Chronic Intermittent Hypoxia/Reoxygenation Facilitate Amyloid-β Generation in Mice

Satomi Shiota,1,∗, Hidenori Takekawa,1, Shin-ei Matsumoto,1, Kazuya Takeda,1,∗, Fariz Nurwidyanto,1, Yasuko Yoshii,1, Fumiyuki Takahashi,1, Nobutaka Hattori,1, Takeshi Tabira,2, Hideki Mochizuki,∗ and Kazuhisa Takahashi1

1Department of Respiratory Medicine, Juntendo University Graduate School of Medicine, Tokyo, Japan
2Department of Diagnosis, Prevention and Treatment of Dementia, Juntendo University Graduate School of Medicine, Tokyo, Japan
3Department of Neurology, Juntendo University Graduate School of Medicine, Tokyo, Japan
4Department of Immunology, Kanazawa Medical University School of Medicine, Japan
5Department of Neurology, Osaka University Graduate School of Medicine, Osaka, Japan

Handling Associate Editor: Robert Friedland

Abstract. Previous studies have shown a high prevalence of obstructive sleep apnea (OSA) among patients with Alzheimer’s disease (AD). However, it is poorly assessed whether chronic intermittent hypoxia (CIH), which is a characteristic of OSA, affects the pathophysiology of AD. We aimed to investigate the direct effect of intermittent hypoxia (IH) in pathophysiology of AD in vivo and in vitro. In vivo, 15 male triple transgenic AD mice were exposed to either CIH or normoxia (5% O2 and 21% O2 every 10 min, 8 h/day for 4 weeks). Amyloid-β (Aβ) profile, cognitive brain function, and brain pathology were evaluated. In vitro, human neuroblastoma SH-SY5Y cells stably expressing wild-type amyloid-β protein precursor were exposed to either IH (8 cycles of 1% O2 for 10 min followed by 21% O2 for 20 min) or normoxia. The Aβ profile in the conditioned medium was analyzed. CIH significantly increased levels of Aβ42 but not Aβ40 in the brains of mice without the increase in hypoxia-inducible factor 1, alpha subunit (HIF-1α) expression. Furthermore, CIH significantly increased intracellular Aβ in the brain cortex. There were no significant changes in cognitive function. IH significantly increased levels of Aβ42 in the medium of SH-SY5Y cells without the increase in the HIF-1α expression. CIH directly and selectively increased levels of Aβ42 in the AD model. Our results suggest that OSA would aggravate AD. Early detection and intervention of OSA in AD may help to alleviate the progression of the disease.

Keywords: Alzheimer’s disease, amyloid-β peptide, hypoxia, obstructive sleep apnea

INTRODUCTION

Obstructive sleep apnea (OSA) is the most common form of sleep-disordered breathing (SDB) and is a major public health problem because of its high prevalence in morbidity and mortality. OSA is characterized by repetitive episodes of upper airway obstruction during sleep associated with intermittent hypoxia (IH). Patients affected by chronic IH (CIH)
and the accompanying sleep fragmentation suffer from its negative effects, such as cognitive dysfunction [1–3]. Ancoli-Israel et al. found a strong correlation between the amount of SDB and cognitive impairment, showing that patients with severe dementia are more likely to have severe SDB than those with milder dementia [3]. Although the pathophysiology of OSA is multifactorial, one of its major features, intermittent episode of hypoxia and reoxygenation during sleep, is highly suggested to associate with cognitive dysfunction. Indeed IH during sleep, not sleep fragmentation, is associated with mild cognitive impairment [4]. The exposure of IH for 4 weeks demonstrated a significant decrease in the N-acetyl aspartate/creatine (NAA/Cr) ratio, which is a reliable marker of neuronal integrity in the hippocampus and thalamus in mice, while mice exposed to 4 weeks of constant hypoxia did not demonstrate any differences in their NAA/Cr ratios from controls in these brain regions [5].

Alzheimer’s disease (AD) is the most common cause of cognitive dysfunction and is another major public health problem worldwide. A small study showed OSA and SDB in more than 40% of AD patients [6, 7] compared to only 4.3% in the age-matched healthy controls [6]. Besides epidemiologic studies, continuous positive airway pressure, which is a standard treatment for OSA [6], has shown to slow or even improve cognitive impairment in patients with mild-moderate AD with OSA, has shown to slow or even improve cognitive impairment in patients with mild-moderate AD with OSA [8–10]. Nevertheless, to the best of our knowledge, there have been no previous studies evaluating the direct effect of IH on AD pathophysiology. We hypothesize that CIH may aggravate the clinical course of AD.

