

## The Excitement of Multiple Noradrenergic Cell Groups in the Rat Brain Related to Hyperbaric Oxygen Seizure

Minako Arai<sup>a</sup>, Ken Takata<sup>b\*</sup>, Yoshimasa Takeda<sup>a</sup>,  
Satoshi Mizobuchi<sup>a</sup>, and Kiyoshi Morita<sup>a</sup>

<sup>a</sup>Department of Anesthesiology and Resuscitology, Okayama University Graduate School of Medicine,  
Dentistry and Pharmaceutical Sciences, Okayama 700-8558, Japan, and

<sup>b</sup>Department of Anesthesiology and Intensive Care II, Kawasaki Medical School, Kurashiki, Okayama 701-0192, Japan

The mechanism of oxygen toxicity for central nervous system and hyperbaric oxygen (HBO) seizure has not been clarified. Noradrenergic cells in the brain may contribute to HBO seizure. In this study, we defined the activation of noradrenergic cells during HBO exposure by *c-fos* immunohistochemistry. Electroencephalogram electrodes were pre-implanted in all animals under general anesthesia. In HBO seizure animals, HBO was induced with 5 atm of 100% oxygen until manifestation of general tonic convulsion. HBO non-seizure animals were exposed to 25 min of HBO. Control animals were put in the chamber for 120 min without pressurization. All animals were processed for *c-fos* immunohistochemical staining. All animals in the HBO seizure group showed electrical discharge on EEG. In the immunohistochemistry, *c-fos* was increased in the A1, A2 and A6 cells of the HBO seizure group, and in the A2 and A6 cells of the HBO non-seizure group, yet was extremely low in all three cell types in the control group. These results suggest the participation of noradrenaline in HBO seizure, which can be explained by the early excitement of A1 cells due to their higher sensitivity to high blood pressure, hyperoxia, or by the post-seizure activation of all noradrenergic cells.

**Key words:** hyperbaric oxygen, seizure, noradrenergic cells, immunohistochemistry

**H**yperbaric oxygen (HBO) therapy has been performed as a unique treatment for carbon monoxide poisoning [1-3], caisson sickness [4], and gas gangrene [5-7]. In addition, it has been reported that HBO improves the outcome in cerebral infarction as well [2, 8]. In spite of those benefits of HBO treatment, the clinical application of HBO is extremely limited due to oxygen (O<sub>2</sub>) toxicity to the central nervous system (CNS) and respiratory system. The specific symptoms of CNS O<sub>2</sub> toxicity at 2 or 3 atm pure

oxygen are characterized as generalized tonic convulsion. A higher oxygen pressure or longer exposure period increases the incidence of the convulsion [9, 10].

In spite of recent trials to elucidate the nature of O<sub>2</sub> toxicity, the mechanism of HBO seizure has not been clarified yet. The possible causes are considered to be production of reactive oxygen species (ROS) including nitric oxide (NO) [11, 12], decrease of gamma-aminobutyric acid (GABA) [13, 14], and collapse of the catecholamine system in the brain [15, 16].

The contribution of catecholamines, especially noradrenaline, has received attention after a report

that administration of a  $\beta$ -adrenergic blockade reduces the incidence of HBO-induced seizures [17]. From this result, we hypothesized that the noradrenergic cells in the rat brain would contribute to HBO seizure.

In this study, we examined the activation of noradrenergic cell groups (A1, A2, and A6) in the rat brain during HBO exposure by measuring Fos expression, a major immunohistochemical marker for neuronal excitation in seizure models.

### Materials and Methods

Fifteen male Wistar rats (Charles River Japan, Yokohama, Japan) were used in this study. The animals were fed *ad libitum* before the experiments. All experiments were performed in accordance with the National Institutes of Health animal care guidelines and were approved by the Animal Research Control Committee of Okayama University Medical School.

**General procedures.** All animals were implanted with EEG electrodes under anesthesia with a mixture of 2% halothane and 50% oxygen/50% nitrogen a day before HBO procedures. The continuously measured EEGs (S1516, Nihon Kohden Kogyo Co. Ltd., Tokyo, Japan) were collected and analyzed using an analog-digital system (AxoScope and Digidata 1200B; Axon Instruments, Inc., Foster City, CA, USA).

After the HBO procedure, EEG data was collected and typical wave signals were analyzed for 3 periods—pre-pressurization as control; just after plateau pressure was reached, and during seizure—using power-analyzing software (Origin Pro 8, Origin Lab Corporation, Northampton, MA, USA).

