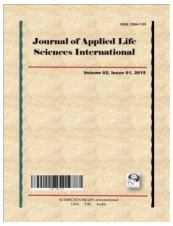

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Mild Hyperoxia Stimulation Increases Regional Tissue Oxygen Pressure in Rat Hippocampus via Oxygen Radical

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Authors' contributions

This work was carried out in collaboration among all authors. Author HY designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors OK, SA and RO managed the analysis of the study. Authors YH and YY managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Aims: The purpose of this study is to examine a rise of the local tissue oxygen pressure in hippocampus (Hip-pO2) which means neuronal activation by mild hyperoxia through oxygen radical.
Study Design: Study was an animal experiment with rat.
Place and Duration of Study: Department of Department of Life Science and Applied Chemistry, Nagaya Institute of Technology, between January 2014 and January 2018.
Methodology: Rats were exposed to air or mild oxygen gas. At the same time, Local tissue oxygen pressure in hippocampus (Hip-pO_2) were measured for 20 min with or without treatment of two type of radical scavengers.

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Results: The Hip-pO_2 levels were significantly increased by mild hyperoxia exposure (50-60% above resting level). The mild hyperoxia-induced enhancement of the Hip-pO_2 levels were inhibited by MnTMPyP (radical scavenger), but not by NADPH oxidase (NOX) inhibitor Apocynin.
Conclusion: These findings suggested that mild hyperoxia could activate hippocampus through generation of oxygen radicals.

Keywords: Mild hyperoxia; oxygen gas; reactive oxygen species; MnTMPyP; apocynin; Hip-pO_2; neural activation; clark-type electrode.

ABBREVIATIONS

Hip-pO_2 : The Local Tissue Oxygen Pressure in Hippocampus
ROS : Reactive Oxygen Species
NOX : NADPH Oxidase
MAO : Monoamine Oxidase
NOS : NO Synthase
O_2- : Superoxide

1. INTRODUCTION

Excess high oxygen environment generates reactive oxygen species (ROS) in the tissue, It acts directly on the cell and gives damage by peroxidation [1-3]. For example, as a result of exposure of 80% oxygen gas for 5 days to neonatal rats, increase in apoptosis and decrease in neuronal density was confirmed in hippocampal CA1 and DG tissues [4]. In addition, exposure to 95% oxygen gas for 2 hours in neonatal rats increased expression of Bcl-X in the cerebral cortex and cell death in the cortex [5]. Moreover, the damage caused by ROS due to hyperbaric oxygen irritation affects brain stem nerve cells, which disrupts brain stem function and causes hyperventilation [6,7]. From the above, as the oxygen becomes high pressure / high concentration, the damage due to ROS tends to be increased.

Meanwhile, the research results indicating beneficial effects on biological function have been reported with 30 to 40% O2 exposure or short term stimulation of 100% O2 inhalation, which is considered to be relatively mild oxygen stimulation conditions [8-11]. In human studies, Chung SC, et al. [8,9] reported that spatial recognition testing improves by inhaling 30 to 40% O2 during testing. Moss.MC and Scholey A.B [10,11] reported that the memory and learning effects by inhalation of 100% oxygen gas for 1 to 2 minute immediately before testing. These reports suggest that relatively mild high oxygen gas stimulation may activate the brain, especially the hippocampus. In vitro experiments using hippocampal slices showed that exposure of oxygen of 2.84 ATA or 4.54 ATA after exposure of oxygen at 0.95 ATA (absolute atmospheric pressure) causes neuronal activation in CA1 [12]. Similar nerve excitation was also observed when switching from 0ATA or 0.6 ATA oxygen exposure to 0.95 AT oxygen exposure [13]. At this time, tissue oxygen content in the hippocampal slice has been observed to increase as the pressure increases. From this result, it is considered that excitement of nerve cells may be induced when the tissue oxygen amount increases due to high pressure oxygen gas exposure. Also, neuronal activation may be induced when the tissue oxygen amount increases due to hyperbaric oxygen gas exposure. D'Agostino DP [14] observed a concentration-dependent manner increase in ROS production exposure to 20%, 40%, 60%, 95% oxygen gas to hippocampal slices. In addition, it is reported that the amount of SOD mRNA in hippocampal slices increases with 100% oxygen gas exposure [15]. In an in vitro experiment, the hypothesis is that the increase in tissue oxygen pressure generates active oxygen and causes neuronal excitation. However, there is no report showing this causal relationship. In addition, there are many uncertainties as to whether or not the regional hippocampal tissue oxygen pressure (Hip-pO2) increases by inhalation of oxygen gas in vivo, and further whether hippocampal neurons are activated or not. Therefore, in this study, we investigate activation of hippocampal nerve cells is examined by measuring the Hip-pO2 by relatively mild hyperoxia gas (oxygen concentration 32±0.5%) exposure *in vivo*.

