

Inhibitive Effects of Low Oxygen and Glucose Deprivation on Brain-Pancreas Relative Protein Expression via Hypoxia-Inducible Factor-1 Pathways

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Key Words

Brain-Pancreas Relative Protein • Hypoxia-inducible factor-1 α • Low oxygen and glucose deprivation • [Ca²⁺]_i • p38 MAPK

Abstract

Background: Brain-Pancreas Relative Protein (BPRP), a novel protein discovered in our lab, was decreased in ischemic rat brain. However, the mechanisms regulating BPRP expression during ischemia need further investigation. **Methods:** In the present study we cultured PC12 cells with low oxygen and glucose deprivation (LOGD, a model of ischemia *in vitro*) media, then examined the signal transduction pathways of BPRP expression under LOGD. **Results:** It was found that LOGD significantly decreased BPRP expression, but increased the intracellular Ca²⁺ concentration ([Ca²⁺]_i), p38 mitogen-activated protein kinases (MAPK) phosphorylation and hypoxia inducible factor 1 α subunit (HIF-1 α) expression. However, BAPTA-AM (an intracellular calcium chelator), SB 203580 (an inhibitor of p38) and HIF-1 α antisense significantly inhibited the [Ca²⁺]_i, p38 MAPK phosphorylation and HIF-1 α expression respectively. Our results also showed that p38 MAPK

phosphorylation was reduced by BAPTA-AM, and HIF-1 α expression was inhibited by SB203580 and BAPTA-AM, suggesting that calcium, p38 MAPK and HIF-1 α are in the same signal transduction pathways during LOGD. Noticeably, reduced BPRP expression by LOGD can be recovered by SB203580, BAPTA-AM and HIF-1 α antisense. **Conclusion:** All together, our observations suggest that calcium, p38 MAPK activation and HIF-1 α are necessary for LOGD-reduced BPRP expression in PC12 cells.

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Introduction

By using the sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis, we found a significant reduction in a 260 kD protein expression in rat ischemic brain. Further immunohistochemical study demonstrated that this protein mainly expressed in the brain neurons and pancreatic islet cells, so we named this protein Brain-Pancreas Relative Protein (BPRP) [1]. BPRP was analyzed by peptide mass fingerprinting (PMF) and matrix assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF-MS), which results

suggest that BPRP is a novel protein and matches highly with microtubule-associated protein 1A (MAP1A) [1]. Further study supported that BPRP may be one of the MAPs or an analog of MAPs [2]. The putative functions of MAP have been proposed to play important roles in the outgrowth of neuronal processes, synaptic plasticity and neuronal cell death [3], indicating that BPRP may play an important role during ischemia. Therefore, it deserves to further investigate the signal transduction pathways regulating BPRP expression during ischemia.

Cerebral ischemia, caused by the occlusion of blood supply to part of or the entire brain, is a major cause of brain damage. Mammalian cells, neurons in particular, are extremely sensitive to hypoxic/ischemic insult, and are subject to substantial injury [4]. Hypoxic condition contributes to the generation of reactive oxygen species (ROS), which, in excess, could be cytotoxic and often cytotoxic [5]. Our previous results showed that the enhanced formation of intracellular ROS was involved in low oxygen and glucose deprivation (LOGD)-induced reduction in BPRP expression in PC12 cells [6]. There is evidence that adaptive responses to ischemia/hypoxia involve the regulation of gene expression by hypoxia inducible factor 1 (HIF-1) which is a heterodimer transcription factor consisting of HIF-1 α and HIF-1 β subunits [7]. HIF-1 β is ubiquitously overexpressed, but HIF-1 α is a mammalian transcription factor expressed uniquely in response to physiologically relevant hypoxic conditions and found at low levels under normoxic conditions due to ubiquitination and proteasomal degradation [8-10]. HIF-1 regulates the expression of at least 180 genes involved in metabolism, cell survival, erythropoiesis and vascular remodeling [11] by accumulation in the nucleus where HIF-1 α dimerizes with HIF-1 β and binds to the core DNA sequence 5'-RCGTG-3' [12, 13, 8]. Thus, HIF-1 α confers sensitivity and specificity for hypoxic induction. However, the precise cellular mechanisms underlying ischemia-induced reduction in BPRP expression over the course of ischemia still require extensive exploration.

Cellular effects of ischemia/hypoxia on excitable cells have been shown to be mediated by O₂-sensitive voltage-dependent ion channels. Such cells respond to ischemia/hypoxia with membrane depolarization and Ca²⁺ influx [14, 15]. Calcium, a second messenger, has been proposed as an essential trigger for cell activation [16]. It was reported that release of intracellular Ca²⁺ ([Ca²⁺]_i) from internal stores is required for mitogen-activated protein kinase (MAPK) activation [17]. MAPK plays an essential role in the transduction of a variety of extracellular stimuli

to the nucleus [13]. MAPK family consists of three commonly recognized subgroups: extracellular signal-regulated kinase (ERK), c-jun-N-terminal kinase (JNK) and p38 kinase [18]. In response to extracellular stimuli, MAPK is activated by phosphorylation on both adjacent threonine and tyrosine residues [19]. For example, JNK and p38 kinase are activated in response to cellular stresses like ischemia in the heart, kidney and brain [20-23].

The purpose of this study is to further reveal the biological regulating mechanisms by which ischemia led to the reduction in BPRP expression. For this purpose, LOGD was applied as a model of ischemic brain damage *in vitro*. LOGD is an *in vitro* model for ischemia/stroke since cessation of blood flow deprives the brain of essential components, oxygen and glucose [24-27]. Then the possible roles of [Ca²⁺]_i, MAPK and HIF-1 in BPRP expression under LOGD were analyzed in PC12 cells, which has been extensively used for cell signaling studies, various types of neurochemical studies and *in vitro* model for neural cells [28-30]. At the same time BPRP was found to express in PC12 cells [6].