**HBO procedure.** A 300-liter chamber for HBO (PHC-special products, TABAI, Tokyo, Japan) was used. Before HBO administration on animals, the chamber was flushed twice with pure oxygen up to 2 atmospheres absolute (atm abs) pressure. In pressurized groups, each animal was pressurized to 5 atm abs with 100% oxygen. After the pressure of 5 atm abs was reached, continuous O<sub>2</sub> flow of 0.5L/min was administered to prevent accumulation of CO<sub>2</sub> in the chamber. The temperature inside the chamber was kept at 24 ± 2°C with a heated water blanket. During HBO, animals were visually observed through an illuminator on the chamber and all changes in their

behavior were recorded.

Rats were randomly divided into 3 groups (n = 5 per group): HBO seizure, HBO non-seizure, and controls. Animals in the HBO seizure group were pressurized for 120 minutes or until manifestation of wild running, a severe and fatal type of HBO seizure. During wild running, rats show running and jumping motions followed by general tonic seizure. Animals in the HBO non-seizure group were exposed to 25 min of HBO with 100% oxygen. The period of 5 atm abs in this procedure was 5 min. The time course of pressurization in the HBO non-seizure group was determined from the preliminary result of the first EEG seizure in HBO. Animals in the control group were put in the chamber for 120 min without pressurization.

**Immunohistochemistry.** Following completion of HBO procedures, all animals were processed for immunohistochemical staining. At 2h after removal from the chamber, animals were transcardially perfusion-fixed with 4% paraformaldehyde in 0.1M phosphate buffer (pH7.4) under urethane anesthesia. Brains were rapidly removed from the skull and post-fixed in the same solution for 12h. Five-mm coronal blocks were cut out and embedded in paraffin wax. A1, A2, and A6 were sectioned using a microtome (microtome RV-240, YAMATO, Asaka, Saitama, Japan) at 5- $\mu$ m thickness and mounted on glass slides. Locations of collecting area were referred to rat brain (Paxinos and Watson, 1997).

Paraffin sections were pretreated with xylene and ethanol in adequate concentrations, rinsed in 0.01M phosphate buffered saline (PBS) (pH7.4, Sigma, St. Louis, MS, USA), placed in 0.1% Triton X-100 for 5 min, rinsed again in PBS, and placed in 5% normal donkey serum in PBS for 1h. Then, they were incubated in a mixture of primary antibodies for 48h at 4°C, rinsed in PBS, incubated in a mixture of secondary antibodies for 4h at room temperature, rinsed in PBS, and coverslipped with an antifade mounting medium (Prolong Gold, Eugene, OR, USA).

A rabbit anti-c-fos polyclonal antibody (sc-52, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and mouse anti-tyrosine hydroxylase polyclonal antibody (MAB318, Chemicon International Ltd., Harrow, UK) were used as the primary antibodies at dilutions of 1:200 and 1:100, respectively.

The secondary antibody, donkey anti-rabbit-IgG

antibody, was labeled by Cy3 (Ab-182C, Chemicon International Ltd., Harrow, UK, 1:50 dilution), and donkey anti-mouse-IgG antibody was labeled by FITC (Ab-192F, Chemicon International Ltd., Harrow, UK, 1:25 dilution). Fos immunoreactivity (IR), which indicates neuronal excitation, was detected as a clear fluorescent-red reaction product confined to the nucleus of immunopositive neurons, and cells containing tyrosine hydroxylase were detected as a pale fluorescent yellowish-green product confined to the cytoplasm.

Sections were examined and photographed using a confocal laser scanning microscope (Zeiss LSM 510, Carl Zeiss Microimaging, Jena, Germany) and LSM image browser. The most heavily labeled sections through each structure of each animal were chosen for cell counting. Cells were counted in a standardized manner under  $200\times$  magnification.

**Statistical analysis.** Values are reported as mean  $\pm$  standard deviation (SD). The rates of Fos-positive cells/tyrosine-positive cells were evaluated by measurement analysis of variance (ANOVA). When indicated by a significant F ratio, the Scheffe post-hoc test was performed. A level of  $p < 0.05$  was considered to be statistically significant.

## Results

**Behavior and EEG.** After the onset of HBO exposure, a decrease in the activity levels of animals was observed, especially just prior to convulsions. Animals stopped moving around and remained in a squatting position in the chamber. On the other hand, some animals showed nervous movements such as biting cables or scratching body parts.

All animals in the HBO seizure group showed at least one general tonic seizure or electrical discharge (EEG seizure) during pressurization, and 3 of 5 animals showed wild running. The average compression time was  $74.4 \pm 26.5$  min, and the average time to express EEG seizure, generalized convulsion and wild running were  $17.41 \pm 19.63$ ,  $20.98 \pm 26.71$  and  $46.92 \pm 7.92$  min after reaching a plateau pressure, respectively.

During HBO, EEG amplitude in both the HBO seizure and HBO non-seizure groups was decreased to 71.7% and 75.3% of the value of the control group, although these differences were not significant ( $p =$

0.084, 0.109, respectively). EEG amplitude during HBO was significantly reduced compared to the pre-HBO value only in the HBO non-seizure group ( $p = 0.043$ ).

