. However, in the peripheral ischemic area, where collateral blood flow can buffer the degree of damage, cell apoptosis predominantly develops (11,12). Evidence suggests that activation of apoptotic pathways occurs in the peripheral cells of the ischemic area, in a caspase-dependent and caspase-independent manner (13,14). Therefore, the apoptotic cascades during the ischemia/hypoxia-induced damage to CNS/PNS are reversible and are a major target of therapeutic interventions (15,16).

A variety of transcription factors have been reported to be involved in regulating the genes responsible for the metabolic responses to hypoxia-ischemia (17,18). A key component among these factors is hypoxia-inducible factor 1 (HIF-1), existing as a heterodimer which is composed of a constitutively expressed HIF-1β subunit and an oxygen sensitive HIF-1α subunit. Following activation by hypoxia, the HIF-1α/HIF-1β dimer (19) binds to a conserved DNA consensus on the promoters of its target genes, known as the hypoxia-responsive
element (20, 21), and induces a group of gene products that are crucial for hypoxic adaptation (22). An anti-apoptotic and a pro-apoptotic effect have been confirmed for HIF-1α and/or hypoxia in hypoxia-ischemia. Severe or prolonged hypoxia induces apoptosis, at least in part, whereas activated HIF-1α, along with other molecules, protects neural cells from apoptosis (23). HIF-1α can prevent apoptosis by activating the phosphoinositide 3-kinase (PI3K)/Akt pathway (24) or by promoting the expression of survivin (25) in acute hypoxia, or by increasing the expression of glycolytic enzymes, p21 and erythropoietin to antagonize the effect of the hypoxia- and/or hypoglycaemia-induced expression of tumor suppressor gene p53 (26) or BNIP3 (27). However, other molecules involved in the pro- or anti-apoptotic effects of HIF-1α in hypoxia-ischemia damage in the CNS/PNS require investigation.

In the present study, in order to investigate the role of HIF-1α and Ras homolog gene family, member A (RhoA) in hypoxic-ischemic damage to the CNS/PNS, PC12 neuroblastoma cells were examined following hypoxia treatment. The expression levels of HIF-1α and RhoA, which belongs to the Rho small GTPase family and is reported to be implicated in hypoxia adaptation were assessed. Subsequently, gain-of-function and loss-of-function strategies were adopted to manipulate the expression levels of HIF-1α or RhoA in PC12 cells, and the expression of hypoxia-induced RhoA and cell apoptosis were determined again.

Materials and methods

Reagents, cell culture and treatment. The PC12 rat pheochromocytoma cell line was provided by the National Platform of Experimental Cell Resources for Sci-Tech (Beijing, China) and were cultured in F12K medium (Invitrogen Life Technologies, Carlsbad, CA, USA) containing 15% fetal calf serum (Invitrogen Life Technologies) at 37°C under 5% CO₂. F12K medium supplemented with 2% fetal bovine serum was used for the cell maintenance. For hypoxia treatment, the cells at 85% confluence were placed in a hypoxia incubator (Innova 49; New Brunswick Scientific, Inc., Edison, NJ, USA) and infused with a gas mixture of 5% CO₂ and nitrogen to obtain 3% oxygen concentration, at 37°C for 0, 2, 4, 6, 12, 24 or 48 h. The oxygen concentration was monitored continuously (Forma 3130; Thermo Fisher Scientific, Rockford, IL, USA). To overexpress HIF-1α in the PC12 cells, the wild-type HIF-1α coding sequence was amplified with Pfu DNA polymerase (Promega, Madison, WI, USA) and cloned into a pcDNA3.1 (+) vector (Invitrogen life Technologies), with HindIII and BamHI (New England Biolabs, Ipswich, MA, USA) as restriction endonucleases. The HIF-1α pcDNA3.1 (+) or control CAT-pcDNA3.1 (+) plasmids were then transfected, respectively, into PC12 cells using Lipofectamine 2000 (Invitrogen Life Technologies), and 25 or 50 nM small interfering (si) RNA-RhoA or siRNA-Control (Sangon Biotech, Co., Ltd., Shanghai, China) were transfected into the cells using Lipofectamine 2000 to knockdown the expression of RhoA.