AD is caused by deposition of amyloid-β peptide (Aβ) in brain tissue. During the sequential endoproteolytic cleavages of the precursor molecule amyloid-β protein precursor (AβPP) operated by the β-secretase1 (BACE1) followed by γ-secretase, Aβ, a 39–42-amino acid peptide is generated [11]. Studies have shown that Aβ42 appears more fibrillogenic and toxic than other Aβ [12]. Cerebral imbalance between Aβ1-42 peptide production and degradation/clearance plays a causal role in AD. Intraneuronal Aβ accumulation is more important in AD progression than extracellular Aβ (senile plaques) depositions [13, 14]. Furthermore, intraneuronal Aβ42, but not Aβ40, accumulation with AD pathology has now been reported [15, 16]. Therefore, to test our hypothesis we undertook an in vivo study to examine the effect of CIH in transgenic AD mice on their Aβ generation. We also developed an in vitro model by exposing cells to IH exposure and investigated the profiles of Aβ generation.

MATERIALS AND METHODS

Animals and CIH exposure

All animal procedures were approved by the animal care committee of Juntendo University. We used male homozygous triple-transgenic model of AD (3xTg-AD) mice [17] (6 months of age, n = 15). Mice were kept on a 12-h light and 12-h dark schedule. CIH was applied (n = 9) by exposing to alternating 5% O2 and 21% O2 every 10 min for 8 h per day during daytime for 8 weeks in a chamber (370 × 260 × 250 mm, 26 L, Sibata Scientific Technology Ltd, Tokyo, Japan). Oxygen concentration in the chamber was continuously recorded by O2 analyzer (XP-3180, New Cosmos Electronic Co, Osaka, Japan) (Fig. 1A). Ambient CO2 in the chamber was maintained at less than 0.03%. Control mice (n = 6) were kept under normoxia and touched by human hands once a day to balance out their stress through direct human contact.

After 8 weeks of exposure to CIH, spatial memory retention in 3xTg-AD mice was analyzed in a Morris water maze (MWM) using a DV-Track Video Tracking System (Muromachi Kikai, Tokyo, Japan). The MWM analysis was similar to those described in a previous report [13, 18]. The main parameter of the probe trial was percentage time spent in the quadrant where the platform had been located. Brain tissues were cut and divided into two hemispheres. One hemisphere was immersed in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) over 24 h and used for enzyme-linked immunosorbent assay (ELISA). The other was kept frozen for immunohistochemistry. ELISA for Aβ levels in brain tissue lysate was performed as described previously [19]. Aβ levels were measured by Human Amyloid-β (x–40) Kit or Human Amyloid-β (x–42) Assay Kit (IBL, Gunma, Japan). The supernatants were diluted with standard dilution buffer at 1:2000 (Aβ40) or 1:400 (Aβ42). AβPP and BACE1 protein levels were analyzed by western blotting (WB) as described previously [20, 21]. WB analysis for hypoxia-inducible factor (HIF)-1α was performed as described previously [22]. We used LLC (mouse lung cancer) cells by exposure to sustained hypoxia (1% O2 24 h) as the positive control for HIF-1α expression. Frozen hemisphere was cut in thickness of 16 μm. It was treated with 99% formic acid at room temperature for 5 min and incubated after being washed with Aβ antibody (4G8; Millipore, Bedford, MA, 1:3,000) followed by Mouse to Mouse HRP (DAB) Staining System (ScyTek Laboratories, Logan, UT). The specimens of both the experimental and
control groups were stained in the same staining chamber in exactly the same condition. Areas containing intracellular Ab-positive cells were first identified by scanning tissue sections. Cell count was determined in a total of 4 areas (1 area per mouse) in CIH and control groups under ×200-magnification. The area of intracellular Ab-positive cells was quantified with the use of Image J software [23].

Cell culture and IH exposure

AbPP was transfected a human neuroblastoma SH-SY5Y cells as already described [20]. IH condition was applied by exposing cells to 8 cycles of 1% O2 for 10 min followed by 21% O2 for 20 min. During the hypoxic period, the plates were placed in a modular incubator chamber (Billups Rothenberg, Inc., Del Mar, CA) with a gas mixture of 1% O2, 5% CO2, and balance N2. Under normoxic conditions, the plates were placed in the other incubator (Forma Steri Cycle, Thermo Scientific, Waltham, MA) with a gas mixture of 21% O2, 5% CO2, and balance N2. The continuous concentration of oxygen in the chamber and incubator was recorded. The representative partial pressure oxygen (PO2) values in the medium were measured by Rapid Point 400 (SIEMENS Healthcare Diagnostics, PA) and added to the oxygen profile in the chamber (Fig. 1B). The temperature was kept at 37°C throughout the exposure. ELISA for Ab levels and WB analysis for HIF-1α in the brain tissues was performed as described in the in vivo study.