**Fos expression.** The regional expression of Fos in each group is shown in Fig. 1. Fos IR was remarkably increased in the HBO seizure group and slightly increased in the HBO non-seizure group, but extremely low in the control group. More specifically (Fig. 2), the HBO seizure group showed significantly higher Fos IR in A1, A2 and A6 than that of control group ( $p < 0.001$ , 0.001, 0.001; respectively), and higher Fos IR in A2 and A6 than that of the HBO non-seizure group ( $p < 0.001$ ,  $p = 0.034$ ; respectively). The HBO non-seizure group exhibited higher Fos IR in A1 than the control group ( $p < 0.001$ ).

## Discussion

In this study, we observed the reaction of the brain noradrenergic system against HBO exposure and examined its contribution to the manifestation of HBO seizure.

In the HBO non-seizure group, only A1 noradrenergic cells showed significantly higher Fos IR than the control group, which suggests the earlier activation of A1 cells by HBO exposure. This early excitement of A1 cells can be explained by their higher sensitivity to high blood pressure and hyperoxia. HBO exposure causes a significant increase of blood pressure and decrease of heart rate [18]. A1 cells receive the projection from arterial baroreceptors [19, 20] as well as from vagal cardiopulmonary volume receptors [21]. It is also well known that the noradrenergic cells of the caudal ventrolateral medulla (CVLM; A1 cells) modulate cardiovascular responses [19, 22, 23]. In the immunohistochemistry, the HBO seizure group showed significantly higher Fos IR in A2 and A6 cells than the HBO non-seizure group. This result suggests that a longer HBO period or HBO seizure itself leads to enhancement of Fos activation in this area or that the excitement of noradrenergic cells in this area may participate in causing or propagating HBO seizure.

The contribution of noradrenergic reaction to HBO seizure has been suggested by the previous finding that administration of a  $\beta$ -adrenergic blocker had an inhibitory effect on HBO seizure [17]. The concen-