Materials and Methods

PC12 cells culture

The undifferentiated PC12 cells which derived from rat pheochromocytoma were plated on poly-L-lysine-coated petri dishes and grown in Dulbecco's modified Eagle's medium (DMEM) (GIBCO) supplemented with 5% horse serum, 10% heated-inactivated fetal bovine serum with 100 U/ml penicillin and 100 μ g/ml streptomycin. Cells were routinely cultured at 37°C in a humidified 95% air-5% CO₂ chamber. Cells were subcultured up to ten passages. Cellular viability was determined with Trypan Blue exclusion, only cell preparations with a 95% viability or greater were used.

LOGD treatment

To induce LOGD in PC12 cells, the DMEM culture media were replaced with glucose-free Earle's solution containing (in mM): NaCl 143, KCl 5.4, MgSO₄ 1.0, NaH₂PO₄ 1.0, CaCl₂ 1.8, Hepes 2.4, and deoxygenated reagent of 0.5 mmol/l sodium dithionite (SD) (Na₂S₂O₄) was supplemented. The inhibitors and antisense oligonucleotides of HIF-1 α were pretreated with PC12 cells for 30 min prior to 12 h of LOGD exposure. Sodium dithionite is a very strong reducing agent and can remove oxygen from the media, despite its short half-life. By using sodium dithionite in aqueous solutions, precise and graded oxygen concentrations could be achieved. Cytotoxicity was directly proportional to the oxygen concentration of the medium [31]. In order to check whether sodium dithionite itself has effect on BPRP expression, sodium dithionite was dissolved in DMEM media 24 h before incubating with PC12 cells, because

the lack of oxygen will last for a time it takes atmospheric oxygen to diffuse back into solution.

Neurotoxicity assay

PC12 cells culture supernatant was collected, then the lactate dehydrogenase (LDH) activity was measured using a colorimetric LDH assay kit (Nanjing Jiancheng Bioengineering Institute, China). The remanent PC12 cells were also collected and lysed, then the superoxide dismutase (SOD) activity was measured using a SOD assay kit (Nanjing Jiancheng Bioengineering Institute, China), and malondialdehyde (MDA) content was measured using a MDA assay kit (Nanjing Jiancheng Bioengineering Institute, China).

Western blot analysis

PC12 cells were collected and washed with ice-cold 0.1M phosphate buffer saline (PBS) solution for 3 times, then centrifuged at 800 rpm for 5 min each time at 4°C. Pellet was resuspended with a solution containing: 25 mM Tris-HCl (pH 8.5), 0.2% SDS, 10 mM EDTA, 10 mM EGTA, 2 mM 2-mercaptoethanol, 1 mM PMSF and 10 U/ml aprotinin and 1% (v/v) Triton X-100. Suspension was kept at 4°C for 20 min to extract protein. The extract was then centrifuged at 12,000 rpm for 30 min at 4°C. Protein concentration of each sample was measured by the Bradford method [32].

The expression of BPRP and β -actin as a loading control was estimated by Western blot analysis. Separating (7.5%) and stacking (4%) polyacrylamide gels containing 0.1% SDS were used. Protein was suspended in a sample buffer containing 2% SDS, 0.1 M Tris-HCl (pH 6.8), 10% glycerol (v/v) and 0.1 M dithiothreitol (DTT), and then heated at 100°C for 5 min in a water bath. Protein (20 μ g) was loaded in each lane of the polyacrylamide gel, which was electrophoresed at 150 V for 1 h. Protein was transferred electrophoretically at 50 mA to a polyvinylidene difluoride (PVDF) membrane (0.45- μ m pore size: Millipore Corp, Bedford, MA) in a transfer buffer (pH 8.3) composed of 25 mM Tris-HCl, 192 mM glycine and 20% methanol at 4°C overnight. The membrane was incubated in Tris-buffered saline [TBS: 100 mM Tris-HCl, 0.9% NaCl (pH 7.5)] solution containing 5% skim milk for 1 h at room temperature and subsequently incubated with the BPRP antibody (1:1000) and β -actin antibody (Santa Cruz, CA, USA) (1:5000) and shaken on a rotator at room temperature for 2 h. The membrane was washed three times with TBS containing 0.1% Tween-20 (TBS-T) and then incubated with streptavidin AP-conjugated antibody (Santa Cruz, CA, USA) (1:3000) in TBS containing 2.5% skim milk for 1 h at room temperature. The membranes were washed three times with TBS-T. The blots were developed using nitro blue trazolium/5-Bromo-4-chlor-3-indolyl phosphate salt (BCIP/NBT) system. The bands were visualized using Gel Doc 2000 (Bio-Rad). The BPRP expression was normalized by the levels of β -actin and expressed as a percentage of the control.

Flow cytometer

Quantification of BPRP, HIF-1 α , p38, phospho-p38 expression was performed by flow cytometer (FCM) (Becton Dickson). In brief, PC12 cells (>10⁶ cells/ml) were collected after

being treated as required, then washed with cold 0.1 M PBS (3x10 min) and centrifuged at 800 rpm for 5 min at 4°C. After that, cells were fixed with 70% ethanol for at least 18 h at 4°C. The cells were washed with cold 0.1M PBS (3x10 min) and centrifuged at 800 rpm for 5 min at 4°C, then incubated with primary antibody which was diluted at 1:1000 in PBS/1.5% bovine serum albumin (BSA) for 2 h at room temperature (BPRP raised in our lab; HIF-1 α , p38, phospho-p38 from Santa Cruz, CA, USA). After being washed and centrifuged as above, the cells were incubated with fluorescein isothiocyanate (FITC)-conjugated IgG (Santa Cruz, CA, USA) which was diluted at 1:2000 in PBS/1.5% BSA for 1.5 h at room temperature. The cells then were analyzed with FCM after being washed with cold 0.1M PBS (3x10 min) and centrifuged at 800 rpm for 5 min at 4°C. Negative controls were set without the presence of the primary antibody. Proteins expression was analyzed and compared to the internal standard by the software automatically in FCM. The data were expressed as percentage of the control.

