Mild hyperoxia stimulation increases regional tissue oxygen pressure in rat hippocampus via oxygen radical

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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.
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

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

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36 studies, Chung S. C. et al. [8, 9] reported that spatial recognition testing improves by inhaling
37 30 to 40% O2 during testing. Moss.MC and Scholey A.B [10, 11] reported that the memory
38 and learning effects by inhalation of 100% oxygen gas for 1 to 2 minute immediately before
39 testing. These reports suggest that relatively mild high oxygen gas stimulation may activate
40 the brain, especially the hippocampus. In vitro experiments using hippocampal slices
41 showed that exposure of oxygen of 2.84 ATA or 4.54 ATA after exposure of oxygen at 0.95
42 ATA (absolute atmospheric pressure) causes neuronal activation in CA1 [12]. Similar nerve
43 excitation was also observed when switching from 0ATA or 0.6 ATA oxygen exposure to
44 0.95 AT oxygen exposure [13]. At this time, tissue oxygen content in the hippocampal slice
45 has been observed to increase as the pressure increases. From this result, it is considered
46 that excitement of nerve cells may be induced when the tissue oxygen amount increases
47 due to high pressure oxygen gas exposure. Also, neuronal activation may be induced
48 when the tissue oxygen amount increases due to hyperbaric oxygen gas exposure.
49 D'Agostino DP [14] observed a concentration-dependent manner increase in ROS
50 production exposure to 20%, 40%, 60%, 95% oxygen gas to hippocampal slices. In addition,
51 it is reported that the amount of SOD mRNA in hippocampal slices increases with 100%
52 oxygen gas exposure [15]. In an in vitro experiment, the hypothesis is that the increase in
53 tissue oxygen pressure generates active oxygen and causes neuronal excitation. However,
54 there is no report showing this causal relationship. In addition, there are many uncertainties
55 as to whether or not the regional hippocampal tissue oxygen pressure (Hip-pO2) increases
56 by inhalation of oxygen gas in vivo, and further whether hippocampal neurons are activated
57 or not. Therefore, in this study, we investigate activation of hippocampal nerve cells is
58 examined by measuring the Hip-pO2 by relatively mild hyperoxia gas (oxygen concentration
59 32±0.5%) exposure in vivo.
60

61 **2. MATERIAL AND METHODS / EXPERIMENTAL DETAILS / METHODOLOGY**

62 **2.1 Animals**

63 All animal procedures were approved by the Nagoya Institute of technology's Laboratory
64 Animal Care and Use Committee. Male Sprague-Dawley （SD）rats were purchased from
65 SLC (Shizuoka, Japan). Rats were housed under a 12 hours light/dark cycle and maintained
66 at 23±1℃ with *ad libitum* access to standard rodent chow and water. 8 weeks old rats were
67 used for all experiments.
68

69 **2.2 Habituation**

70 Before the surgery, rats were habituated to gas chamber for 4 consecutive days to minimize
71 the effect of stress from environment (60, 90, 120 and 120 minutes at each day). Rats were
72 placed on the gas chamber (cylindrical acrylic chamber (43 cm × 24 cm × 18 cm, 4 slit with
73 25 cm x 1.5 cm) in an acrylic cage (50 cm × 30 cm × 20 cm)) refluxed with air. Air (oxygen
74 concentration, 21±0.5%) was supplied to the cage at a flow rate of 8 l/min using an air
75 charger (α1500, manufactured by Nippon Tankan Industrial Co., Ltd. and HIBLOW AIR
76 POMP, manufactured by Techno Takatsuki and MS-X 2, National), and oxygen gas (oxygen
77 concentration, 32±0.5%) was delivered at a same flow rate to air.
78

79 **2.2 Stereotaxic surgery for cannulation**

80 After habituation period, rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.),
81 and a stainless steel guide cannula (O.D. 0.8mm, Unique Medical Co., Tokyo, Japan) was
82 stereotaxically implanted into the left dorsal hippocampal region (co-ordinates:
83 anteroposterior +1.5mm, mediolateral 3.6 mm from the bregma, and dorsoventral -2.0 mm
84 from the dura). The guide cannula was fixed to the skull with an anchor screw using dental
85 cement (Shofu Co., Tokyom, Japan). After surgery, antibiotics (100 U penicillin and100 µg
86 streptomycin/kg BW.) were administered subcutaneously (s.c.). Rats were housed
87 individually and allowed to recover for two days at least.