2. MATERIALS AND METHODS

2.1 Animals

All animal procedures were approved by the Nagoya Institute of technology's Laboratory Animal Care and Use Committee. Male Sprague-Dawley （SD） rats were purchased from SLC (Shizuoka, Japan). Rats were housed under a 12 hours light/dark cycle and maintained at 23±1℃

with *ad libitum* access to standard rodent chow and water. 8 weeks old rats were used for all experiments.

2.2 Habituation

Before the surgery, rats were habituated to gas chamber for 4 consecutive days to minimize the effect of stress from environment (60, 90, 120 and 120 minutes at each day). Rats were placed on the gas chamber (cylindrical acrylic chamber (43 cm × 24 cm × 18 cm, 4 slit with 25 cm x 1.5 cm) in an acrylic cage (50 cm × 30 cm × 20 cm)) refluxed with air. Air (oxygen concentration, 21±0.5%) was supplied to the cage at a flow rate of 8 l/min using an air charger (α1500, manufactured by Nippon Tankan Industrial Co., Ltd. and HIBLOW AIR POMP, manufactured by Techno Takatsuki and MS-X 2, National), and oxygen gas (oxygen concentration, 32±0.5%) was delivered at a same flow rate to air.

2.3 Stereotaxic Surgery for Cannulation

After habituation period, rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.), and a stainless steel guide cannula (O.D. 0.8 mm, Unique Medical Co., Tokyo, Japan) was stereotaxically implanted into the left dorsal hippocampal region (co-ordinates: anteroposterior +1.5 mm, mediolateral 3.6 mm from the bregma, and dorsoventral -2.0 mm from the dura). The guide cannula was fixed to the skull with an anchor screw using dental cement (Shofu Co., Tokyom, Japan). After surgery, antibiotics (100 U penicillin and 100 μg streptomycin/kg BW.) were administered subcutaneously (s.c.). Rats were housed individually and allowed to recover for two days at least.

2.4 Hip-pO_2 Measurement

Hip-pO_2 was measured by using improved Clark-Type electrodes (U0E-04TS, Unique Medical Co., Tokyo, Japan) composed with a sensor at the tip (diameter 0.4 mm, length 10 mm of Teflon tube coating) and followed by a 35 mm stainless steel coating. Each electrode was connected to a digital pO_2 monitor (POG-203, Unique Medical Co., Tokyo, Japan). The details are described in previous our report [16]. Rats were stabilized in acryl chamber cage for 10 min, meantime, the electrode sensor was calibrated in water that was saturated with 20.9% O2-N2 balance, air and 0% O2-N2 gas. After calibration, the electrode sensor tip was heparinized, then inserted into the hippocampal region through the guide cannula and fixed with rocking nut. The tip of sensor protruded 1.0 mm from the end of the guide cannula.

2.4.1 Experiment 1: Hip-pO_2 changes during oxygen gas exposure

Rats were placed on the gas chamber flowing with air (rate, 1.0 L/min) for 10 minutes and the heparinized electrode was inserted through the cannula. After wait for stabilization, Hip-pO_2 level was measured for 80 minutes flowing schedule: air (10 min) – 30% oxygen gas (20 min) – air (20 min) – 30% oxygen gas (20 min) – air (20 min).