Calcium measurement with Fluo-3/AM

The calcium-sensitive dye fluo-3/AM (Biotium, CA, USA) was dissolved in dimethyl sulfoxide (DMSO) (initially dissolved at 1 mM in DMSO) associated with pluronic F-127 (Biotium, CA, USA) which was also prepared with DMSO (25%, w/v). PC12 cells were loaded with 1 μ M fluo-3/AM for 30 min at 37°C and then rinsed with freshly prepared 0.1M PBS. The changes of [Ca²⁺]_i in PC12 cells were examined by confocal laser scanning microscopy (CLSM) (LEICA). The software of CLSM analyzed the average fluorescence values of 30 cells.

Antisense oligonucleotides of HIF-1 α

Antisense oligonucleotides of HIF-1 α was designed and synthesized according to Caniggia et al [33]. Oligonucleotides of 15 bp targeted against sequences adjacent to the AUG initiation codon of HIF-1 α mRNA were synthesized. The sequences of the antisense and sense (as control) HIF-1 α oligonucleotides were: 5'-GCC GGC GCC CTC CAT-3' and 5'-ATG GAG GGC GCC GGC-3' respectively. Oligonucleotides were dissolved in water and their concentrations were estimated by optical density at OD₂₆₀. 10 μ M antisense or sense oligonucleotides were added in the PC12 cells medium 30 min before LOGD.

Reverse transcription polymerase chain reaction (RT-PCR) analysis

Total RNA was extracted from PC12 cells following the manufacturer's instructions with the addition of an extra acid phenol/chloroform extraction followed by RNA precipitation, and reverse transcribed (Dingguo Biology, Beijing, China). We devised primers as follows: HIF-1 α : forward 5'-GTT TAC TAA AGG ACA AGT CAC C-3'; reverse 5'-TTC TGT TTG TTG AAG GGA G-3' (193 bp; T_m 81.3). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH): forward 5'-CAGAAGACT GTG GAT GG-3'; reverse 5'-GCT TCA CCA CCT TCT TG-3' (237 bp; T_m 88.7). GAPDH was used as the internal control. The conditions of the PCR amplification were as follows: 94°C for 2 min followed by 25 cycles of 94°C for 45 sec, 55°C for 45 sec, 72°C for 45 sec, then a final extension step was performed at 72°C for 5 min, and

