Focus on orexin-A in obese diabetes rats: 
upregulation of orexin-A receptor in the diabetic brain

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ABSTRACT — Diabetes mellitus and brain toxicity are closely linked, and oxidative stress, obesity, insulin resistance, and glucose toxicity can affect the brain. Orexin-A, also known as hypocretin-1, through its activated receptor, participates in many physiological processes. Orexin-A has been associated with feeding behavior, obesity, and pathogenesis of Alzheimer's disease. We have recently established that high-dose thiamine in obese diabetic Otsuka Long–Evans Tokushima Fatty (OLETF) rats leads to reduced obesity and metabolic disorders. Additionally, we found that plasma orexin-A levels in OLETF rats can be modulated by thiamine supplementation under conditions of oxidative stress. Here, we focused on orexin-A in obese diabetic OLETF rats, which at 58 weeks of age and as expected, showed an increase in body weight and blood glucose levels. Plasma orexin-A was measured by ELISA and tended to be higher in obese diabetic OLETF rats than in non-obese diabetic control rats. We evaluated hypocretin receptor 1 (Hcrtr1, also orexin-A receptor) gene expression in the brain of diabetic OLETF rats by reverse transcription (RT)-polymerase chain reaction (PCR) and show that, compared to controls, diabetic OLETF rats exhibited greater orexin-A receptor gene expression in the brain. The results presented here are expected to provide a better understanding of the role of orexin-A and its contribution to brain toxicity in obese diabetic rats.

Key words: Diabetic brain, Orexin-A receptor expression, Plasma orexin-A level, Obese diabetic OLETF rat, RT-PCR products

INTRODUCTION

Diabetes is the most common metabolic disease, and its prevalence is increasing (Ng et al., 2014; WHO, 2016, 2017; Afshin et al., 2017; Kohda, 2018). It is associated with an increased risk of mild cognitive impairment, dementia, and stroke (Pasquier et al., 2006; Roriz-Filho et al., 2009; VanElderen et al., 2010; Bornstein et al., 2014; Gaspar et al., 2016; Bogush et al., 2017). Although the exact mechanisms by which diabetes affects the brain remain unclear, changes to brain vasculature, disturbances in cerebral insulin signaling, and alterations in amyloid metabolism are all thought to be involved (Pasquier et al., 2006; Roriz-Filho et al., 2009; VanElderen et al., 2010; Bornstein et al., 2014; Gaspar et al., 2016; Bogush et al., 2017).

Orexin, also known as hypocretin, is produced in the hypothalamus, and orexinergic neurons project into several brain areas (DeLecea et al., 1998; Sakurai et al., 2009; VanElderen et al., 2010; Bornstein et al., 2014; Gaspar et al., 2016; Bogush et al., 2017).
There are two known isoforms, namely, orexin-A and orexin-B (DeLecea et al., 1998; Sakurai et al., 1998). By activating its receptor, orexin-A participates in many physiological processes, such as sleep/wake regulation, modulation of energy metabolism, feeding, learning, and memory (Sakurai et al., 1998, 2010; DeLecea, 2012). Diabetes and obesity are among the modificable risk factors for Alzheimer’s disease, and research has indicated that, compared to non-diabetics, people with either type 1 or type 2 diabetes may be at greater risk of developing Alzheimer’s disease (Boles et al., 2017; Jiang et al., 2017; Pugazhenthi et al., 2017; Arnold et al., 2018; Tumminia et al., 2018; Ebrahimpour et al., 2020). However, the mechanisms underlying diabetes-related brain toxicity remain elusive.