88

2.3 Hip-pO_2 measurement

89
90 Hip-pO_2 was measured by using improved Clark-Type electrodes (U0E-04TS, Unique
91 Medical Co., Tokyo, Japan) composed with a sensor at the tip (diameter 0.4 mm, length 10
92 mm of Teflon tube coating) and followed by a 35 mm stainless steel coating. Each electrode
93 was connected to a digital pO_2 monitor (POG-203, Unique Medical Co., Tokyo, Japan). The
94 details are described in previous our report [16]. Rats were stabilized in acryl chamber cage
95 for 10min, meantime, the electrode sensor was calibrated in water that was saturated with
96 20.9%O2-N2 balance, air and 0% O2-N2 gas. After calibration, the electrode sensor tip was
97 heparinized, then inserted into the hippocampal region through the guide cannula and fixed
98 with rocking nut. The tip of sensor protruded 1.0 mm from the end of the guide cannula.
99

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

100
101 Rats were placed on the gas chamber flowing with air (rate, 1.0 L/min) for 10 minutes and
102 the heparinized electrode was inserted through the cannula. After wait for stabilization, Hip-
103 pO_2 level was measured for 80 minutes flowing schedule: air (10 min) – 30% oxygen gas (20
104 min) – air (20 min) – 30% oxygen gas (20 min) – air (20 min).
105

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

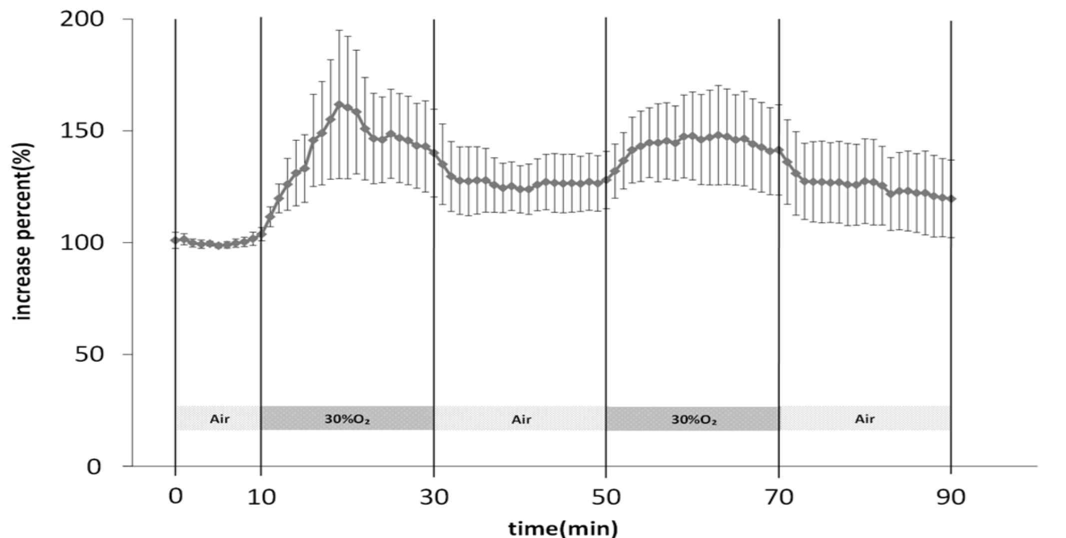
106
107
108 Overall experimental conditions were identical to experiment 1. MnTMPyP (CALBIOCHEM.
109 purchased from Sigma-Aldrich, JAPAN) was prepared in a physiological saline to a
110 concentration of 5 mg/kg.B.W. Apocynin (Toronto Research Chemicals Inc., Canada.
111 purchased from FUJIFILM, JAPAN) was prepared in a physiological saline and ethanol to a
112 concentration of 4 mg/kg.B.W (0.5% ethanol). Each reagent was administered by i.p. 20
113 minutes before the experiment. Hip-pO_2 level was measured for 45 minutes flowing
114 schedule: air (15 min) – 30% oxygen gas (15 min) – air (15 min)
115

2.4 Statistics

116
117 The data were analyzed by one- or two-way ANOVA, followed by a post-hoc test (Fisher's
118 PLSD) for comparison among means. All data were expressed as means ± SD.
119

3. RESULTS AND DISCUSSION

120



121

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)

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 (Figure 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.

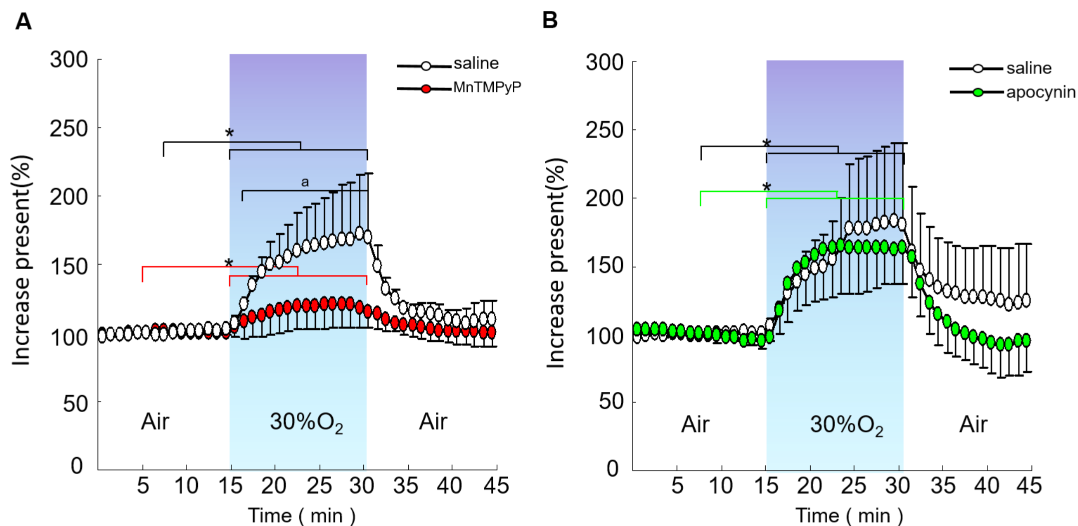


Fig. 2. Effect of the inhibitor or scavenger administration on pO_2 changes induced by mild hyperoxia.