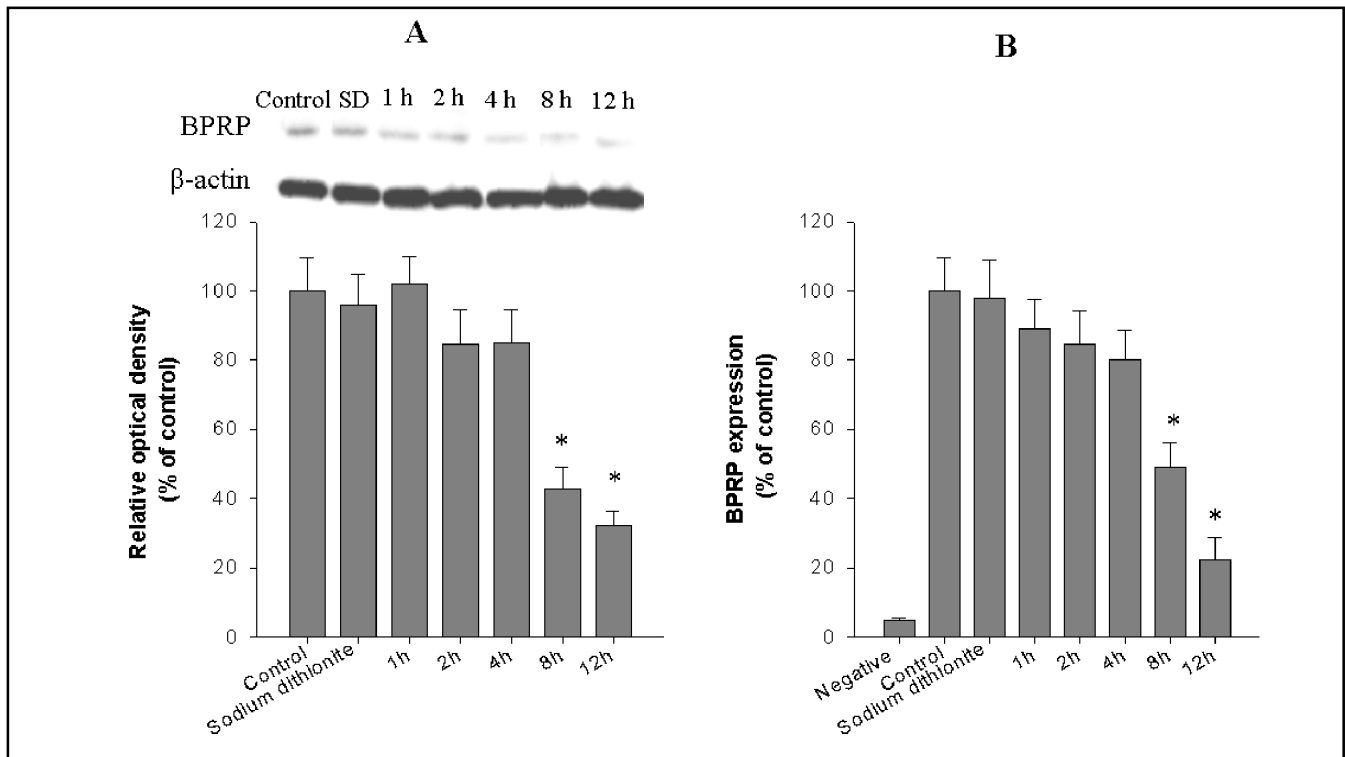


Fig. 1. Effects of low oxygen and glucose deprivation (LOGD) on BPRP expression. PC12 cells were exposed to LOGD for 1 h, 2 h, 4 h, 8 h and 12 h, then BPRP expression in PC12 cells were analyzed by Western blot and FCM. A, effects of LOGD on BPRP expression measured by Western blot; B, effects of LOGD on BPRP expression measured by flow cytometer. The data were represented as the means \pm S.E. Values were obtained from three separate cultures. * $P < 0.05$ vs control (normal oxygen and DMEM culture medium).

finally cooled down to 4°C. PCR product was electrophoresed through 2.0% agarose gel and stained with EB. The sizes of amplified DNA fragments were determined by comparing with DL2000 DNA ladder marker (TaKaRa, Shiga, Japan). The intensity of the amplified bands was quantified with Gel Doc2000 Image System (Bio-Rad, USA). No signals were detected without the initial addition of reverse transcriptase.

Statistical analysis

Data were evaluated for statistical significance with one way ANOVA followed by Duncan's analysis with the SPSS software (version 12.0). All data are represented as the means \pm S.E. accompanied by the number of experiments performed independently. P -values lower than 0.05 were considered as statistically significant.

Results

LOGD reduced BPRP expression

Both Western blot and FCM were applied to examine the effects of LOGD on BPRP expression. As shown in Figure 1A, LOGD induced a significant reduction in BPRP expression in PC12 cells after 8 h ($P < 0.05$). Reduction

in BPRP expression measured by Western blot was further confirmed by the results from FCM (8 h, 12 h, $P < 0.05$) (Figure 1B). These changes were consistent with our previous study which showed that ischemia led to a decrease in BPRP expression [1]. Further experiment showed that sodium dithionite, which had been dissolved in DMEM medium for 24 h before incubating with PC12 cells, had no significant effect on BPRP expression (Figure 1A and 1B).

Neurotoxicity of LOGD on PC12 cells

The amount of LDH release is a marker of structural damage. SOD is an important antioxidant enzyme that can quench excess free radicals such as superoxide anion in cells. As shown in Figure 2, LOGD significantly increased the LDH release in a time-dependent manner (1 h, 2 h, $P < 0.05$; 4 h, 8 h, 12 h, $P < 0.01$) (Figure 2A), while LOGD significantly decreased the SOD activity after 2 h in PC12 cells ($P < 0.05$) (Figure 2B). Moreover, the aversive effects of LOGD on PC12 cells were accompanied by increased lipid peroxides. As shown in Figure 2C, intracellular MDA, a suitable biomarker for

