Alterations of Hypoxia-Inducible Factor-1 Alpha in the Hippocampus of Mice Acutely and Repeatedly Exposed to Hypoxia

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Key Words

Hypoxic preconditioning · Hippocampus, hypoxia-inducible factor-1 alpha alterations · Hypoxia-inducible factor-1 alpha, hippocampus

Abstract

This work aims at investigating the effects of hypoxic preconditioning on hypoxia-inducible factor-1 alpha (HIF-1 α) expression in the hippocampus of mice during acute and repeated hypoxic exposures. The mice were randomly divided into three groups and exposed, respectively, to hypoxia for 4 runs (group H4), 1 run (group H1), and 0 run (group H0). Reverse transcription-polymerase chain reaction (RT-PCR), Western blot, electrophoretic mobility shift assay (EMSA), and chromatin immunoprecipitation were used to examine the HIF-1 α responses in the mouse hippocampus following exposure to hypoxia. The tolerance of mice to hypoxia increased significantly following acute and repetitive exposure to autoprogressive hypoxia. Total mRNA, total protein, and nuclear protein were extracted from the hippocampus for RT-PCR, Western blot, and EMSA, respectively. The HIF-1 α mRNA levels were found to be in-

creased in group H1 and decreased in group H4. The HIF-1 α protein levels and HIF-1 DNA-binding activities were increased in group H1 and markedly increased in group H4. One of the HIF-1 target genes, vascular endothelial growth factor, increased in group H4. HIF-1 activation is thought to be involved in the protection of the brain of hypoxic preconditioned mice.

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Introduction

Over the last decade, a lot of reports have demonstrated that in rodents a phenomenon called hypoxic preconditioning can be induced in the brain. Hypoxic preconditioning is defined as a period of sublethal hypoxia insult which can induce a protection from an otherwise lethal insult. Our previous work [1] showed that the tolerance to hypoxia in adult mice was significantly increased by acute and repetitive exposure to autoprogressive hypoxia. The quantities or activities of some chemical materials and gas/energy metabolism in the brain were found to be changed during the preconditioning in our model [2, 3].

The mechanism of hypoxic preconditioning is still unclear, but is believed to trigger endogenous cellular adaptation. The complex cellular signaling cascades and downstream genes and protein regulatory processes were suggested to be factors involved in the preconditioning [4].

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As a key molecule in hypoxia, hypoxia-inducible factor-1 (HIF-1) regulates the activities of genes that have in common one or more binding sites for HIF-1. HIF-1 is a member of the basic helix-loop-helix family and consists of an oxygen-regulated α subunit and a constitutively expressed β subunit [5]. Under normoxic conditions, HIF-1 α is hydroxylated by the HIF prolyl hydroxylase family [6, 7], and the hydroxylation reaction requires molecular oxygen and Fe(II) [8, 9]. The von Hippel-Lindau tumor suppressor protein, a ubiquitin protein ligase, recognizes HIF-1 α that has been modified at the two proline residues and binds to a region of HIF-1 α named oxygen-dependent degradation domain [10]. Thus HIF-1 α is damaged through the ubiquitin path. Under hypoxic conditions, HIF-1 α escapes the damage and dimerizes with HIF-1 β and binds to the hypoxic response element of many target genes, leading to the transcriptional activation of several dozen genes such as erythropoietin (EPO), vascular endothelial growth factor (VEGF), and so on [11]. EPO and VEGF have been proved to have the role of neuroprotective factors [12, 13].

The purpose of this study was to investigate the change of HIF-1 expression, when mice were exposed to hypoxia acutely and repetitively. In addition, the induction of VEGF, one of the HIF-1 target genes, was also measured during the preconditioning.

Materials and Methods

Animal Model

Male adult BALB/C mice (weighing 16.0–22.0 g) were randomly separated into three groups: blank control group with no exposure to hypoxia (H0), hypoxia control group exposed to hypoxia once (H1), and hypoxia preconditioning group exposed to hypoxia for four runs (H4). The procedure of the hypoxic experiment was performed as previously described [2]. Briefly, the animal was placed into a 125-ml jar with fresh air, and the jar was sealed with a rubber plug. The animal was removed from the jar as soon as the first gasping breath appeared and was switched to another fresh-air-containing jar of similar volume. This procedure was performed once (group H1) or repeated four times (group H4). The appearance of the first gasping was regarded as the tolerance limit in each trial. The time period between the beginning of airtightness and the appearance of the first gasping was termed ‘tolerance time’ for each run.