2.4.2 Experiment 2: Effect of ROS scavenger and NOX inhibitor on oxygen gas exposure

Overall experimental conditions were identical to experiment 1. MnTMPyP (CALBIOCHEM. purchased from Sigma-Aldrich, JAPAN) was prepared in a physiological saline to a concentration of 5 mg/kg.B.W. Apocynin (Toronto Research Chemicals Inc., Canada. purchased from FUJIFILM, JAPAN) was prepared in a physiological saline and ethanol to a concentration of 4 mg/kg.B.W (0.5% ethanol). Each reagent was administered by i.p. 20 minutes before the experiment. Hip-pO_2 level was measured for 45 minutes flowing schedule: air (15 min) – 30% oxygen gas (15 min) – air (15 min).

2.5 Statistics

The data were analyzed by one- or two-way ANOVA, followed by a post-hoc test (Fisher's PLSD) for comparison among means. All data were expressed as means ± SD.

3. RESULTS AND DISCUSSION

3.1 Mild Hyperoxia Increases Hip-pO_2

After switch air to 30% oxygen gas, Hip-pO_2 was increased to 60% above resting level. Surprisingly, this high level was maintained after switch to air again. In addition, 48% increase of Hip-pO_2 was observed in the second 30% oxygen gas exposure and maintained after switch to air again (Fig. 1.). Since rats were restrained in the chamber during experiment, possibility that restraint stress could affect our results remained. However, we did not observe over-excitement of animals. Therefore, it was

shown that the change in Hip-pO2 in this experiment was simply a result of high oxygen gas stimulation.

3.2 Hypothesis of Hip-pO2 Increase by Mild Hyperoxia

The reasons for the increase in local tissue oxygen pressure in brain under high oxygen gas environment are as follows: 1) the blood oxygen amount increases due to an increase in the amount of oxygen in inspiration, and 2) an increase in blood flow due to neuronal activation is considered [17-19]. Regards 1), oxygen present in the blood are divided into hemoglobin-bound oxygen and dissolved oxygen, and most of oxygen exists as hemoglobin-bound oxygen. However, when air is normally inhaled under atmospheric pressure, the oxygen saturation of hemoglobin has already reached approximately 98%, and even when exposed to high oxygen gas, the saturation increase of only 2% can be anticipated. Dissolved oxygen that increases by 0.003 mL / dL every 1 mmHg increases only about 0.2% in the case of inhalation of 32±0.5% oxygen gas. From this it can not be explained that the increase in blood oxygen level alone can increase Hip-pO2 by more than 50% by exposure to about 30% oxygen gas. Therefore, it is speculated that local blood flow increase is accompanied. Local cerebral blood flow increases as the neuronal activity at that site increases. For example, it has been reported that local cerebral blood flow in the rat striatum increases when striatum neuron cells are active [17]. In addition, cerebral blood flow in the hippocampus is increased by the treadmill running exercise, reports suggesting that this increase in blood flow is due to an increase in neural activity in the hippocampus [18,19]. For these findings, the main reason for the increase in Hip-pO2 due to the exposure to oxygen gas of about 30% observed in this experiment is that the hippocampal neurons are activated by a slight increase in blood oxygen amount, and it is inferred that this is due to an increase in the local blood flow caused by it.

3.3 Administration of MnTMPyP, but not Apocynin, Suppressed the Mild Hyperoxia-Induced Hip-pO2 Increases

The increase of Hip-pO_2 might be a consequence of increase of ROS activity. Therefore, MnTMPyP (active oxygen scavenger) and Apocynin (NOX inhibitor) were treated to investigate whether ROS was involved in the rise in Hip-pO_2 at 30% oxygen gas exposure. MnTMPyP is a widely used reagent as an active oxygen scavenger and has an effect of reducing oxidative stress [20, 21]. Also, Apocynin is a reagent that specifically inhibits NOX, and it has been found that the effect of reducing nerve cell death and oxidative stress upon NOX activation [22]. Before the experiment, we intraperitoneally injected MnTMPyP or apoxynin and measured change of Hip-pO_2 with 30% oxygen gas exposure (Fig. 2.). At the first, administration of MnTMPyP suppressed increase of Hip-pO_2 by 32% oxygen gas exposure to 10-20% above from resting level (control groups, 50-60% above from resting level). However, Apocynin showed no suppressive effect on Hip-pO_2 increase by 30% oxygen gas exposure (both of control and Apocynin group, 50-60% above from resting level).

