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Research Report

The temporal profile of genomic responses and protein synthesis in ischemic tolerance of the rat brain induced by repeated hyperbaric oxygen

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ABSTRACT

Repeated hyperbaric oxygen (HBO) exposure prior to ischemia has been reported to provide neuroprotection against ischemic brain injury. The present study examined the time course of neuroprotection of HBO (3.5 atmosphere absolute, 100% oxygen, 1 h for 5 consecutive days) and the changes of gene/protein expression in rats. First, at 6 h, 12 h, 24 h, and 72 h after HBO sessions, rats were subjected to forebrain ischemia (8 min). Histopathological examination of hippocampal CA1 neurons was done 7 days after ischemia. Second, temporal genomic responses and protein expression were examined at the same time points after HBO sessions without subjecting animals to ischemia. HBO significantly reduced loss of hippocampal CA1 neurons that normally follows transient forebrain ischemia when the last HBO session was 6 h, 12 h, or 24 h before ischemia (survived neurons 55%, 75%, and 53%, respectively), whereas if there was a 72-h delay before the ischemic insult, HBO was not protective (survived neurons only 6%). Statistical analysis on microarray data showed significant upregulation in 60 probe sets including 7 annotated genes ($p75^{NTR}$, *C/EBPδ*, *CD74*, *Edg2*, *Trip10*, *Nrp1*, and *Igf2*), whose time course expressions corresponded to HBO-induced neuroprotection. The protein levels of $p75^{NTR}$, C/EBPδ, and CD74 were significantly increased (maximum fold changes 2.9, 2.0, and 7.9, respectively). The results suggest that HBO-induced neuroprotection against ischemic injury has time window, protective at 6 h, 12 h and 24 h but not protective at 72 h. Although the precise interaction is to be determined, the genes/proteins relevant to neurotrophin and inflammatory-immune system may be involved in HBO-induced neuroprotection.

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Abbreviations: ANOVA, analysis of variance; ATA, atmosphere absolute; C/EBP, CCAAT enhancer binding protein; Egr-1, early growth response protein 1; HBO, hyperbaric oxygen; HSP, heat shock protein; MABP, mean artery blood pressure; MAP, mitogen-activated protein; NGF, nerve growth factor; NGFI-B, nerve growth factor-induced B; NMDA, *N*-methyl-D-aspartate; $p75^{NTR}$, p75 neurotrophin receptor; SOD, superoxide dismutase; Trk, tyrosine kinase

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1. Introduction

Accumulated data show that central nervous system acquires tolerance against ischemic insult by a prior exposure to a brief period of ischemia (Kitagawa et al., 1990; Kirino et al., 1991). Furthermore, various diverse environmental changes and various substances, such as non-lethal hyperthermia (Chopp et al., 1989; Kitagawa et al., 1991), oxidative stress (Ohtsuki et al., 1992), tumor necrosis factor (Nawashiro et al., 1997), and interleukin 1β (Ohtsuki et al., 1996) have been shown to induce tolerance, which has been designated 'cross-tolerance'. Among the various preconditioning stimuli that might induce cross-tolerance, hyperbaric oxygen (HBO) is attractive because it has already been used safely for various disorders. Several reports demonstrated that repeated HBO treatment before cerebral ischemia (Wada et al., 1996, 2001; Prass et al., 2000) or spinal cord ischemia (Dong et al., 2002; Nie et al., 2006) reduced ischemia-induced neuronal damage. Furthermore, a recent study has demonstrated that single dose HBO preconditioning provide an equivalent neuroprotective effect to that with hypoxic preconditioning in neonatal rats (Freiberger et al., 2006). However, in these studies, the time course of neuroprotection by HBO has not been well determined.

Concerning the mechanism of neuroprotection induced by HBO, some candidate mRNA/proteins have been suggested (Wada et al., 2001; Nie et al., 2006). Assuming that a cellular defense function against ischemia may be inherent to neurons by posttranslational modification of proteins or by expression of new proteins, exploring the correlation of time course of neuroprotection and temporal profile of comprehensive gene/protein expression is required to elucidate the mechanism of HBO-induced tolerance.