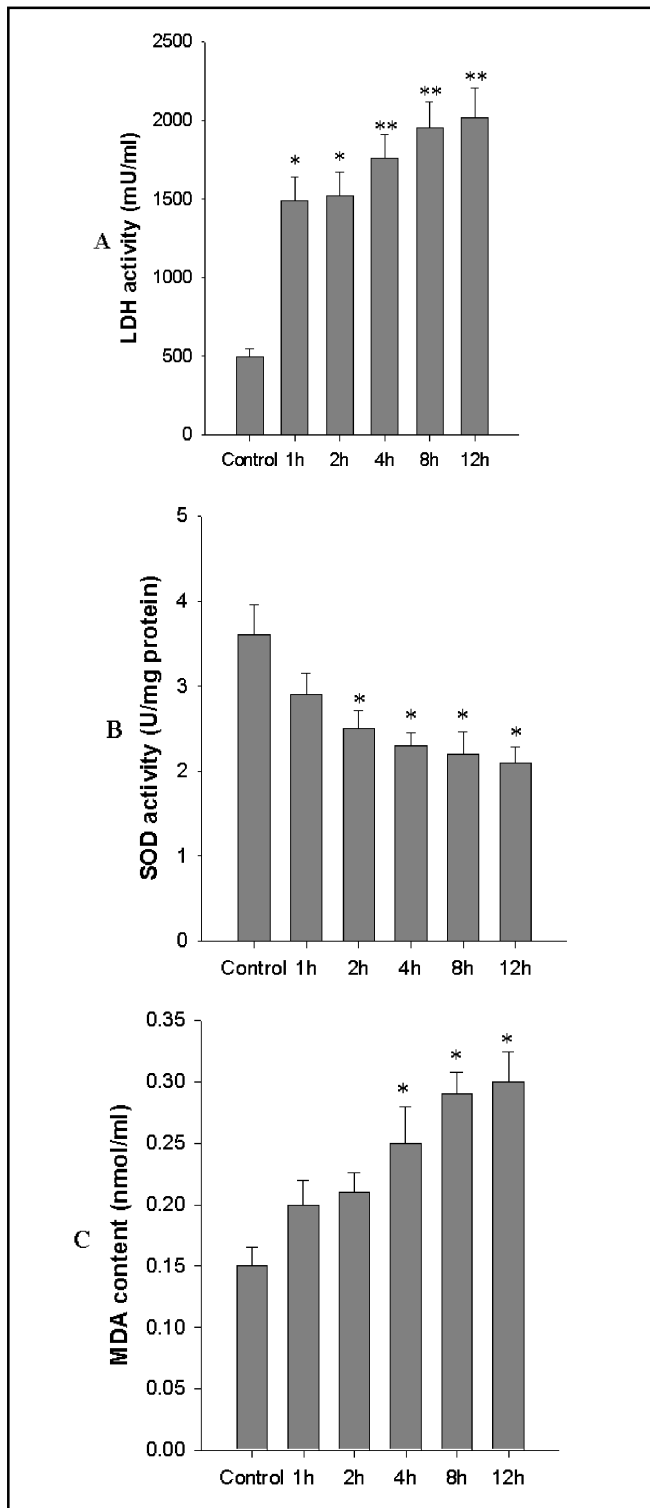


Fig. 2. Effects of low oxygen and glucose deprivation (LOGD) on the levels of LDH, SOD and MDA. PC12 cells were exposed to LOGD for 1 h, 2 h, 4 h, 8 h and 12 h. A, effects of LOGD on the LDH activity; B, effects of LOGD on the SOD activity; C, effects of LOGD on the MDA content. All data were represented as the means \pm S.E. and the values were obtained from six separate cultures. * $P < 0.05$, ** $P < 0.01$ vs control (normal oxygen and DMEM culture medium).

lipid peroxidation induced by oxidative damage, was significantly increased after PC12 cells exposed to LOGD for 4 h ($P < 0.05$).

Involvement of $[Ca^{2+}]_i$ in LOGD-induced reduction of BPRP expression

$[Ca^{2+}]_i$ overload plays a significant role in the development of several brain pathologies including ischemia/hypoxia conditions. In order to determine whether $[Ca^{2+}]_i$ is involved in the LOGD-induced reduction in BPRP expression, we examined the effects of LOGD on $[Ca^{2+}]_i$, then investigated the $[Ca^{2+}]_i$ pathways in BPRP expression. As shown in Figure 3A and 3B, exposure PC12 cells to LOGD for 12 h significantly increased the concentration of $[Ca^{2+}]_i$ ($P < 0.05$), whereas pretreatment with BAPTA-AM (10^{-6} M, intracellular Ca^{2+} chelator) for 30 min significantly suppressed the increase of $[Ca^{2+}]_i$ induced by LOGD ($P < 0.05$). LOGD significantly decreased BPRP expression in PC12 cells ($P < 0.05$) (Figure 3C). However, when $[Ca^{2+}]_i$ concentration was inhibited by 10^{-6} M BAPTA-AM, BPRP expression was recovered ($P < 0.05$) (Figure 3C), which indicated a negative correlation between BPRP expression and the concentration of $[Ca^{2+}]_i$.

Involvement of MAPKs in LOGD-induced reduction of BPRP expression

To evaluate the biological significance of MAPK activation under LOGD, we tested the effects of LOGD on MAPK activation and the effects of MAPK inhibitors on BPRP expression. As shown in Figure 4A, LOGD significantly increased the p38 phosphorylation after 2 h ($P < 0.05$) while had no obvious effect on p38 expression. Further study showed that pretreatment PC12 cells with 5×10^{-7} M SB203580 (a specific inhibitor of p38-MAPK pathway) and 10^{-6} M BAPTA-AM (intracellular Ca^{2+} chelator) for 30 min prior to 12 h of LOGD exposure almost inhibited the increase of phospho-p38 induced by LOGD ($P < 0.05$) (Figure 4B). Accordingly, inhibition of phospho-p38 expression recovered BPRP expression ($P < 0.05$) (Figure 4C). Although LOGD also increased the phosphorylation of ERK, inhibitor of ERK-MAPK pathway (PD98059) had no significant effect on BPRP expression (data not shown).

Involvement of HIF-1 α in LOGD-induced reduction in BPRP expression

Man and other mammals adapt to hypoxia/ischemia by a number of physiologically appropriate responses, such

Fig. 3. Involvement of $[Ca^{2+}]_i$ in low oxygen and glucose deprivation (LOGD)-induced reduction of BPRP expression. PC12 cells were pretreated with BAPTA-AM (10^{-6} M, intracellular Ca^{2+} chelator) for 30 min prior to 12 h of LOGD exposure, then $[Ca^{2+}]_i$ was measured by confocal laser scanning microscopy (CLSM) and BPRP expression was assayed by flow cytometer (FCM). A, images of $[Ca^{2+}]_i$ assayed by LSCM ($\lambda_{ex} = 500$ nm, $\lambda_{em} = 530$ nm); B, DCF fluorescence intensity of $[Ca^{2+}]_i$ conducted by LSCM; C, BPRP expression measured by FCM. Data were represented as the means \pm S.E. Values were obtained from three separate cultures (FCM) (for $[Ca^{2+}]_i$ 30 PC12 cells were calculate in each culture). * $P < 0.05$ vs control (normal oxygen and DMEM culture medium), # $P < 0.05$ vs LOGD.