RNA Isolation and Semiquantitative Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Analysis

Total RNA was prepared from mouse hippocampus using the Rneasy minikit (Qiagen, Valencia, Calif., USA). Total RNA (1 μ g) was reverse transcribed with Superscript II reverse transcriptase (Invitrogen, Carlsbad, Calif., USA). One microliter of the resulting cDNAs was subjected to amplification in a total volume of 20 μ l

containing 10 $\times$ buffer, 1.5 mmol/l $MgCl_2$, 0.2 mmol/l of each dNTP, 1 U Taq polymerase (Takara, Otsu, Japan), and a pair of specific primers (0.2 μ mol/l each). Primers for HIF-1 α and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were designed using Primer Premier 5.0 software, and both sequences were obtained from GenBank: GAPDH forward primer: 5'-CCCTTCATTG-ACCTCAAC-3', reverse primer: 5'-TTCACACCCATCACAAC-3'; HIF-1 α forward primer: 5'-TATAAACCTGGCAATGTCTCC-3', reverse primer: 5'-GATGCCTTAGCAGTGGTCGT-3'. The PCR temperature for HIF-1 α was as follows: cycles at 92°C for 30 s, at 53°C for 30 s, and at 72°C for 60 s, total 29 cycles, followed by a final extension period at 72°C for 5 min. The PCR temperature for GAPDH was: cycles at 92°C for 40 s, at 50°C for 40 s, and at 72°C for 45 s, total 20 cycles, followed by a final extension period at 72°C for 5 min. PCR products (9 μ l) were separated by 1.0% agarose gel electrophoresis and stained with ethidium bromide. The RT-PCR products were 672 bp for HIF-1 α and 301 bp for GAPDH.

Western Blot Analysis

Total proteins from mouse hippocampus were extracted according to a previously described protocol [14]. The protein concentrations were determined using the bicinchoninic acid method. Total cell lysate of mouse hippocampus (80 g) was separated by SDS (8%)/PAGE (12%) at 30 mA for 2.5 h and then blotted onto a nitrocellulose membrane. The membrane was then incubated for 1 h in blocking buffer (Tris-buffered saline containing 10% skimmed-milk powder) at room temperature. Next, the membrane was incubated for 16 h at 4°C with the primary antibodies. Then, the membrane was incubated with secondary antibodies for 1 h at room temperature. After each incubation, the membrane was washed three times thoroughly with Tris-buffered saline containing 0.05% Tween 20. Protein signals were detected by an electrochemiluminescence detection system (Pierce Biotechnology, Rockford, Ill., USA), in which the membrane was exposed to the detection solution for 5 min.

Preparation of Nuclear Protein Extracts

The whole hippocampus was broken into single cells with cold phosphate-buffered saline after hypoxia exposure or not. The cell pellet was centrifuged, and the resulting pellet was lysed in lysis buffer A (10 mmol/l HEPES, 10 mmol/l KCl, 1.5 mmol/l $MgCl_2$, 1 mmol/l EDTA, 1 mmol/l dithiothreitol, 1 mmol/l phenylmethylsulfonyl fluoride (PMSF), and 1 μ g/ml protease inhibitors in distilled water). Ten minutes later, the cells were homogenized on ice and investigated by microscope using trypan blue. The samples were centrifuged for 15 min at 3,000 rpm at 4°C. The supernatants (cytosolic extracts) were discarded, and the resulting pellet (nucleus) was resuspended in buffer B (25% glycerol, 20 mmol/l HEPES, 20 mmol/l KCl, 1.5 mmol/l $MgCl_2$, 1 mmol/l EDTA, 1 mmol/l dithiothreitol, 1 mmol/l PMSF, and 1 μ g/ml protease inhibitors in distilled water), then high-salt buffer C (20 mmol/l HEPES, 1 mmol/l EDTA, 1.5 mmol/l $MgCl_2$, 25% glycerol, 1.2 mol/l KCl, 1 mmol/l dithiothreitol, 1 mmol/l PMSF, and 1 μ g/ml protease inhibitors in distilled water) was added dropwise while stirring gently. The suspension was mixed vigorously with tips and incubated on ice for 30 min. The samples were centrifuged for 10 min at 14,000 rpm at 4°C, and the supernatants (nuclear extracts) were kept at -80°C.