RNA isolation and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total cellular RNA was isolated from the cells using a PureLink® RNA Mini kit (Invitrogen Life Technologies), according to the manufacturer's instructions. The mRNA expression levels of HIF-1α and RhoA were quantified by RT-qPCR using a Takara One Step RT-PCR kit (Takara Biotechnology, Co., Ltd., Dalian, China) and paired primers as follows: HIF-1α, forward 5′-aac ctaacaaaaacatcca-3′, and reverse 5′-tattgaagatgacatgaag-3′; RhoA, forward 5′-gtgccagatatcaggtga-3′ and reverse 5′-aat ctctgtgagctgtg-3′ and β-actin, forward 5′-tgcctccatccgca agatg-3′ and reverse 5′-tgtaagctcgctctga-3′ (Sangon Biotech, Co., Ltd., China). Equal quantities of mRNA sample (1 µl) were used for quantitative analysis. The RT-qPCR was performed as follows: 65°C for 5 min, 42°C for 40 min and 95°C for 10 sec for the reverse transcription step then 95°C for 5 sec and 60°C for 10 sec (40 cycles) for the PCR reaction. The mRNA samples were amplified using primer/probe sets specific for the genes of interest on a Lightcycler 480 II (Roche Diagnostics, Mannheim, Germany). Relative quantification was determined using the ∆∆Ct method (28), with tubulin as a reference gene (28).

Western blot analysis. To determine protein expression levels, 5x10⁵ PC12 cells were collected and lysed using a cytoplasmic protein extraction kit (TP-001; ZmTech Scientific, Inc., San Jose, CA, USA) and supplemented with a protease inhibitor cocktail (Roche Diagnostics) according to the manufacturer's instructions. All protein samples were quantified using Bradford protein assay reagent (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and each sample with 30 µg (5 µl) was separated on a 8-12% gradient SDS-PAGE gel, followed by being transferred onto a polyvinylidene difluoride membrane and blocked in 5% skimmed milk. Rabbit polyclonal antibodies against caspase 3 (1:1,000; cat. no. ab2302; Abcam, Cambridge, UK), Poly ADP ribose polymerase (PARP; 1:500; cat. no. ab137653; Abcam), cytochrome c (1:1,000; cat. no. 4272; Cell Signaling Technology Inc., Danvers, MA, USA), HIF-1α (1:1,000; cat. no. sc-10790; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), HIF-1β (1:500; cat. no. C15A11, Cell Signaling Technology Inc.), RhoA (1:1,000; cat. no. sc-179, Santa Cruz Biotechnology, Inc.), Rho-associated kinase (Rock) 1 (1:500; cat. no. sc-5560, Santa Cruz Biotechnology, Inc.), Rock 2 (1:1,000; cat. no. sc-5561, Santa Cruz Biotechnology, Inc.), RhoB (1:1,000; cat. no. sc-180, Santa Cruz Biotechnology, Inc.) and β-actin (1:500; cat. no. sc-130656, Santa Cruz Biotechnology, Inc.) were used to quantified the protein level of each molecule via an incubation at 4°C overnight. Goat anti-rabbit IgG conjugated to horseradish peroxidase (1:500; cat. no. 3122, Pierce Biotechnology, Inc., Rockford, IL, USA) and an enhanced chemiluminescence detection system (Super Signal West Femto; Pierce Biotechnology, Inc.) were used for detection and quantification. Prior to each inoculation, samples were washed four times with 1X phosphate-buffered saline with Tween-20.

Apoptosis and caspase 3 assays. The apoptosis of the PC12 cells was examined using an Annexin V-FITC Apoptosis Detection kit (Sigma-Aldrich, St. Louis, MO, USA). Briefly, 4x10⁵ cells were stained with Annexin V-flourescein isothiocyanate (FITC; 5 µl Annexin V-FITC conjugate in 1 ml suspended cells) and propidium iodide (10 µl propidium iodide solution in 1 ml suspended cells), and detected using a FACSFlow cytometer (Bio-Rad Laboratories, Inc.) to analyze the levels of cellular apoptosis. The results were calculated using...
CellQuest™ Pro software (Bio-Rad Laboratories, Inc.) and expressed as the percentage of apoptotic cells in the total cells. The activity of caspase 3 was examined using a Caspase 3 Activity Assay kit (Cell Signaling Technology, Inc.), according to the manufacturer's instructions, with the activity expressed as the relative fluorescence intensity of 7-amino-4-methylcoumarin (AMC), compared with the control.

