Region-specific induction of hypoxic tolerance by expression of stress proteins and antioxidant enzymes

著者	OMATA N, MURATA T, TAKAMATSU S, MARUOKA N, YONEKURA Y, FUJIBAYASHI Y, WADA Y
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Brief communication

**Region-Specific Induction of Hypoxic Tolerance by Expression of Stress Proteins
and Antioxidant Enzymes**

Naoto Omata, Tetsuhito Murata, Shinji Takamatsu, Nobuyuki Maruoka, Yoshiharu
Yonekura, Yasuhisa Fujibayashi and Yuji Wada

Corresponding author. Tel.: +81-776-61-8363; Fax: +81-776-61-8136

E-mail address: tmurata@fmsrsa.fukui-med.ac.jp (Tetsuhito Murata)

Naoto Omata, Tetsuhito Murata, Nobuyuki Maruoka, Yuji Wada

Department of Neuropsychiatry, University of Fukui, Fukui 910-1193, Japan

Shinji Takamatsu, Yoshiharu Yonekura, Yasuhisa Fujibayashi

Biomedical Imaging Research Center, University of Fukui, Fukui 910-1193, Japan

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Figures)]

Abstract

We examined the induction of hypoxic tolerance after hypoxic preconditioning in the frontal cortex, caudate putamen and thalamus using the dynamic positron autoradiography technique and [^{18}F]2-fluoro-2-deoxy-D-glucose with rat brain slices. Hypoxic tolerance induction was confirmed in the frontal cortex, but not in the caudate putamen and thalamus. Next, we compared the gene expression in the frontal cortex and caudate putamen using the ATLAS Rat Stress Array, and found that the expression of 150-kDa oxygen-regulated protein and mitochondrial heat shock protein 70 as stress proteins, and copper-zinc-containing superoxide dismutase and manganese-containing superoxide dismutase as antioxidant enzymes was elevated only in the frontal cortex. These results suggest that the induction of hypoxic tolerance after hypoxic preconditioning is region-specific, and stress proteins and antioxidant enzymes participate in this phenomenon.

Key words: Hypoxic tolerance • Brain slice • Glucose metabolism • Regional differences • Gene expression • Antioxidant enzymes

Introduction

Sublethal hypoxia loading (hypoxic preconditioning) induces tolerance against subsequent lethal hypoxia, and this phenomenon is called hypoxic tolerance [1]. Recent studies suggest that the response against stress or plasticity is not uniform in all brain regions, and these regional differences are related to symptoms of stress-induced disorders [2]. Different gene expression changes are thought to contribute to such regional differences [3]. Therefore, hypoxic tolerance induction may also have regional differences. Thus, it is important to compare the gene expression patterns of brain regions in which hypoxic tolerance is induced and not induced. We developed a dynamic positron autoradiography technique (dPAT) to image metabolic changes in rodent brain slices, and the maintenance of cerebral metabolic rate of glucose (CMR_{glc}) was regarded as the parameter of viability [4, 5]. Recently we reported that the induction of hypoxic tolerance was most clearly in the frontal cortex following hypoxic preconditioning using dPAT and [¹⁸F]2-fluoro-2-deoxy-D-glucose ([¹⁸F]FDG) [6]. Furthermore we revealed using the ATLAS Rat Stress Array (Clontech, Palo Alto, CA) that the gene expression of some molecules, including stress proteins, was elevated in the frontal cortex after hypoxic preconditioning [6].

In this study we examined the induction of hypoxic tolerance after hypoxic preconditioning in the frontal cortex, caudate putamen and thalamus using dPAT and [¹⁸F]FDG with rat whole brain slices to compare these brain regions under the same conditions at the same time. Next, we profiled the gene expression of brain regions in which hypoxic tolerance was induced and not induced respectively, using the ATLAS Rat Stress Array. The comparison of these profiles showed that the expression of 150-kDa oxygen-regulated protein (ORP150) and mitochondrial heat shock protein 70

(mtHSP70) as stress proteins, and copper-zinc-containing superoxide dismutase (Cu-Zn SOD) and manganese-containing superoxide dismutase (Mn SOD) as antioxidant enzymes was elevated only in the hypoxic tolerance induced region. These proteins seemed to be related to region-specific induction of hypoxic tolerance.