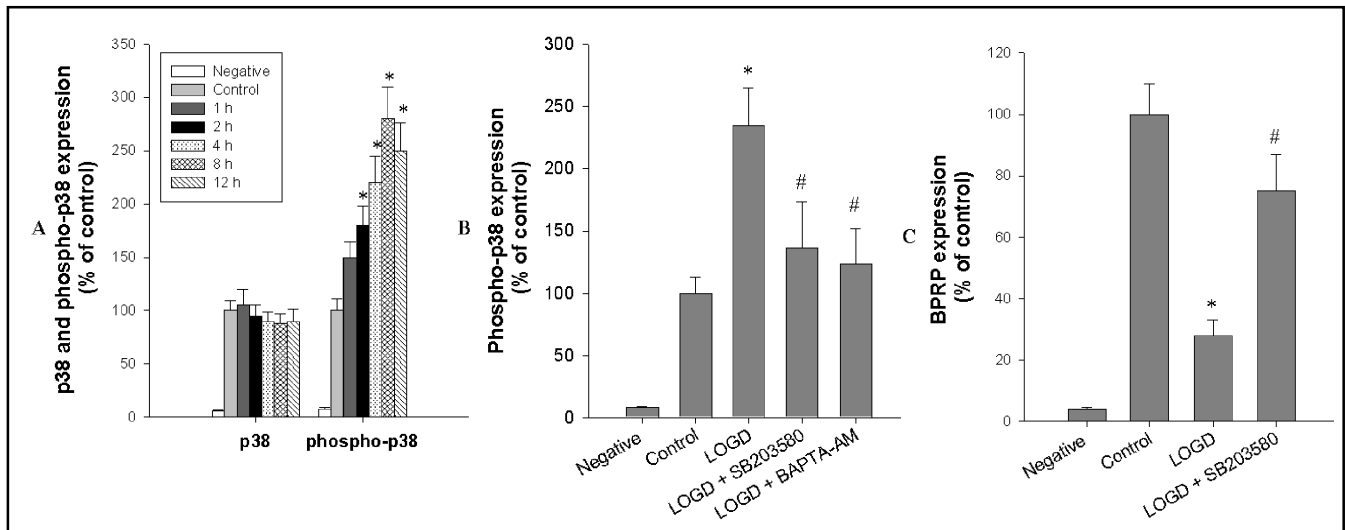
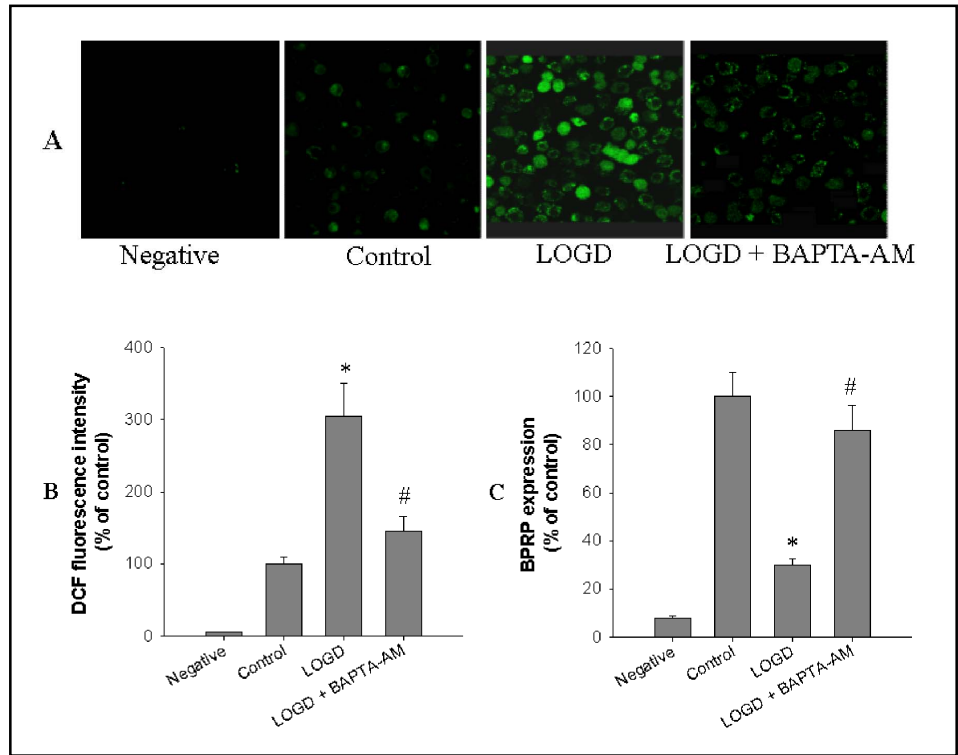
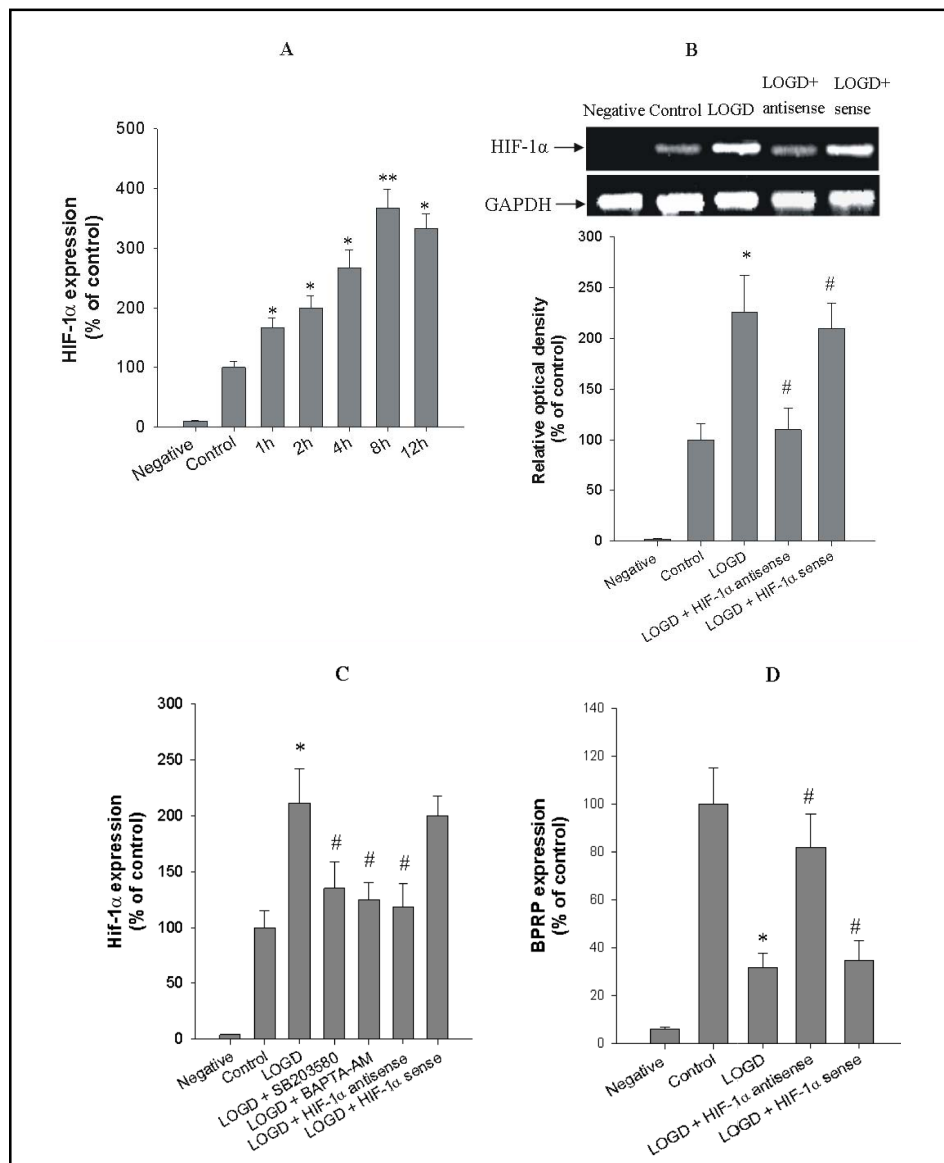