Electrophoretic Mobility Shift Assay (EMSA)

EMSA was performed according to the method of Semenza and Wang [15]. The EPO gene-derived sense oligonucleotide sequence GCCCTACGTGCTGCCTCGCATGGC contains a HIF-1-binding site. Sense and antisense oligonucleotides were annealed in annealing buffer and then were labeled with [γ - 32 P]ATP by T4 polynucleotide kinase (Takara). Unincorporated nucleotides were removed by using the QIAquick nucleotide removal kit (Qiagen). Nuclear protein was added to binding buffer (HEPES 60 mM, EDTA 5 mM, KCl 300 mM, 10% glycerol, dithiothreitol 5 mM, Tris-HCl 20 mM) containing 1.5 μ g poly(dI-dC) (Sigma, St. Louis, Mo., USA), with or without antibody. Then the mixture was incubated on ice for 30 min before addition of 1×10^4 cpm of oligonucleotide probe in a total volume of a 20- μ l solution for 1 additional hour. Samples were run on 4% nondenaturing polyacrylamide gels at 200–250 V for 2–3 h in $0.5 \times$ TBE buffer (Tris-borate-EDTA) at 4°C. Afterwards, the gels were dried and autoradiographed.

Chromatin Immunoprecipitation (ChIP) Assay and Real-Time PCR

The hippocampus of mice was prepared into single-cell supernatants on ice using phosphate-buffered saline. ChIPs were performed following the Upstate Biotechnology (Waltham, Mass., USA) assay kit protocol. Briefly, formaldehyde was added to the cold phosphate-buffered saline at a final concentration of 3%, then the solution was incubated at 37°C for 10 min. Cross-linked DNA was sheared to 200–1,000 bp by sonication. A portion of cell supernatant was kept as the input material, and the remaining solution was immunoprecipitated using an anti-HIF-1 α antibody (Santa Cruz Biotechnology, Santa Cruz, Calif., USA) or an unrelated antibody (IgG) as negative control. The DNA was reversed from the protein-DNA complex and used as template for PCR. The fragments of the housekeeping gene β -actin and EPO 3'-terminal sequence including the HIF-1-binding site were amplified. The primers for PCR of mouse β -actin fragment and EPO 3'-terminal sequence were as follows: β -actin forward primer: 5'-GAACGGTGAAGGCGACAG-3', β -actin reverse primer: 5'-TTGGGAGGGTGAGGGACT-3'; EPO 3' enhancer forward primer: 5'-GTTCGCAAGGCATCAGA-3', EPO 3' enhancer reverse primer: 5'-CTTGTCCTAGCGGGTT-3'. The products were 175 and 187bp, respectively. The PCR condition for both primer sets was as follows: hot start at 95°C for 2 min; 33 cycles, each consisting of 92°C for 25 s, 52°C for 40 s, and 72°C for 25 s and a final extension at 72°C for 5 min. The PCR products were separated on 1% agarose gels and stained with ethidium bromide.

DNA obtained from ChIP was used as template, and the same EPO 3' enhancer HRE primer used in PCR was applied as primer in real-time PCR. The TaqMan probe was: 5'-CTTGAGGCCACAATACCTGC-3'. Thermal cycling conditions were 94°C before 40 PCR cycles (92°C, 30 s; 52°C, 80 s).

Quantification and Statistics

The optical densities (OD) of bands of PCR products, Western blot, and EMSA were obtained through the Gel-Doc system and analyzed by Bandscan software. The data of RT-PCR are presented as relative abundance, and Western blots and EMSA are presented as OD. All data are expressed as mean values $\pm$ SD. Statistical analysis was performed by means of ANOVA and Tukey test using SPSS 10.0 software. $p < 0.05$ was considered to be statistically significant.

Fig. 1. Tolerance time in the different exposure runs ($n = 30$; ** $p < 0.01$ as compared with the preceding runs).

Results

Effects of Acute and Repeated Hypoxia on the Tolerance Time of Mice

The increment of tolerance to hypoxia in each run was approximately in an arithmetic progression. The tolerance time of each run lasted significantly longer and longer, as the exposure run increased. The average tolerance times of runs 1, 2, 3, and 4 were 17.2 ± 2.9 , 37.4 ± 5.7 , 58.5 ± 7.3 , and 78.7 ± 7.9 min, respectively (fig. 1), which was in accordance with our previous findings [1–3].

Effects of Acute and Repeated Hypoxia on HIF-1 α mRNA

The HIF-1 α mRNA was analyzed by RT-PCR immediately at the end of hypoxia exposures. The relative abundance value of HIF-1 α in each group of HIF-1 α mRNA was calculated by the OD ratio of HIF-1 α to GAPDH. The relative abundance values of group H1 (5.67 ± 1.79) were significantly higher than those of group H0 (2.41 ± 0.56 ; $p < 0.05$). However, after repetitive hypoxia exposure, the HIF-1 α values recovered at mRNA level in group H4 (3.76 ± 0.97). A significant difference of the relative quantities of HIF-1 α between group H1 and group H4 was detected ($p < 0.05$), and no difference between H0 and H4 groups was shown. The results of RT-PCR products of HIF-1 α and GAPDH are shown

Fig. 2. Electrophoresis of HIF-1 α and GAPDH RT-PCR products in agarose gel.