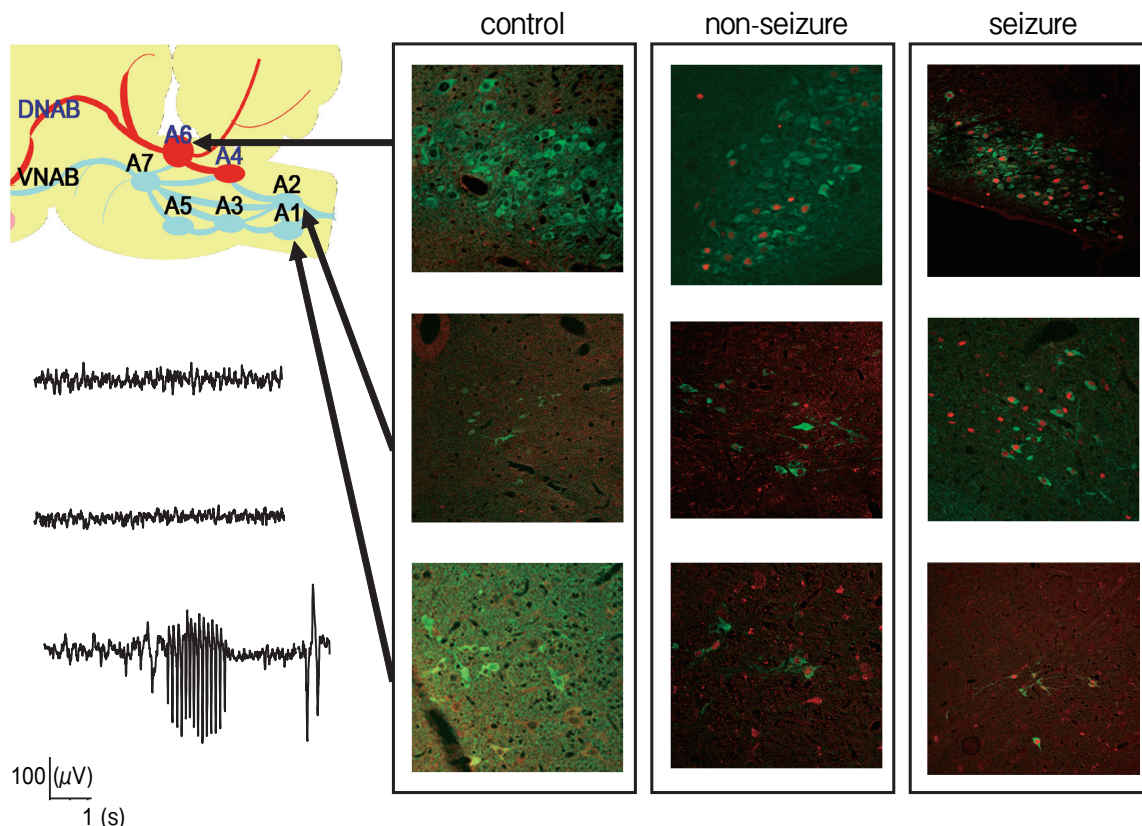


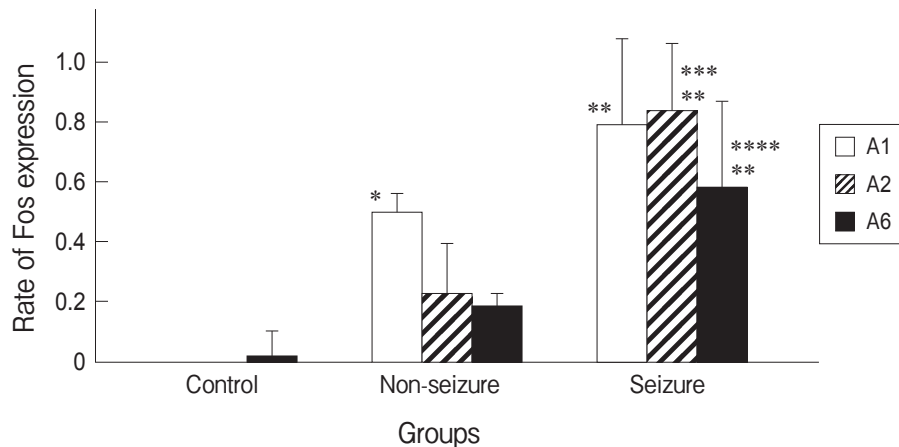
Fig. 1 Representative appearances of regional expression of Fos. Markedly higher Fos IR was observed in all HBO seizure groups. Fos IR was slightly increased in the HBO non-seizure group and extremely low in the control group.

tration of noradrenaline in the brain was also reduced following HBO seizure [24, 25]. This depletion of noradrenaline can be explained by the consequence of rapidly increased consumption of noradrenaline caused by intense activation of a noradrenergic cascade by HBO seizure. On the other hand, there still is the possibility that the noradrenergic reaction induced the HBO seizure. Further experiments are required to clarify this issue.

The mechanism of CNS  $O_2$  toxicity also involves increased production of ROSn [8], depletion of GABA pool [13, 14], and changes in cortical amino acid and  $NH_3$  levels [15, 16]. Available evidence supports the toxicity of  $H_2O_2$  during HBO; pre-administration of an MAO inhibitor reduced the oxygen toxicity due to decreased  $H_2O_2$  production and improved mortality, and pretreatment with aminotriazole, a reversible inhibitor of catalase which is a breakdown enzyme of  $H_2O_2$ , reinforced the oxygen toxicity [25].

NO vasodilation, which results in the increase of cerebral blood flow (CBF), also has a role as a mediator of CNS  $O_2$  toxicity [11, 12, 26–28]. Administration of NO synthesis inhibitor reduces CBF and results in the decreased incidence of HBO seizure and reduced mortality [11, 24, 25]. There is an interaction between  $O_2$ -dependent noradrenaline metabolism and NO synthesis in the CNS as well [25]. Since an adequate amount of oxygen is necessary for NO synthesis, the longer exposure to high-concentration oxygen may result in increased NO production.

There still remains a question: why only A2 and A6 cells, and not A1 cells, have the potential for dynamic outbreak of neuronal excitement during HBO seizure. This could be explained by the fact that increased amplitude of focal EEG by electrical stimulation of the nucleus of solitary tract (A2) is synchronized with rCBF increase, and that this neuronal excitement may expand to the whole brain [29, 30]. Several drug-induced seizure studies, which suggested



**Fig. 2** Fos IR expression in areas A1, A2 and A6. The HBO non-seizure group exhibited higher Fos IR in A1 than the control group ( $*p < 0.001$ ). The HBO seizure group showed significantly higher Fos IR in A1, A2 and A6 than the control group ( $**p < 0.001$ ,  $0.001$ ,  $0.001$ ; respectively), and higher Fos IR in A2 and A6 than the HBO non-seizure group ( $***p < 0.001$ ,  $****p = 0.034$ ; respectively).

that certain brain structures were selectively vulnerable, and the earliest and most severe signs of neuropathology would be key sites for the initiation of the seizures [31–33], may also support our findings; Fos expression was observed only in A1 during the pre-convulsion period, and in A2 and A6 post-convulsion.

In summary, we have demonstrated that HBO treatment activated Fos expression in the noradrenergic cells prior to and following HBO seizure. This result suggests that noradrenaline plays an important role in the development of HBO-induced seizure in rats.

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