Fig. 4. Involvement of MAPKs in low oxygen and glucose deprivation (LOGD)-induced reduction in BPRP expression. PC12 cells were pretreated with SB203580 (5×10^{-7} M, a specific inhibitor of p38-MAPK pathway) and BAPTA-AM (10^{-6} M, intracellular Ca^{2+} chelator) for 30 min prior to 12 h of LOGD exposure, then p38, phospho-p38 and BPRP expression was measured by flow cytometer (FCM). A, effects of LOGD on p38 and phospho-p38 expression; B, effects of BAPTA-AM and SB203580 on phospho-p38 expression under LOGD; C, effects of SB203580 on BPRP expression. Data were represented as the means \pm S.E. Values were obtained from three separate cultures. * $P < 0.05$ vs control (normal oxygen and DMEM culture medium), # $P < 0.05$ vs LOGD.

as HIF-1 α . In order to ensure that LOGD applied in this study induced typical cell responses to hypoxic and hypoglycemic stress, the changes of HIF-1 α expression at different times (0-12 h) were observed by FCM.

As shown in Figure 5A, a significant increase in HIF-1 α expression was examined during 1 h-12 h LOGD exposure, and the maximal level appeared at 8 h (1 h, 2 h, 4 h, 12 h, $P < 0.05$; 8 h, $P < 0.01$).

Fig. 5. Involvement of HIF-1 α in low oxygen and glucose deprivation (LOGD)- induced reduction in BPRP expression. PC12 cells were pretreated with 10 μ M antisense or sense for 30 min prior to 12 h of LOGD exposure. A, Effects LOGD on HIF-1 α expression. PC12 cells were exposed to LOGD for 1 h, 2 h, 4 h, 8 h and 12 h, then HIF-1 α expression was analyzed by flow cytometer. B, effects of HIF-1 α antisense and sense on HIF-1 α mRNA expression analyzed by RT-PCR; the statistical results of relative optical density of HIF-1 α mRNA were shown; C, PC12 cells were pretreated with 5×10^{-7} M SB203580 (a specific inhibitor of p38-MAPK pathway) and 10^{-6} M BAPTA-AM (intracellular Ca^{2+} chelator) for 30 min prior to 12 h of LOGD exposure, then HIF-1 α expression was measured by flow cytometer; D, effects of HIF-1 α antisense and sense on BRRP expression. Data were represented as the means \pm S.E. Values were obtained from three separate cultures. * $P < 0.05$ vs control (normal oxygen and DMEM culture medium), # $P < 0.05$ vs LOGD.



To further determine the effects of HIF-1 α signal pathway on BPRP expression under LOGD, HIF-1 α antisense and sense were designed. As shown in Figure 5B, LOGD significantly increased the HIF-1 α mRNA expression, while pretreatment PC12 cells with 10 μ M antisense for 30 min prior to 12 h of LOGD exposure significantly decreased HIF-1 α mRNA expression ($P < 0.05$), accompanied by a reduction in HIF-1 α protein expression ($P < 0.05$) (Figure 5C) and recovery in the BPRP expression ($P < 0.05$) (Figure 5D), while HIF-1 α sense had no effect on HIF-1 α mRNA, HIF-1 α and BPRP expression. Furthermore, pretreatment PC12 cells with 5×10^{-7} M SB203580 (a specific inhibitor of p38-

MAPK pathway) and 10^{-6} M BAPTA-AM (intracellular Ca^{2+} chelator) for 30 min prior to 12 h of LOGD exposure also reduced HIF-1 α protein expression ($P < 0.05$) (Figure 5C).

Discussion

Our previous study reveals that disturbance between the oxidant and antioxidant balance plays an important part of the BPRP reduction mechanism under LOGD [6]. In the present study the increase in LDH and MDA and the decrease in SOD further confirm that ROS is

accumulated during LOGD. Mainly, the present experimental evidence firstly demonstrates that Ca^{2+} -dependent activation of p38 MAPK and HIF-1 α are involved in the LOGD-induced reduction in BPRP expression in PC12 cells. Reversely, inhibition of calcium, p38 phosphorylation and HIF-1 α expression recovers BPRP expression.