3.4 ROS Mediates the Increase of Hip-pO_2 By Mild Hyperoxia

In this study, we showed that the rise in Hip-pO2 due to mild hyperoxia is mediated by reactive oxygen species (ROS) from experiments using radical scavenger (MnTMPyP). In vitro experiments using hippocampal slices reported that ROS increases in a concentration dependent manner with 40 to 60% oxygen gas [14]. In the culture medium without blood flow, it is considered that active oxygen ROS was generated due to an increase in the amount of tissue oxygen due to an increase in dissolved oxygen. Subsequently, it has been reported that ROS production was induced to excite the hippocampal nerve cells in many cases [14,23-25]. Even with a slight increase in blood or tissue oxygen level, ROS production occurs, and as a result of this ROS causing neuronal activation in hippocampus, could accompanie by an increase in blood flow. This is surmised to be cause of the greatly Hip-pO2 rise as our results have shown.

Four possible sources of ROS production are mitochondria, NADPH oxidase (NOX), Monoamine oxidase (MAO), and NO synthase (NOS) [23]. NOX is a major ROS production department in blood vessels [26-29], and it is also expressed in the brain [30,31]. It is thought that oxygen ingested is the first to act due to the fact that the production of ROS (O2-) is the main function and because NOX localized on the cell membrane. However, a NOX inhibitor, Apocynin could not suppress the mild hyperoxia-induced Hip-pO_2 increases. Furthermore, MAO and NOS are enzymes that do not generate ROS as a by-

product or directly use oxygen [23], therefore, these would be hard to be considered as a source of high oxygen-dependent ROS. Consequently, mitochondria are likely to be the source of ROS production by mild hyperoxia stimulation. Under hypoxic conditions, it is known that ROS is increased by decreasing electron transfer chain by inhibiting oxidative phosphorylation [32-35]. In hyperoxic conditions, an increase in dissolved oxygen and a concomitant increase in mitochondrial respiratory chains may be driving an increase in ROS. However, further studies with mitochondrial superoxide scavengers are needed to clarify the mechanisms of the mild hyperoxia-induced ROS production.

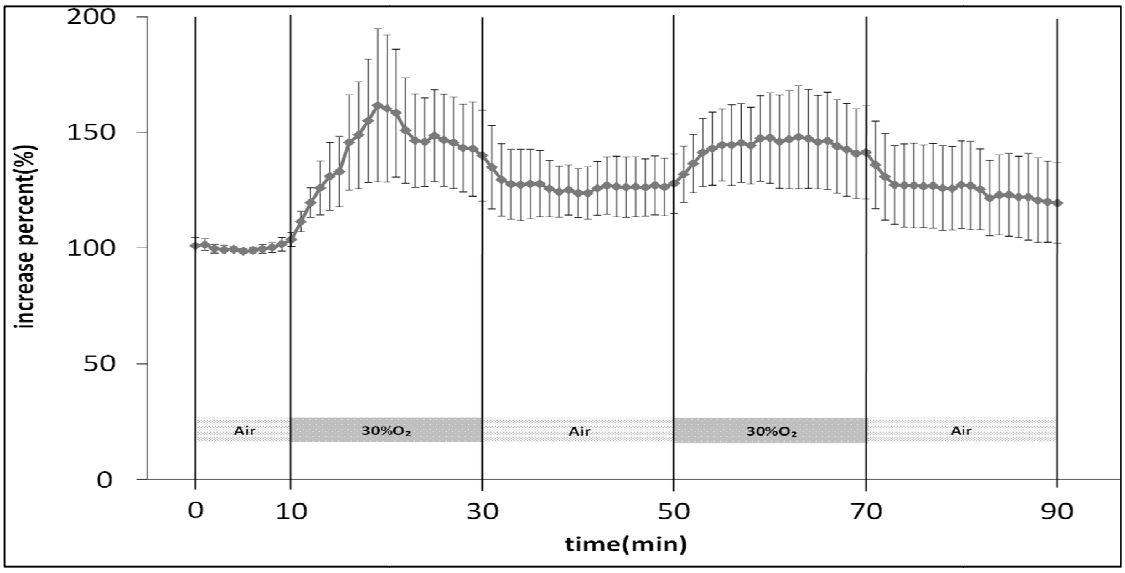

Fig. 1. Mild hyperoxia increases hippocampal tissue oxygen pressure with sustained pattern