Data are presented as means ± S.D. Unpaired two-tailed t-tests were used to compare values of control groups with CIH or IH group. Statistical significance was set at p < 0.05.

RESULTS

In vivo study

To examine the effect of CIH for 8 weeks in 3xTg AD mice, we measured the Ab levels in the brain tissue lysate by ELISA and examined the amyloid plaques on the brain tissues by immunostaining assay. Compared to control (n = 6), the CIH group (n = 9) showed a significant increase in Ab1-42 levels (457.9 ± 69.44 pg/ml and 635.7 ± 113.6 pg/ml, p = 0.010, Fig. 2A), while Ab1-40 levels remained unchanged (21391.7 ± 1003.2 pg/ml and 20995.2 ± 2327.2 pg/ml, p = 0.948, Fig. 2B). As a result, the Ab1-42/Ab1-40 ratio increased significantly in the CIH group compared to the control group (0.0214 ± 0.003 and 0.0303 ± 0.0048, p = 0.005, Fig. 2C). To investigate the process of Ab1-42 generation in 3xTg AD mice, we examined the AbPP and BACE1 expression levels in the brain tissues of the mouse by WB analysis. The levels of AbPP and BACE1 showed no significant difference between CIH and control group. We also examined the HIF-1α protein level in the brain tissues of the mouse. HIF-1α protein did not accumulate in the CIH group compared to the control group (Fig. 3). Amyloid plaques were not detected in both groups, however, intracellular Ab-containing neurons were more frequently detected in the brain cortex of the CIH group compared to the control group (Fig. 4A). A quantitative immunohistochemical analysis demonstrated that the intracellular Ab positive area significantly increased in the CIH group compared to the control group (5.74 ± 1.59% and 2.28 ± 1.35%, p = 0.028, Fig. 4B). To investigate the cognitive function with regards to spatial memory retention, we performed the MWM test. The percentage time spent...
Fig. 2. Recorded levels of Aβ42, Aβ40, and Aβ42/Aβ40 in the brain tissue lysate between chronic intermittent hypoxia (CIH) group and control group. Compared to control, CIH significantly increased the level of Aβ42 (A) and Aβ42/Aβ40 (C), while the level of Aβ40 was unchanged (B).

Fig. 3. Western blot analysis showing the expression levels of amyloid-β protein precursor (AβPP), β-secretase (BACE1), and hypoxia-inducible factor 1 alpha subunit (HIF-1α) of mouse brain tissues. There were no significant differences in the protein expression levels of AβPP and BACE1 between chronic intermittent hypoxia (CIH) group and control group. The levels of actin served as internal controls for loading equal amounts of protein in each lane (A). The bar graph shows the protein expression fold change of AβPP (B) or BACE1 (C), calculated by setting the ratio of AβPP or BACE1 protein/Actin protein band intensities in the control group to 1. HIF-1α protein was not accumulated in the mouse brain tissues in both groups (D). Lysate of hypoxia-exposed Lewis lung cancer (LLC, mouse lung cancer) cells was used as the positive control of HIF-1α expression by western blot analysis.

In vitro study

To determine the direct effect of IH on Aβ generation, we used SH-SY5Y cells stably expressing...
wild-type AβPP in vitro. Compared to the control group, the IH group showed a significant increase of the Aβ42 levels in the medium (89.81 ± 32.2 pg/ml and 135.2 ± 43.1 pg/ml, p = 0.028, Fig. 6A), while the Aβ40 levels remained unchanged (2805.7 ± 872.1 pg/ml and 3084.5 ± 1169.8 pg/ml, p = 0.940, Fig. 6B). Compared to the control group, the Aβ42/Aβ40 ratio in the IH group showed an increased tendency but did not reach statistical significance (0.035 ± 0.016 and 0.047 ± 0.017, p = 0.070, Fig. 6C). The HIF-1α protein level was not upregulated in the IH group, although it was markedly accumulated in the SH group (Fig. 6D).

**DISCUSSION**

In the present in vitro and in vivo study, we made novel observations. Firstly, we demonstrated that the 8 weeks of CIH exposure in the AD mice model significantly increased the level of brain toxic Aβ, Aβ42 and Aβ42/Aβ40 and revealed the accumulation of intraneuronal Aβ compared to control mice without either a worsening of cognitive function or amyloid plaque deposition in the brain. Secondly, the increase in brain Aβ42 was not accompanied by an increase in both the AβPP and BACE1, and in the HIF-1α expression. Thirdly, 8 cycles (4 hours) of IH exposure to human neuroblastoma SH-SY5Y cells stably expressing wild-type AβPP also significantly increased Aβ42 and increased the tendency of Aβ42/Aβ40 in the culture medium without the HIF-1α expression. These support that CIH is a risk factor for AD progression.