Materials and methods

Male Wistar rats weighing 190–230 g were used. All protocols met with institutional approval and were consistent with the NIH policy on the use of animals in experimental research. Hypoxic preconditioning was achieved as follows [6]. Rats were individually placed in 5-liter air-tight jars and N₂ gas was delivered into the jars via inlet and outlet portals at a flow rate of 3 liters/min. Three-min hypoxic loading was performed at 1-h intervals three times daily for three days. dPAT and gene expression profiling were performed on the 4th day.

dPAT was performed as previously described [4, 5]. Briefly, sagittal brain slices (300 μ m in thickness) were prepared from rats treated by hypoxic preconditioning (preconditioning group) and untreated rats (control group) using a Microslicer (DTK-2000; Dosaka EM, Kyoto, Japan). They were incubated in Krebs Ringer solution (36°C pH 7.3–7.4 and bubbled with 95%O₂/5%CO₂ gas) containing [¹⁸F]FDG. The exposed imaging plate (BAS-MP 2040S, Fuji Photo Film Co., Tokyo, Japan; serially replaced by another one every 10 min) was scanned using a BAS-1500 (Fuji Photo Film Co., Tokyo, Japan). Loading and relief of hypoxia were conducted by exchanging the solution for another one bubbled in advance with either 95%N₂/5%CO₂ gas or 95%O₂/5%CO₂ gas with the same amount of [¹⁸F]FDG. A three-compartment model using the Gjedde-Patlak graphical method was applied to the image data for

determination of the fractional rate constant for phosphorylation of [^{18}F]FDG ($= k_3^*$, proportional to the CMR_{glc}) [4, 5]. The k_3^* is estimated from the slope of the linear portion of the graph, $\text{Ci}^*(t)/\text{Cp}^*(t)$ (vertical Y-axis) versus time (horizontal X-axis), where $\text{Ci}^*(t)$ is the total brain tissue radioactivity and $\text{Cp}^*(t)$ is the input function [4, 5]. Data were analyzed using a Macintosh computer and MacBAS software version 2 (Fuji Photo Film, Co.). The presented values are mean $\pm$ SD of a total eight slices from four rats obtained in two experiments. For statistical analysis, the Mann-Whitney U-test was used. Significance was regarded as $p < 0.01$.

Poly A⁺ RNA was extracted from the brain region in which hypoxic tolerance was induced and not induced respectively using a Micro-FastTrack 2.0 Kit (Invitrogen, San Diego, CA), and gene expression was profiled using the ATLAS Rat Stress Array. ^{33}P -labelled cDNA probes were prepared from poly A⁺ RNA, and were hybridized to the ATLAS membranes. Genes were judged to exhibit elevated expression in the preconditioning group if their expression was increased at least 2-fold compared to that of the control group.

Results

Fig. 1 shows the Gjedde-Patlak plots in the frontal cortex and caudate putamen with 20-min hypoxia loading. In the control group, the k_3^* values (estimated from the slope of the linear portion of the graph, $\times 1,000$) after reoxygenation (frontal cortex; 0.89 ± 0.82 , caudate putamen; 0.64 ± 0.64) were significantly lower than before hypoxia loading (frontal cortex; 5.87 ± 0.41 , $p < 0.01$, caudate putamen; 5.54 ± 0.39 , $p < 0.01$). In the frontal cortex of the preconditioning group, the k_3^* value after reoxygenation (5.60 ± 0.91) did not significantly differ from that before hypoxia loading (5.76 ± 0.45 ,

$p = 0.93$). However, in the caudate putamen of preconditioning group, the $k3^*$ value after reoxygenation (0.36 ± 0.84) was significantly lower than that before hypoxia loading (5.63 ± 0.37 , $p < 0.01$). Similar findings were obtained in the thalamus (data not shown).

Next, we profiled the gene expression in the frontal cortex and caudate putamen, and compared them (Fig. 2). As the molecules whose expression was elevated only in the frontal cortex, we found ORP150 and mtHSP70 as stress proteins, and Cu-Zn SOD and Mn SOD as antioxidant enzymes.