Fig. 4. Western blot analysis of HIF-1 α in the hippocampus of the mice in groups H0, H1, and H4.

Fig. 3. Ratio of HIF-1 α mRNA to GAPDH mRNA in H0, H1, and H4 groups (n = 6; * p < 0.05 vs. group H1).

Fig. 5. Expression of HIF-1 α protein in mouse hippocampi (n = 6; ** p < 0.01 vs. groups H0 and H1).

in figure 2, and the relative abundance of HIF-1 α to GAPDH is shown in figure 3.

Effects of Acute and Repeated Hypoxia on HIF-1 α Protein

HIF-1 α protein was detected in three groups by Western blot (fig. 4), using a rabbit polyclonal antibody (Santa Cruz Biotechnology). A faint band of about 120 kDa was seen in group H0 (OD 1,577 $\pm$ 366), while a more distinct band of HIF-1 α protein was seen at the corresponding location in groups H1 and H4. The HIF-1 α protein level was increased in both group H1 (OD 3,817 $\pm$ 1,723) and group H4 (OD 14,478 $\pm$ 7,199). The HIF-1 α protein levels in group H4 were significantly higher than those seen

in groups H0 and H1 (p < 0.01 vs. H0 and H1, n = 6; fig. 5). The increase of the protein level was parallel to the increase of the tolerance time.

Effects of Acute and Repeated Hypoxia on the DNA-Binding Activities of HIF-1

The in vitro hippocampal DNA-binding activities were analyzed by EMSA (fig. 6). The DNA-binding activities of HIF-1 were constitutively expressed under normal conditions. A 20- to 28-fold increase was seen in group H1 (vs. group H0). The activity in group H4 was even higher than that in group H1 (1.2- to 1.8-fold). The in vivo hippocampal DNA-binding activities of HIF-1 were analyzed by ChIP (fig. 7). The DNA obtained by

Fig. 6. DNA-binding activities of HIF-1 on the EPO hypoxia response element (CREB-1) in the different groups. Lane 1: protein free. EMSA using crude nuclear extract was prepared from group H0 (lanes 2, 5, 8), group H1 (lanes 3, 6, 9), and group H4 (lanes 4, 7, 10). For supershift assays (lanes 5–7), binding reactions contained 2 μ l antiserum raised against HIF-1 α (ATF-1). For competition assays (lanes 8–10), binding reactions included 100-fold excess of unlabeled oligonucleotides ($n = 3$).

ChIP was used as a template for RT-PCR and real-time PCR. The results of *in vivo* binding were similar to those of *in vitro* binding. The DNA-binding activities of HIF-1 could be detected in the three groups by RT-PCR. The Ct values were 31.51, 30.65, and 28.65 in groups H0, H1, and H4, respectively ($n = 6$). Both *in vitro* and *in vivo* binding tests showed that the HIF-1 DNA-binding activities were increased in group H1 and markedly increased in group H4.

Effects of Acute and Repeated Hypoxia on VEGF Protein

VEGF protein was detected in the three groups by Western blot using a mouse monoclonal antibody (Santa Cruz Biotechnology). The β -actin protein was used as internal control. The relative abundance value of VEGF protein in each group was calculated by the OD ratio of VEGF protein to β -actin protein. As shown in figure 8, no significant difference was found between group H0 (0.208 ± 0.05) and group H1 (0.200 ± 0.08), while there were marked changes in group H4 (0.387 ± 0.08). The VEGF protein levels were increased almost twofold in group H4 ($p < 0.01$ vs. groups H0 and H1, $n = 6$; fig. 9).

Fig. 7. Electrophoresis of ChIP PCR products in agarose gel. Samples from group H0 (a), group H1 (b), and group H4 (c). M = Marker; HBS = hypoxia binding site.

Fig. 8. Western blot analysis of VEGF and β -actin in the hippocampus of mice in groups H0, H1, and H4.

Fig. 9. Ratio of VEGF protein to β -actin protein in H0, H1, and H4 groups ($n = 6$; ** $p < 0.01$ vs. groups H0 and H1).

Discussion

Acute and chronic preconditioning procedures are accepted as two mechanistically distinct kinds of protection afforded by hypoxia, and the mechanism of acute and chronic preconditioning is thought to be different [16]. However, acute and chronic preconditioning may have similar biochemical components [17]. Most of the researchers focus on the role of HIF-1 in chronic preconditioning in immature animals [14, 18]. It is unclear whether HIF-1 is induced in adult animals after repeated exposure to acute hypoxia within a short period of time. Our study showed that this might be true.