Rats in gas chamber were exposed to 32% of oxygen gas and air according to following schedule: Air (10 min) – O2 gas (20 min) – Air (20 min) – O2 gas (20 min) – Air (20 min). The Hip-pO2 was introduced to pre-implanted cannula, and measured during all gas exposure experiment. Data are mean ± SD. (n=7)

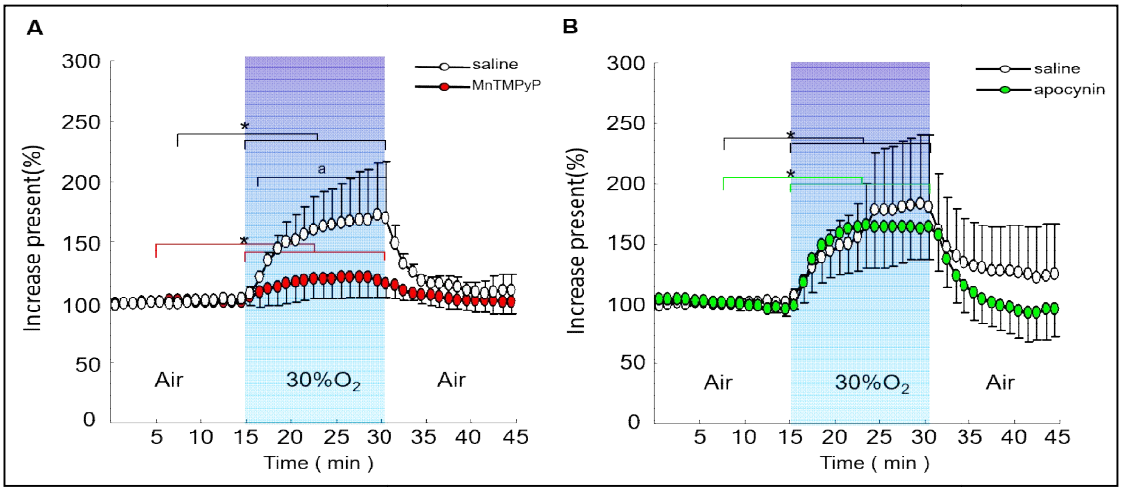

Fig. 2. Effect of the inhibitor or scavenger administration on pO_2 changes induced by mild hyperoxia

*Drug was applied during 30% oxygen gas exposure: (A) MnTMPyP (5 mg/kg I.P) (n=5), saline control (n=6), (B) Apocynin (4 mg/kg I.P) (n=4), saline control (n=6). Data are mean ± SD. *: P<0.01 vs Air control, a: P<0.05 MnTMPyP vs saline control*

4. CONCLUSION

We were able to investigate the reactivity of the Hip-pO2 to O2 gas stimulus in real time. It began to react in one minute after the start of the stimulation, reached the peak after 6 minutes.

Our findings suggested that relatively mild hyperoxia could fully active local hippocampal neuron through ROS production. Nagatomo F [36] found that oxidative metabolites in the blood did not increase even if a gas with oxygen concentration of 35% or less was inhaled for 24 hours under atmospheric pressure in rats. However, more than 40% O2 inhalation for 24 hours induced oxidative stress. From this, it is conceivable that relatively mild hyperoxia about 30% (strictly 32 ± 2%) oxygen used in this study generates ROS causing neuronal activition, but it does not greatly damage the brain. Relatively mild hyperoxia stimulation has the possibility of expecting beneficial neuronal activation effect without oxidative stress disorder.

ETHICAL APPROVAL

As per international standard written ethical permission has been collected and preserved by the author(s).

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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