Statistical analysis. Statistical analyses were performed using SPSS 16.0 software (SPSS, Inc., Chicago, IL, USA). Comparison of the mRNA and protein expression levels of HIF-1α and RhoA, the percentage of apoptotic cells and the activity of caspase 3 between the two groups were analyzed using Student's t-test. P<0.05 was considered to indicate a statistically significant difference.

Results

Hypoxia induces the apoptosis of PC12 cells. In the present study, flow cytometric analysis was used to measure the level of apoptosis of cells of the PC12 rat pheochromocytoma cell line, induced by hypoxia. When the cells were exposed to hypoxia for 12 h, 24 h or 48 h, significantly more PC12 cells underwent apoptosis, compared with the cells under normoxic conditions (P<0.05 or P<0.01; Fig. 1A). Procaspase 3 undergoes cleavage and activation in apoptosis (29), and hypoxia
treatment also induced higher levels of caspase 3 activity in the present study, which was evaluated by measuring the fluorescence intensity of AMC, compared with the control PC12 cells (P<0.01 or P<0.001; Fig. 1B). The cleavage of procaspase 3 in the PC12 cells following hypoxia treatment was also examined (Fig. 1C). As shown in Fig. 1D, a higher level of procaspase 3 was cleaved into its 17 and 12 kDa subunits in the soluble protein in the PC12 cells, compared with the control (P<0.01). To further determine whether PC12 cell death by hypoxia was mediated by caspase-3 activation, the cleavage of poly(ADP-ribose) polymerase (PARP) by activated caspase 3 was examined. The results demonstrated that, following exposure to hypoxia for 48 h, PARP cleavage was significantly promoted (P<0.05; Fig. 1C and E). The present study also determined the release of cytochrome c, which is translocated between the mitochondria and the cytosol where it assists in activating caspases. As shown in Fig. 1C and F, hypoxia also significantly enhanced the release of cytochrome c (P<0.05).

**HIF-1α antagonizes hypoxia-induced apoptosis of PC12 cells.** To determine whether HIF-1α alters hypoxia-induced apoptosis in PC12 cells, an plasmid overexpressing the HIF-1α gene was transfected into PC12 cells, and the mRNA and protein levels of HIF-1α and HIF-1β were evaluated using RT-qPCR and western blot analysis. As shown in Fig. 2A, the expression of HIF-1α was over four times higher in the HIF-1α-overexpressing plasmid-transfected cells (P<0.01),
compared with the control plasmid-transfeted cells. The protein level of HIF was also increased in the HIF-1α overexpression-plasmid-transfected cells (P<0.01 or P<0.001; Fig. 2B and C), whereas the expression of HIF-1β was not regulated by HIF-1α overexpression (Fig. 2B and D). Subsequently, to determine whether HIF-1α alters hypoxia-induced apoptosis, the present study re-evaluated the activity of caspase 3 by measuring the AMC fluorescence intensity of the PC12 cells with or without HIF-1α transfection. As shown in Fig. 2E, HIF-1α overexpression inhibited caspase 3 activity from 24 h post-transfection, and less AMC fluorescence intensity was observed in the HIF-1α-overexpressed PC12 cells (P<0.05) 24 or 48 h post-transfection. In addition, flow cytometric analysis revealed that the overexpression of HIF-1α inhibited the hypoxia-induced apoptosis of the PC12 cells (P<0.05) 24 or 48 h post-transfection; Fig. 2F). Taken together, these findings indicated that the overexpression of HIF-1α attenuated hypoxia-induced PC12 cell apoptosis.