Reduction of oxygen induced HIF-1 α protein accumulation, and reoxygenation induced a rapid decay of HIF-1 α protein. In our model, the mice were exposed repeatedly to hypoxia and normoxia. The HIF-1 α protein level should change in this process. HIF-1 α and HIF-1 DNA-binding activities in the hippocampus were found to be markedly increased after repeated exposure to hypoxia. The changes of the DNA-binding activities of HIF-1 were closely parallel to the HIF-1 α protein level. This result supports our hypothesis that HIF-1 may contribute to the hypoxia tolerance of mice. Our previous findings [3] demonstrated that the energy metabolism might depend on anaerobic glycolysis after preconditioning. HIF-1 regulates the transcriptional activation of target genes such as EPO, VEGF, and glucose transporter (GLUT) and glycolytic enzymes [18–20]. The expression of these genes in response to hypoxia may be a protectively adaptive change. Al-

though only one target gene of HIF-1, VEGF, was studied in this context, some other target genes may also be induced during this process for the accumulation of HIF-1 α and the increase of the DNA-binding activities of HIF-1.

HIF-1 α and HIF-1 DNA-binding activities can be detected in the hippocampus of mice under normoxic conditions. HIF-1 α protein detection in the brain has been reported under normoxic conditions [21]. To our knowledge, this work is the first reporting that the transcription factor HIF-1 is constitutively activated in the hippocampus of mice under normoxic condition using an *in vivo* binding test (ChIP). It has been suggested and supported [21] that the physiological oxygen tension in normal tissue presents a low-level HIF-1 protein to induce target genes to provide the cellular energy requirements. The basal activation of HIF-1 may be necessary to regulate target genes to provide cellular energy and signal in normal brain tissue. This was supported by a mouse model lacking the HIF-1 α gene in neural cells, where the animals exhibited hydrocephalus accompanied by a reduction in neural cells and an impairment of spatial memory [22].

In the present study, the level of VEGF was found to be increased after acute and repeated exposure to hypoxia, and no change was found after a single exposure to hypoxia. *In vitro* and *in vivo* studies reported that VEGF plays a neuroprotective role [23, 24]. The anti-apoptotic signal can be enhanced, and the apoptotic gene may be inhibited through VEGF associated with the PI(3)K/Akt pathway [25, 26]. Other factors may be involved in this pathway to promote the survival of neurons under hypoxic conditions. Hypoxia preconditioning-induced VEGF may thus protect the brain and promote its tolerance to hypoxia.

The change of HIF-1 α mRNA expression *in vivo* under hypoxia and hypoxia preconditioning has been studied [14]. A significant induction of HIF-1 α mRNA was found 19 h after the onset of ischemia [27]. In the present study, the HIF-1 α mRNA levels were found to be increased rapidly and transiently in the H1 group and decreased to the basal level after repeated exposure to hypoxia in the H4 group. This finding is different from the results obtained by Bergeron et al. [14]. This discrepancy could be explained by the difference of the methods for preconditioning. Our model belongs to whole-body hypoxia preconditioning, by which an intrinsic protective potential can be induced within a short period of time [4]. As a key molecule, HIF-1 should play an important role in this process. In our present work, the results of the studies of the HIF-1 α protein level and the HIF-1 DNA-binding activities demonstrated that this may be true. The HIF-1 α mRNA contains an internal ribosome entry site that makes effi-

cient translation equal under normoxic and hypoxic conditions [28]. So the increase of HIF-1 α mRNA means the increase of HIF-1 α protein. The change in group H1 implies that the increase in HIF-1 α mRNA as template for protein synthesis is necessary at the beginning of preconditioning. After preconditioning, energy-consuming processes of cells are depressed, and the cells change to hypometabolic states. Both translation and transcription are energy-consuming processes. The transcription of HIF-1 α mRNA is, therefore, depressed, so that the level of HIF-1 α mRNA decreased in group H4. It is likely that the adaptation involves changes in mRNA transcription, but is mainly controlled at the posttranscriptional level.

No direct evidence for the roles of HIF-1 and its target gene in the increase of tolerance to hypoxia is provided in the present study. The changes of HIF-1 and VEGF in the process of acute and repeated hypoxia present an indirect evidence for these possible functions. In conclusion, HIF-1 α activation in autoprogressive hypoxia plays an important role in developing hypoxic preconditioning. Some molecules, such as VEGF, which are HIF-1s downstream and are regulated by it, can be expected to increase in expression. These changes may protect the brain from hypoxic injury.

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