HIF-1 α is used as a reference for classical hypoxia-regulated genes that is up-regulated after global brain ischemia [34]. We show that LOGD increases HIF-1 α expression, suggesting low oxygen condition is generated with LOGD model and an adaptive response is triggered. Cell adaptive responses to the reduction of oxygen supply minimize the deleterious effects of hypoxia via HIF-1 to regulate gene expression. HIF-1 mediates the adaptation of cells to hypoxia and hypoglycemia by regulating the expression of genes involved in erythropoiesis, angiogenesis, glucose transport, glycolysis and cell proliferation/survival [35, 36]. Consistent with our *in vivo* results [1], LOGD decreases BPRP expression, inhibition of HIF-1 α recovers BPRP expression, suggesting the reduction of BPRP is an adaptive response to LOGD and HIF-1 α regulates BPRP expression during LOGD. However, it is unclear so far whether BPRP is a neuron-protective or a pro-death molecule. Nevertheless, all the evidence indicates that the LOGD model is good and the BPRP antibody works as it supposes.

Different from the effects of hypoxia on HIF-1 α , Kwon and Lee shows that glucose deprivation inhibits the accumulation of HIF-1 α in human pancreatic cancer MiaPaCa-2 and human prostatic cancer DU-145 cells under hypoxic conditions by disrupting translational processes rather than transcriptional or proteasomal degradation processes [37], suggesting glucose deprivation partially counteracts the effect of oxygen deprivation on HIF-1 α expression. Nevertheless, our previous results show that low and free glucose decreases BPRP expression in primary cultured hippocampal neurons [38], indicating that the mechanisms underlying the effects of hypoxia and hypoglycemia on BPRP expression are different. Textor et al show that hypoglycemia triggers the expression of vascular endothelial growth factor (VEGF) independent of HIF-1 in mouse embryonic fibroblasts, while HIF-1 mediates the expression of VEGF under hypoxia [39]. Then a question arises, why inhibition of HIF-1 α with antisense can recover BPRP expression under LOGD in which both hypoxia and hypoglycemia coexist? In our opinion, different from cells such as human pancreatic cancer MiaPaCa-2 and human prostatic cancer DU-145 cells and proteins like VEGF, in PC12

cells there is a common mechanism for hypoxia and hypoglycemia to regulate the expression of BPRP. Both of hypoxia and hypoglycemia are oxidative stress and lead to mitochondrial dysfunction [40] and then result in HIF-1 signal pathway activation [41]. For example, ROS is accumulated in our study during LOGD [6] and under hypoglycemia [42] in PC12 cells.

HIF is not only induced by oxygen deprivation and other stimuli can stabilize HIF and its translocation to the nucleus. For example, there is an interaction between ROS and HIF-1 α , and ROS and prooxidants like arsenite stabilize HIF-1 α and induce HIF-1 DNA binding [43]. Another example could be insulin, which activates the PI3K-AKT pathway and promotes nuclear accumulation of HIF-1 α in *Drosophila* [44]. These findings suggest the dynamic functions of HIF-1 in regulating protein expression and may provide insight into the role in regulating BPRP expression.

The hypoxic signal needs to be transmitted to the nucleus where changes in gene expression occur. Several critical signaling pathways including the cAMP-protein kinase A, Ca^{2+} -calmodulin, p42/44 MAPK, p38 MAPK and the phosphatidylinositol 3-kinase-AKT that regulate a complex gene expression profile in PC12 cells during hypoxia are identified [45]. Among them the MAPK pathways play a critical role in response to cellular stress and promote changes in gene expression [46]. The MAPKs are known to mediate their effects via the activation of HIF-1 α [47]. The p38 is activated primarily by noxious environmental stimuli. Some results provide genetic evidence that p38 MAPK signaling is essential for HIF-1 activation [48]. As shown in this study, LOGD upregulates the expression of phosphor-p38 MAPK and HIF-1 α , inhibition of p38 with SB203580 decreases HIF-1 α expression but recovers BPRP expression. In general, hypoxia regulates the activity of the MAPK signal pathways and consequently regulates HIF-1 α transcriptional activity.

PC12 cell line is an excitable cell line, exposure PC12 cells to hypoxia results in membrane depolarization and Ca^{2+} influx [45, 49]. Changes in gene expression mediated by HIF-1 α are potentiated by Ca^{2+} via calmodulin and the MAPK pathway [50]. Blocking of calcium with BAPTA-AM results in a reduction in p38 activation and HIF-1 α expression. The critical role of Ca^{2+} is specific to these excitable cell lines. Furthermore, there is a correlation between $[\text{Ca}^{2+}]_i$ and ROS levels. ROS levels during oxidative stress are at least partly dependent upon $[\text{Ca}^{2+}]_i$ concentration, $[\text{Ca}^{2+}]_i$ levels may be influenced by ROS as well [51]. Lowering of $[\text{Ca}^{2+}]_i$ during hypoxia

may reduce ROS levels and thereby possibly ameliorate the severity of cells damage [52]. Therefore, antioxidant [6] and inhibitor of $[Ca^{2+}]_i$ mitigates the aversive effects of LOGD and recovers BPRP expression, suggesting a negative relationship between $[Ca^{2+}]_i$ and BPRP expression in PC12 cells.

In conclusion, the present data firstly demonstrate that activation of multiple signaling mediators including Ca^{2+} , p38 MAPKs and HIF-1 α are involved in the inhibitive effects of LOGD on BPRP expression in PC12 cells. However, the possible roles of BPRP in the pathophysiology of ischemia need further study. Attempt

to over-express or down-regulate BPRP to assess the relevance of this protein in primary hippocampal neurons is underway.

Acknowledgements

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