Hypoxia induces RhoA in PC12 cells. RhoA has been reported to be activated by chronic hypoxia in the lungs (30,31), as two of the key downstream effectors of RhoA, Rock 1 and Rock 2, have been confirmed to be involved in the lung response to hypoxia (32). In present study, to determine possible role of RhoA, Rock 1, Rock 2 and RhoB in hypoxia-induced PC12 cell apoptosis, the present study examined the expression levels of the three molecules in PC12 cells. As shown in Fig. 3A and B, the expression of RhoA was markedly upregulated by >10-fold, between 2 and 6 h post-hypoxia (P<0.001). The expression of Rock 1 was also significantly upregulated in the PC12 cells from 2 h post-hypoxia exposure, with a ~30-fold elevation in the expression of Rock 1 following hypoxia stimulation (P<0.01 or P<0.001; Fig 3C). Notably, the expression levels of Rock 2 appeared to change minimally on hypoxia stimulation, and began to increase 4 h post-hypoxia exposure, with an elevation of only three-fold (Fig. 3D). RhoB, an inhibitor of Rho activity, was unresponsive to hypoxia.

Figure 3. Hypoxia induces RhoA in PC12 cells. The PC12 cells were exposed to hypoxic conditions for 0, 2, 4, 6 and 8 h and were analyzed for the expression of RhoA, Rock 1 and Rock 2. (A) Western blot analysis of the expression levels of RhoA, Rock1, Rock2 and RhoB in hypoxia-treated PC12 cells. Percentage of (B) RhoA, (C) Rock1, (D) Rock2 or (E) RhoB to β-actin in the hypoxia-treated PC12 cells. All experiments were performed in triplicate and data are presented as the mean ± standard error of the mean. *P<0.01 and **P<0.001. Rho, Ras homolog gene family, member A; Rock, Rho-associated kinase; ns, no significance; H.P.T, hours post-treatment.
hypoxia stimulation, with no significant change between 2 and 6 h post-hypoxia exposure (Fig.3E). Therefore, RhoA signaling was significantly promoted by hypoxia in the PC12 cells, and RhoA became the focus of subsequent experiments to investigate levels of expression and to investigate the molecular mechanisms used by the Rho proteins to regulate hypoxic responses.

**Hypoxia-induced HIF-1α is RhoA-dependent.** The present study used RhoA specific siRNA to investigate the effect...
of RhoA on the promotion of HIF-1α. First, the mRNA and protein levels of RhoA were measured using RT-qPCR and western blotting following the transfection of siRNAs specific for RhoA (25 or 50 nM) into the PC12 cells. It was observed that 25 or 50 nM anti-RhoA siRNA significantly inhibited the mRNA and protein expression levels of RhoA (Fig. 4A and B). It was also demonstrated that the mRNA expression of HIF-1α under hypoxia treatment also reduced following anti-RhoA siRNA transfection in the PC12 cells between 12 and 24 h, which suggested that hypoxia-induced HIF-1α was RhoA-dependent (P<0.05). The present study also examined whether transfection with anti-RhoA siRNA regulated the protein expression of HIF-1α. As shown in Fig. 4D and E, compared with the siRNA control-transfected PC12 cells, siRNA targeting RhoA significantly inhibited hypoxia-induced the protein expression of HIF-1α at 12, 24 h (P<0.05) and 48 h (P<0.01) post-hypoxia.

RhoA knockdown abrogates the antagonism of HIF-1α in hypoxia-induced apoptosis of PC12 cells. As shown in Fig. 2, HIF-1α was observed to antagonize hypoxia-induced apoptosis of the PC12 cells, whereas the overexpression of HIF-1α in the PC12 cells was inhibited by RhoA knockdown by siRNA transfection. The present study investigated whether RhoA knockdown can reverse this change. Following co-transfection with anti-RhoA siRNA and the pcDNA-HIF-1α plasmid, the PC12 cells were treated with hypoxia for 12, 24 and 48 h. The fluorescence intensity of AMC revealed that the transfection with RhoA siRNA significantly improved hypoxia-induced caspase 3 activity with HIF-1α treatment (Fig. 4E). Flow cytometric analysis also indicated that more apoptotic cells were formed in the RhoA siRNA transfected PC12 cells, compared with the control siRNA group (Fig. 4F). In conclusion, knockdown of RhoA in the PC12 cells abrogated the antagonism of HIF-1α to hypoxia-induced apoptosis.