*Drug was applied during 30% oxygen gas exposure: (A) MnTMPyP (5mg/kg I.P) (n=5), saline control (n=6), (B) Apocynin (4mg/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*

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

159 example, it has been reported that local cerebral blood flow in the rat striatum increases
160 when striatum neuron cells are active [17]. In addition, cerebral blood flow in the
161 hippocampus is increased by the treadmill running exercise, reports suggesting that this
162 increase in blood flow is due to an increase in neural activity in the hippocampus [18, 19].
163 For these findings, the main reason for the increase in Hip-pO2 due to the exposure to
164 oxygen gas of about 30% observed in this experiment is that the hippocampal neurons are
165 activated by a slight increase in blood oxygen amount, and it is inferred that this is due to an
166 increase in the local blood flow caused by it.
167

168 **3.3 Administration of MnTMPyP, but not Apocynin, suppressed the mild**
169 **hyperoxia-induced Hip-pO2 increases.**

170 The increase of Hip-pO_2 might be a consequence of increase of ROS activity. Therefore,
171 MnTMPyP (active oxygen scavenger) and Apocynin (NOX inhibitor) were treated to
172 investigate whether ROS was involved in the rise in Hip-pO_2 at 30% oxygen gas exposure.
173 MnTMPyP is a widely used reagent as an active oxygen scavenger and has an effect of
174 reducing oxidative stress [20, 21]. Also, Apocynin is a reagent that specifically inhibits NOX,
175 and it has been found that the effect of reducing nerve cell death and oxidative stress upon
176 NOX activation [22]. Before the experiment, we intraperitoneally injected MnTMPyP or
177 apoxynin and measured change of Hip-pO_2 with 30% oxygen gas exposure (Figure 2.). At
178 the first, administration of MnTMPyP suppressed increase of Hip-pO_2 by 32% oxygen gas
179 exposure to 10-20% above from resting level (control groups, 50-60% above from resting
180 level). However, Apocynin showed no suppressive effect on Hip-pO_2 increase by 30%
181 oxygen gas exposure (both of control and Apocynin group, 50-60% above from resting level).
182

183 **3.4 ROS mediates the increase of Hip-pO_2 by mild hyperoxia.**

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

195 Four possible sources of ROS production are mitochondria, NADPH oxidase (NOX),
196 Monoamine oxidase (MAO), and NO synthase (NOS) [23]. NOX is a major ROS production
197 department in blood vessels [26-29], and it is also expressed in the brain [30, 31]. It is
198 thought that oxygen ingested is the first to act due to the fact that the production of ROS
199 (O2-) is the main function and because NOX localized on the cell membrane. However, a
200 NOX inhibitor, Apocynin could not suppress the mild hyperoxia-induced Hip-pO_2 increases.
201 Furthermore, MAO and NOS are enzymes that do not generate ROS as a by-product or
202 directly use oxygen [23], therefore, these would be hard to be considered as a source of high
203 oxygen-dependent ROS. Consequently, mitochondria are likely to be the source of ROS
204 production by mild hyperoxia stimulation. Under hypoxic conditions, it is known that ROS is
205 increased by decreasing electron transfer chain by inhibiting oxidative phosphorylation [32-
206 35]. In hyperoxic conditions, an increase in dissolved oxygen and a concomitant increase in
207 mitochondrial respiratory chains may be driving an increase in ROS. However, further
208 studies with mitochondrial superoxide scavengers are needed to clarify the mechanisms of
209 the mild hyperoxia-induced ROS production.

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.

ACKNOWLEDGEMENTS

The authors wish to thank our colleagues at the Nagoya Institute of Technology for kind technical assistance and valuable advice.

COMPETING INTERESTS

The authors declare that they have no conflict of interests.

AUTHORS' CONTRIBUTIONS

Yoshizato H. designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Kwon O., Ato S., Ogasawara R. managed the analyses of the study. Hanai Y., Yoshimura Y. managed the literature searches. All authors read and approved the final manuscript.

Ethical Disclaimer:

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

Consent: NA

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364 **DEFINITIONS, ACRONYMS, ABBREVIATIONS**

365 **Hip-pO_2**: the local tissue oxygen pressure in hippocampus
366 **ROS**: reactive oxygen species
367 **NOX** : NADPH oxidase
368 **MAO**: Monoamine oxidase
369 **NOS**: NO synthase
370 **O_2-** : superoxide
371

372 **APPENDIX**

373