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Proteomic analysis of the mouse brain after repetitive exposure to hypoxia

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ABSTRACT

Hypoxic preconditioning (HPC) is known to have a protective effect against hypoxic damage; however, the precise mechanisms involved remain unknown. In this study, an acute and repetitive hypoxia mouse model, two-dimensional fluorescence difference gel electrophoresis (2D-DIGE) coupled with matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF/TOF-MS), and Western blot experiments were used to identify the differential expression of key proteins in the mouse brain during HPC. Approximately 2100 2D-DIGE spots were observed following gel imaging and spot detection. Significant differences ($p < 0.05$) in the expression of 66 proteins were observed between the 3× HPC treatment group and the control group, 45 proteins were observed between the 6× HPC treatment group and the control group, and 70 proteins were observed between the 3× HPC treatment group and the 6× HPC group. Consistent results among Western blot, 2D-DIGE and MS methods were observed for the proteins, ATP synthase subunit alpha, malate dehydrogenase, guanine nucleotide-binding protein subunit beta-1 and proteasome subunit alpha type-2. The proteins associated with ATP synthesis and the citric acid cycle were down-regulated, while those linked to glycolysis and oxygen-binding were up-regulated. This proteomic analysis of the mouse brain after HPC furthers understanding of the molecular pathways involved in the protective effect of HPC and these findings provide new insight into the mechanisms of hypoxia and HPC.

1. Introduction

Oxygen, a terminal electron acceptor in the respiration chain, is indispensable for all aerobic organisms. In addition to the many environmental challenges of life, oxygen deprivation (i.e., hypoxia) is of particular concern. Hypoxia can cause dysfunction, damage and death to cells, tissues and organisms, and is associated with many pathophysiological processes and diseases such as stroke, asthma, emphysema, angina pectoris, myocardial infarction and tumors [1,2]. Hypoxic preconditioning (HPC) has evolved as a very effective intrinsic mechanism of cell protection that motivated by repetitive exposure to hypoxia [3]. Adaptation to decreased oxygen availability (hypoxia) is crucial for proper cell function and survival. HPC is described here as the intrinsic adaptation against hypoxia that results in an increased acclimatization to a more severe hypoxic exposure. Previous studies have shown that the survival time of HPC mice is longer than that of normoxic mice, indicating that HPC has a protective effect against hypoxia [4]. Although the protective effect of HPC is known, the mechanisms involved remain unclear, particularly at the molecular and proteomic levels.

At the molecular level, metabolomic and genomic studies have been performed to profile patterns of small molecule metabolites and gene expression in mouse brain tissue exposed to hypoxia [1]. In addition, proteomics provides the potential to identify changes in protein levels following hypoxic exposure and may provide important insights into the mechanisms involved in HPC. Some results have showed that the decreased expression of myosin

Abbreviations: 2D-DIGE, two-dimensional fluorescence difference gel electrophoresis; GPD1L, glycerol-3-phosphate dehydrogenase 1-like protein; GNBP-1, guanine nucleotide-binding protein subunit beta-1; HPC, hypoxic preconditioning; LGL, lactoylglutathionelyase; MALDI-TOF/TOF-MS, matrix-assisted laser desorption ionization-time of flight mass spectrometry; PAGE, polyacrylamide gel electrophoresis; PS-2, proteasome subunit alpha type-2; SD, standard deviation; SDS, sodium dodecyl sulfate; SBP1, syntaxin-binding protein 1; VDAC1, voltage-dependent anion-selective channel protein-1.

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light polypeptide 3, and the increased expression of nucleoside diphosphate kinase and calreticulin induced by HPC may be involved in protection via decreasing contractibility of cells, activating G protein, and decreasing calcium concentration [5]. The regulated proteins during chronic hypoxia were mainly involved in cell metabolism, apoptosis, Ca^{2+} signaling, pentose phosphate pathway, protein mediated signaling cascades and protein degradation [6,7]. The resolution and reproducibility of proteomic experiments can be enhanced by two-dimensional fluorescence difference gel electrophoresis (2D-DIGE) [8,9]. In 2D-DIGE, the protein samples are first labeled with fluorescent dyes and are then separated by 2D-polyacrylamide gel electrophoresis (PAGE). In addition, improvements in mass spectrometry and Western blot techniques continue to improve detection limits and increase the accuracy of protein identification [10]. In our study, changes in protein levels between HPC and normal mouse brains were identified using 2D-DIGE, matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF/TOF-MS) and Western blot methods. The purpose here was to screen and identify the key proteins that are differentially expressed between HPC and normal mouse brains to provide mechanistic insight into the protective role of HPC. The proteomic changes induced by HPC may also indicate how organisms acclimatize to hypoxia.

2. Materials and methods

2.1. Chemicals and reagents

CHAPS, IPG buffer (pH 3–10), IPG dry strip (pH 3–10, 24 cm), iodoacetamide, thiourea and the fluorescent dyes Cy2, Cy3 and Cy5 were purchased from the GE Healthcare. All other chemicals and reagents were of analytical grade unless indicated otherwise.