Discussion

Hypoxic conditions regulate several metabolic enzymes and transcription factors, which are involved in cancer, ischemia and pulmonary diseases (33). HIF-1 is a transcription factor induced by hypoxia, which possesses two subunits, HIF-1α and HIF-1β (34). Several studies have demonstrated that reactive oxygen species (ROS) generation is necessary for the transcriptional response induced by hypoxia (35,36). The addition of diphenylene iodonium, an inhibitor of ROS, or the use of cells depleted in mitochondria can eradicate the hypoxic induction of HIF-1α, erythropoietin, glycolytic enzymes and vascular endothelial growth factor (37). Analysis of the events upstream of HIF-1α has suggested the importance of the generation of ROS in hypoxia via mitochondria as a sensor of oxygen levels, and that ROS may be involved in stabilizing HIF-1α (36,38).

RhoA belongs to the Rho small GTPase family, within the Ras-like protein superfamily, which also includes the Ras, Rab, Arf and Ran families (39). Rho molecules are highly conserved between eukaryotes and mammals (40). Following activation, Rho GTPases trigger a signaling cascade to direct a variety of cellular responses (41-43). Rho GTPases have been reported to contribute to the majority of steps of cancer initiation and progression, including unlimited proliferation potential and evasion from apoptosis, and several Rho GTPases are upregulated in certain types of human tumor, including RhoA, RhoC, Rac1 (44,45). The upregulated Rho GTPases exert their pro-oncogenic effects via the stimulation of cell cycle progression and regulation of gene transcription (46). Certain Rho GTPases are considered to be able to regulate the release of pro-angiogenic factors to promote neovascularisation (47). Interacting with the plasma membrane, RhoA modulates downstream signaling pathways to regulate cell cycle and survival (48,49). In addition, RhoA is essential in cancer cell migration and invasion (50-52). RhoA has been suggested to be involved in several anti-apoptotic pathways in the suppression of apoptosis. The expression of RhoA promotes activation of extracellular signal-regulated kinase (ERK) and facilitates glomerular epithelial cells survival (53). RhoA also upregulates the expression of anti-apoptotic Bcl-2 in T cells (54), vascular smooth muscle cells (55) and osteosarcoma cells (56). In addition, Rho inhibition induces p53 in human endothelial cells (57).

Apoptosis during CNS/PNS damage is reversible and is regarded as a major target of therapeutic interventions (15,16). In the present study, hypoxia was demonstrated to induce apoptosis of PC12 cells. Hypoxia had a significant effect on PC12 cell apoptosis (Fig. 1), including caspase 3 activation and cytochrome c release. Secondly, transfection of an expression plasmid into PC12 cells revealed that the overexpression of HIF-1α inhibited hypoxia-induced PC12 cell apoptosis (Fig. 2). To improve understanding of the mechanisms involved in the upregulation of RhoA, the present study investigated its expression in hypoxia-induced PC12 cells. The promotion of HIF-1α and RhoA, which is reported to be involved in hypoxia adaptation, was observed in the neuroblastoma PC12 cells following exposure to hypoxia (Fig. 3). By inducing the overexpression and inhibition of RhoA, the present study manipulated the level of HIF-1α in the PC12 cells, and revealed that hypoxia induced RhoA upregulation and cell apoptosis. The results further confirmed the protective role of HIF-1α and RhoA in hypoxia-induced PC12 cell apoptosis, and that the upregulation of RhoA by hypoxia was HIF-1α-dependent (Fig. 4).

In conclusion, the present study demonstrated that HIF-1α upregulation protected PC12 neuroblastoma cells from hypoxia-induced apoptosis in a RhoA-dependent manner, and the promotion of HIF-1α and RhoA may be a valuable strategy for therapeutic intervention for hypoxic-ischemic damage to the CNS/PNS.

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