Using DNA microarray technology several studies investigated the expression changes of thousands of genes in a single hybridization assay in various brain ischemic/hypoxic models (Jin et al., 2001; Keyvani et al., 2002; Soriano et al., 2000; Kawahara et al., 2004), and hypoxic models (Gilbert et al., 2003). Recent study by Rickhag et al. (2006) has demonstrated dynamic ischemia-induced gene expression patterns, revealing a biphasic activation of genes in surviving tissue. They indicate the importance of analyzing many time points to obtain a comprehensive gene expression profiling following stress/insult to the brain.

In the present study, we sought to investigate the time course of HBO-induced tolerance and to explore the temporal profile of comprehensive gene/protein expression in order to determine the plausible underlying mechanisms for the tolerance.

2. Results

2.1. Physiological variables

Pericranial temperature was maintained close to 37 °C before and during ischemia. Mean arterial blood pressure (MABP) as well as arterial PCO_2 and PO_2 were maintained in the normal ranges (except for MABP during ischemia), and there were no significant differences among the groups in any of these variables (data not shown).

2.2. Effects of HBO on ischemic neuronal damage in hippocampal CA1

Hippocampal CA1 neurons were selectively and extensively damaged in the untreated group (Figs. 1A and a), exhibiting only limited number of normal neurons (1.0±1.5%). In HBO-6h, HBO-12h (Figs. 1B and b), HBO-24h groups, neuronal damage was significantly less, exhibiting normal neurons 55±27%, 75±13%, and 53±13% of those in the normal rats group, respectively ($p<0.01$). In HBO-72h group (Figs. 1C and c), neuronal damage was similar to the untreated group, exhibiting normal neurons only 5.7±11%. The surviving neurons was significantly larger in HBO-6h, HBO-12 h, and HBO-24h groups compared with HBO-72h group ($p<0.01$) (Fig. 2).

2.3. Genomic responses in hippocampal CA1

Fig. 3 shows the results of *K*-means cluster analysis (8 clusters), showing time-dependent changes of expression. The *K*-means algorithm emphasizes the shape and pattern of expression over time course. Clusters 1, 4 and 7 display patterns with apparent increased expression (Cluster 1, elevated at 12 h, declined to basal level at 24 h and remained to be invariant at 72 h; Cluster 4, elevated at 12 h and declined over 72 h; Cluster 7, displays elevated expression with two phases, at 12 h or 24 h). Clusters 2 and 6 display decreased expression with two phases, at 12 h or 24 h. The other clusters display multiple phases of up- or downregulated gene expression. Statistical Group Comparison (GeneSpring software) applied to all genes (excluded flagged spots) revealed significant upregulation in 60 probe sets including 7 annotated genes and significant downregulation in 58 probe sets including 20 annotated genes. Of 7 upregulated and annotated genes, *p75*^{NTR}, *C/EBPδ*, *CD74*, *Nrp1*, and *Trip10* belong to Cluster 4, *Edg2*, to Cluster 1, and *Igf2* to Cluster 7, respectively (Table 1). All these up- and downregulated genes are deposited in supplement files.

2.4. RT-PCR and Western blot analysis

mRNA levels of p75^{NTR} and C/EBPδ were significantly elevated, peaking at 12 h (respectively 2.1-, and 2.2-fold change compared with untreated group) (Fig. 4), which followed microarray data and well corresponded to the time course of neuroprotection. The results of Western blot analysis of p75^{NTR}, C/EBPδ, and CD74 were presented in Fig. 5. Significant increases in these three proteins were observed, the time of peak increase being varied slightly, namely at 24 h, 24 h, and 12 h for p75^{NTR} (2.9-fold), C/EBPδ (2.0-fold), and CD74 (7.9-fold), respectively. Detection of Nrp1 and IGF2 in Western blots was unable because of low protein concentrations (data not shown).

3. Discussion

3.1. Effects of HBO on neuronal damage in hippocampal CA1

The present study demonstrated in rats that repeated exposure to 3.5 atmosphere absolute (ATA)-HBO (1 h once daily for 5

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