Discussion

In all brain regions analyzed by dPAT, the $k3^*$ values of the control group after reoxygenation were significantly lower than those before hypoxia loading, so 20-min hypoxia loading was thought to be lethal. In the frontal cortex of the preconditioning group, the $k3^*$ value after reoxygenation did not significantly differ from that before hypoxia loading, thus displaying maintenance of CMR_{glc} after hypoxic loading and suggesting the induction of hypoxic tolerance. However, in the caudate putamen and thalamus of the preconditioning group, the $k3^*$ values after reoxygenation were significantly lower than those before hypoxia loading, so hypoxic tolerance was not induced. Therefore it was suggested that the induction of hypoxic tolerance after hypoxic preconditioning was region-specific. Previously we reported the induction of hypoxic tolerance following 3-nitropropionic acid (3-NPA) pretreatment [7]. 3-NPA causes chemical hypoxia by inhibiting the mitochondrial electron transport chain. In that study the induction of hypoxic tolerance was confirmed in the frontal cortex, but not in the caudate putamen and thalamus, so the brain regions in which hypoxic

tolerance was induced and not induced were the same as those suggested in this study. Therefore, our series of studies have revealed that the response of the frontal cortex to hypoxia was different from that of the caudate putamen and thalamus.

By comparing gene expression in the frontal cortex and caudate putamen, it was found that the expression of ORP150 and mtHSP70 as stress proteins, and Cu-Zn SOD and Mn SOD as antioxidant enzymes was elevated only in the frontal cortex. Of course, ORP150 and mtHSP70 are known to be molecular chaperons and they protect proteins from various stresses including hypoxia [8, 9]. Hypoxia/reoxygenation produce free radicals [5]. Cu-Zn SOD and Mn SOD are thought to play a major role in scavenging processes [10].

The expression of the genes encoding the proteins mentioned above was elevated only in the hypoxic tolerance induced region. So, these region-specific changes in gene expression may contribute to the region-specific induction of hypoxic tolerance. It is interesting and logical that all these proteins work to protect against hypoxia/reoxygenation. The overexpression of these proteins in many brain regions may reduce the symptoms of stress-induced disorders [8]. To our knowledge, this is the first reported study that examined the hypoxic tolerance induction in several brain regions under the same conditions at the same time, and that addressed the molecular biological mechanism of region-specific induction.

In summary, the present study using dPAT revealed the region-specific induction of hypoxic tolerance after hypoxic preconditioning. Furthermore, analyses using the ATLAS Rat Stress Array suggested that stress proteins and antioxidant enzymes seemed to be related to this induction. In the future, we are planning to examine the expression changes of genes identified in this study before and after the

hypoxic tolerance induction. The genes included in ATLAS Rat Stress Array are limited, so further studies will be needed to understand the total mechanism of hypoxic tolerance induction.

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Figure legends

Fig. 1. Effect of hypoxic preconditioning on the Gjedde-Patlak plots of [^{18}F]FDG uptake in the frontal cortex and caudate putamen. Ordinate: $[\text{Ci}^*(t)/\text{Cp}^*(t)]$ expressed in terms of the radioactivity signal ratio on the imaging plate, where $\text{Ci}^*(t)$ is the total brain tissue radioactivity and $\text{Cp}^*(t)$ is the input function, versus Abscissa: time in minutes. Time zero is when [^{18}F]FDG was added to the bathing medium containing brain slices. The point at which hypoxia or reoxygenation was applied is shown in the figure. Values are the means $\pm$ SD obtained in eight slices (SD is shown for only the uppermost and lowermost lines).

Fig. 2. Gene expression profiles of the frontal cortex and caudate putamen in the control group and preconditioning group using an Atlas Rat Stress Array. In the frontal cortex the expression ratios of ORP150, mtHSP70, Cu-Zn SOD and Mn SOD in the preconditioning group compared to the control group were 7.3, 9.5, 3.4 and 8.6, respectively. On the other hand, the expression ratios of these genes in the preconditioning group compared to the control group in the caudate putamen were 1.3, 1.1, 1.1 and 1.0, respectively.