Accumulation of intraneuronal Aβ has been observed in both the human brains of AD patients [16, 24] and in transgenic AD mice [16, 17, 25, 26] before amyloid plaque deposition. Furthermore, several studies have implicated intraneuronal Aβ in the toxic processes of AD [27]. In addition, when we consider intraneuronal Aβ accumulation, dominant accumulation of Aβ42 but not Aβ40, has been reported in both AD transgenic mice [16, 26] and in human AD brain [16]. Therefore, in our results, this suggests that the intracellularly accumulated Aβ is Aβ42 rather than Aβ40 in the CIH group.

Although an increase of Aβ42 levels and an accumulation of intraneuronal Aβ were observed in the brain of 3xTg-AD mice in our results, we did not see any cognitive dysfunction or amyloid plaque after 8 weeks of CIH exposure. Oddo et al. [17], using same transgenic mice as us, reported that extracellular Aβ deposits first appeared in 6-month old mice and were readily evident by 12 months preceding plaque and tangles formation. Later, Billings et al. [13] demonstrated, again using same transgenic mice as us, that the
Fig. 6. The protein level of Aβ42, Aβ40, and the ratio of Aβ42/Aβ40 in the medium, and the hypoxia-inducible factor 1 alpha subunit (HIF-1α) protein level in the SH-SY5Y cells in the intermittent hypoxia (IH) group and control group. Compared to controls, IH significantly increased the level of Aβ42 (A), while the level of Aβ40 was unchanged (B). The ratio of Aβ42/Aβ40 showed a tendency to increase in IH group (C). HIF-1α protein in the SH-SY5Y cells was not detected in both the IH and control group, although the HIF-1α protein level was upregulated in the sustained hypoxia (SH) group by western blot analysis. HCC827 (human lung cancer) cells were exposed to SH (1% O2, 24 h) or normoxia, and protein lysate of hypoxia-exposed HCC827 cells were used as the positive controls of HIF-1α expression (D). RA, room air.

earliest cognitive dysfunction impairment manifested at 4 months as a deficit in long-term retention and correlated with intraneuronal Aβ accumulation. Although our 6-month old transgenic mice showed neither extracellular Aβ deposition nor cognitive impairment and we cannot say what the exact reasons for this are, we speculate that one of the possibilities is our sample size. That is, the earliest pathological and cognitive changes were not observed in our small number of 6-month old transgenic mice even after CIH exposure or normoxia. Nevertheless, our results still suggest that 8 weeks of CIH exposure in 6-month old 3xTg-AD mice is substantial enough to cause a significant increase of intraneuronal Aβ.

The novel clinical implication from this study’s results is that CIH could worsen the progression of AD by inducing earlier Aβ42 generation which precedes cognitive decline. Since CIH during sleep is a characteristic of OSA, OSA may contribute to a further worsening of the progression of the disease in AD patients. A possible interesting future study would be to evaluate the longer effects of CIH on amyloid plaque formation and cognitive decline.

In our study, the direct mechanism(s) of how the increased level of brain toxic Aβ42 results in an accumulation of intraneuronal Aβ remains to be investigated. Nevertheless, several points merit discussion. First, although our in vitro study demonstrated that IH directly increased Aβ42, we should still take into account other possible indirect effects, especially sleep fragmentation secondarily induced by CIH during sleep time as well. In mice, the intermittent hypoxic exposure itself during the light period can cause sleep fragmentation and sleep deprivation [28]. Kang et al. showed using AD transgenic mice that Aβ plaque formation depends on brain interstitial fluid levels of Aβ, which is related to sleep cycles [29]. Even though IH directly increased Aβ42 in our in vitro study, it is still
possible that sleep fragmentation may have had some sort of affect in vivo.

Second, when considering the direct effect of IH on the earlier selective generation of $A\beta_{42}$, it may be important to focus on the degradation/clearance issue. Neprilysin, a membrane metallo-endopeptidase, is thought to be one of the rate-limiting steps in $A\beta$ degradation [30]. The inability of synaptic activity to reduce $A\beta_{42}$ in neprilysin knock-out neurons or thiorphan-treated neurons has been reported [31]. The level of neprilysin at the cell surface remarkably reduced with time in neurons of AD-transgenic mice but not wild-type neurons [32]. Thus, although there have been no previous studies investigating the direct effect of CIH on neprilysin, the reduction of neprilysin caused by CIH may explain the results. The lower levels of neprilysin in the presence of oxidative stress [33] may support this idea, given the high association of oxidative stress with CIH [34].