2.2. Animals and HPC experiments

Five weeks old male ICR mice of weight from 18 g to 22 g were obtained from the Laboratory Animals Center of Capital Medical University (LAC, CMU, Beijing, China). All studies on animals were performed in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals in China. Experiments were carried out in accordance with the current guidelines for the care of laboratory animals and the ethical guidelines for investigations of experiments in conscious animals [11]. The animal experimental protocols employed were approved by the Committee for the Use of Animals, Capital Medical University. Each group of mice were just housed in the isolated cages for experiment after 24 h fasting. Mice were randomly allocated to three groups; each group contained three mice: the control group without hypoxia, the HPC group exposed to hypoxia three times (3× HPC), and the HPC group exposed to hypoxia six times (6× HPC).

Repeated hypoxia exposed models were performed at room temperature (20 ± 1 °C). The repetitive hypoxic mouse model was established according to our previously described method [4]. Briefly, a mouse was placed in a 125 ml airtight jar containing air and $Ca(OH)_2$ to absorb CO_2. The jar was immediately sealed with a rubber stopper and smeared with Vaseline. The mouse was taken out of the jar as soon as the gasping breath appeared (an indicator to end each hypoxic exposure). This is the first time of hypoxic preconditioning treatment. Then the mouse was moved to another new jar with same volume fresh air and the jar was hermetically sealed with rubber plug immediately, and to duplicate a progressive hypoxia environment. The tolerance time of the mouse was recorded and the behavior of the mouse was observed. The tolerance time was recorded when the asthmoid respiration of the mouse appeared, after which the mouse was immediately removed to another jar with an equal volume of air, and the tolerance time was recorded again. The oxygen contents of the experimental mice were determined by the pHOxCO-Oximeter methods.

The above hypoxic preconditioning treatment was repeated three (3× HPC) or six times (6× HPC). After the third or sixth HPC treatment, the mice were quickly sacrificed by cutting head on ice bath to minimize the degradation of brain samples. After being separated on ice bath, the whole brain was putted in pre-cooled (liquid nitrogen) sterile RNase/Dnase-free tube, and stored in liquid nitrogen until use.

2.3. Protein extraction

Brain tissue from each mouse was pulverized on ice and homogenized in extraction buffer (7 M urea, 2 M thiourea, 4% (w/v) CHAPS). To prevent protein degradation, EDTA-free protease inhibitor cocktail tablets (Roche, Switzerland) were added to samples. The homogenate was centrifuged at 15,000 rpm for an hour at 4 °C. Three volumes of chilled 10% (w/v) TCA/acetone were added to each sample and incubated overnight at −20 °C. The samples were centrifuged at 15,000 rpm for 30 min at 4 °C and the pellet was washed with chilled 10% (w/v) TCA/acetone followed by pure chilled acetone. Pellets were lyophilized for subsequent experiments.

2.4. Protein labeling with CyDye

Protein quantification was estimated using the GE Healthcare 2D Quant kit, and proteins were labeled with CyDye DIGE Fluor dyes from GE Healthcare. Minimal labeling was performed according to the manufacturer's recommendations. An internal standard was generated by combining equal amounts of each sample labeled with Cy2 dye. Samples subjected to three and six times hypoxia were labeled with Cy3 or Cy5. Each sample (50 μg) was minimally labeled by incubation with 400 pmol of amine-reactive cyanine dyes at room temperature for 30 min in the dark. The reaction was quenched by addition of 10 mM lysine and incubation on ice for 10 min. The labeled samples were mixed together according to the experimental design before running on the same gel.

2.5. Two-dimensional electrophoresis and image scanning

For two-dimensional electrophoresis, samples were adjusted to rehydration buffer [7 M urea, 2 M thiourea, 30 mM Tris–HCl, 4% (w/v) CHAPS, 50 mM DTT, 0.5% (w/v) IPG buffer and 0.005% (w/v) bromophenol blue] before first-dimension isoelectric focusing. IPG strips (pH 3–10, 24 cm) were used for separation in the first dimension. The first dimension was performed at 20 °C using the IPG phor isoelectric focusing apparatus (GE Healthcare/Amersham Biosciences). Seven steps were conducted, including: 30 V for 6 h, 60 V for 6 h, 200 V for 0.5 h, 500 V for 0.5 h, 1000 V for 1 h, 8000 V for 1 h and the last step was maximum voltage, 8000 V, for a total of 30 kV. After the first dimension, the strips were equilibrated in two steps: 15 min in equilibration buffer supplemented with 1% (w/v) DTT and 15 min in equilibration buffer supplemented with 1.25% (v/v) iodoacetamide. The acrylamide gel for the second dimension was 12.5% sodium dodecyl sulfate (SDS)-PAGE was performed using an Ettan Dalt-six vertical unit. The electrophoresis conditions were 1 W/gel for 30 min, followed by 2.5 W/gel for 12 h at 20 °C in the dark. Five gels were processed simultaneously. After electrophoresis, the gels were immediately scanned using the Typhoon 9400 Variable Mode Imager (GE Healthcare). Acquired images were analyzed using DeCyder2D 7.0 software (GE Healthcare).

The DIA module was used for automatic spot detection, and all of the spots were visually examined to determine whether they