Regarding the over production of $A\beta_{42}$, it is important to look at the role of the sustained hypoxia (SH) effect on $A\beta$ as the comparison of CIH effect. SH markedly induces $A\beta$ production and plaque formation in the central nervous system both in vivo and in vitro [35, 36]. Four weeks of SH (8% O₂ for 16 h/day) in transgenic AD mice increased $A\beta$ generation accompanied by HIF-1α expression and BACE1 facilitation [35]. One mechanism is that binding of HIF-1α to the BACE1 promoter results in $A\beta$ overproduction [37]. Interestingly, SH associated with HIF-1α expression and BACE1 activation resulted in a significant increase of both $A\beta_{42}$ and $A\beta_{40}$ in vivo and in vitro [35]. These results are clearly different from our CIH results. These suggest that CIH and SH have profoundly different effects on transcriptional activation, signaling pathways, and protein expression regarding $A\beta$ alteration. No activation of HIF-1α under intermittent hypoxic exposure has been reported [38], which was consistent with our results. Instead a proinflammatory transcription factor, nuclear factor-kappa B (NF-κB), was selectively activated in their study. They suggested that HIF-1α activation is more sensitive to SH, while NF-κB activation is more sensitive to IH. Tomita et al. reported that in vitro activation of NF-κB leads to secretion of $A\beta_{42}$ but not $A\beta_{40}$ [39]. Therefore, although we did not evaluate NF-κB, it may be involved in our study.

Repetitive episodes of hypoxia-reoxygenation is thought to result in the production of reactive oxygen species by different mechanism from SH [40]. A numbers of studies have produced supportive evidence for the presence of oxidative stress in OSA patients [41–45]; some studies went on to show reversible oxidative stress after treatment of continuous positive airway pressure therapy [41, 42, 44, 45]. It is easily understood that these oxidative stress in OSA is mainly caused by IH. In fact, Yamauchi et al. [34] demonstrated that IH can play a key role in oxidative stress. Oxidative stress is mechanistically and chronologically associated with other key features of AD, namely, metabolic, mitochondrial, metal, and cell-cycle abnormalities [46]. Therefore, it is easy to speculate that the oxidative stress caused by IH in OSA can facilitate $A\beta$ generation in AD, although it is beyond our scope.

The limitation of this study is that our CIH cycle may not fully replicate the apnea condition of OSA patients. Ng et al. [47] applied the oxygen cycle with 21% and 5% every other minute for 8 hours per day for 7 days. The level of total $A\beta$ increased significantly at 3 days and then significantly declined close to the baseline level. The HIF-1α was expressed and transited in a similar way as the level of $A\beta$. It is difficult to compare their results with ours as they did not evaluate the $A\beta$ profile and they applied more frequent IH cycles which might cause the different mean oxygen levels through the IH. Our study differs in that it is the first study to demonstrate the effect of a distinct and longer application of CIH on the $A\beta$ profile. Another limitation is that we could not follow the cognitive dysfunction and amyloid plaque formation for longer durations to evaluate the time needed to induce cognitive decline and amyloid plaque formation. Further study focusing on these points would be our next step.

In summary, this is the first study to demonstrate that CIH directly induces earlier toxic $A\beta_{42}$ generation as well as the accumulation of intraneuronal $A\beta$ in AD transgenic mice. The results of our study show that complications with OSA may be an independent risk factor in the progression of AD. We recommend that detection and treatment of OSA in AD patients be performed as soon as possible in order to help alleviate their AD progression.

ACKNOWLEDGMENTS

We wish to thank Ms. Yukako Hasegawa and Mrs. Etuko Kobayashi for providing excellent technical assistance, Dr. Hiromitsu Yasui and Professor Osamu Inanami for providing us with the hypoxic chamber, and Dr. Mark P. Mattson (National Institute on Aging, Baltimore, MD) and Dr. Frank M. LaFerla (University of California, Irvine, CA) for kindly providing us with the 3xTg-AD mice. SM, KT, NH, and TT have
received the contribution of research funds from Eisai Co., Janssen Co., and Japan Mediphysics Co. All other have no conflicts of interest. This study was supported by a Grant-in-Aid for Scientific Research (C) Grant Number 2159120 and a Grant of Strategic Research Foundation Grant-aided Project for Universities from the Ministry of Education, Culture, Sport, Science, and Technology (NEXT), Japan. Authors’ disclosures available online (http://www.j-alz.com/disclosures/view.php?id=1794).

REFERENCES