We have recently established that continuous thiamine consumption by obese diabetic Otsuka Long–Evans Tokushima Fatty (OLETF) rats leads to a reduction in obesity and metabolic disorders (Tanaka et al., 2010; Kohda et al., 2012, 2017; Kohda, 2020). Thiamine plays an important role in the Wernicke-Korsakoff syndrome, which is a form of amnesia caused by brain damage that occurs in long-term alcoholics who predominantly rely on alcohol for nutrition (Day et al., 2013; Latt and Dore, 2014; Chandrakumar et al., 2018). The acute syndrome is normally reversible as its progress can be arrested by a timely injection of high-dose thiamine; however, it may develop into profound dementia (Day et al., 2013; Latt and Dore, 2014; Gibson et al., 2016; Chandrakumar et al., 2018). We have demonstrated that thiamine modifies cerebral oxidative stress and that thiamine supplementation can modulate plasma orexin-A levels in OLETF rats under oxidative stress conditions (Kohda and Matsumura, 2019). Orexin-A has been linked to feeding behavior, obesity, and pathogenesis of Alzheimer’s disease, and it has been suggested that thiamine may have a beneficial effect on cerebral oxidative stress and Alzheimer’s disease (Karuppagounder et al., 2009; Lu’o’ng and Nguyen, 2011; Gibson et al., 2013, 2020; Liu et al., 2017; Wang et al., 2017). Aging is also a risk factor for obesity, diabetes, insulin resistance, glucose toxicity, and oxidative stress, and can lead to brain toxicity, especially in the obese diabetic brain (Salmon, 2016; Boles et al., 2017). Hence, we focused on orexin-A levels in old OLETF rats (58 weeks old) and evaluated hypocretin receptor 1 (Hcrtr1, also known as orexin-A receptor) gene expression in the rat brain using reverse transcription (RT)-polymerase chain reaction (PCR). The results of this study are expected to provide a better understanding of the role of orexin-A and its contribution to brain toxicity in obese diabetic animals.

**MATERIALS AND METHODS**

**Chemicals**

Orexin-A ELISA kit was purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). Glucose pilot meter and blood glucose test strips (Aventir Biotech, Carlsbad, CA, USA) were used for blood glucose testing. RNAlater solution was purchased from Sigma-Aldrich (St Louis, MO, USA). All other chemicals were of the highest purity available from Nacalai Tesque (Kyoto, Japan).

**Animals and experimental design**

Animals were handled as per the institutional guidelines for animal research, and the experimental work was approved by the Experimental Animal Research Committee of the Osaka Medical and Pharmaceutical University. We chose OLETF rats that exhibit progressive obesity and metabolic disorders due to similarities with the human metabolic syndrome. Male OLETF rats (n = 4; Japan SLC, Inc., Shizuoka, Japan) aged 5 weeks, were used initially, along with Long–Evans Tokushima Otsuka (LETO, n = 4) rats, non-obese/lean counterparts of OLETF rats, also aged 5 weeks. The rats were housed in the animal facility in cages, were provided a standard diet, and had access to water ad libitum. They were housed under controlled temperature and humidity conditions with 12-hr light-dark cycle.

Obesity was assessed based on body weight and diabetes was assessed based on glucose levels in blood collected from the tail vein of the rats. Glucose levels were determined using a blood glucose test strip and random blood glucose levels were measured in obese diabetic OLETF rats and non-obese diabetic LETO rats.

Both obese diabetic and normal rats were sacrificed at 58 weeks of age as follows. Animals were anesthetized with 50 mg/kg pentobarbital and blood samples were collected from the ventral aorta. Whole brain tissue was immediately harvested after the rats were exsanguinated and stored at −80°C in RNAlater solution (Sigma) until RNA preparation. Isolated RNA was converted to complementary DNA (cDNA) by RT, and the cDNA subjected to amplification by PCR.

**Plasma orexin-A level measurement**

Blood samples were collected from the ventral aorta and into heparin tubes. Plasma was separated from whole blood by centrifugation at 3000 rpm for 10 min in a refrigerated bench-top centrifuge (Kubota Corp., Tokyo, Japan), and plasma aliquots were stored at −80°C until analysis. Plasma orexin-A content was measured by
enzyme-linked immunosorbent assay (ELISA) and absorbance was read in a microplate reader (FUJIFILM Wako Pure Chemical Corp.). The kit did not cross-react with orexin-B. Plasma orexin-A assays were performed in duplicate. We also evaluated spike and recovery for the ELISA using plasma samples from each group.

RNA preparation and PCR amplification of the hypocretin receptor 1 (Hcrtr1) targeted region

Diabetic brain tissue was stored at −80°C in RNAlater solution (Sigma) until RNA preparation. Total RNA was extracted using NucleoSpinRNA Plus (Takara Bio Inc., Shiga, Japan). RNA purity and concentration were determined by measuring optical density at 260 nm and 280 nm before use. The optical density ratio at 260/280 ranged from 1.8 to 2.1. For reverse transcription, 1 µg of total RNA was reverse transcribed into cDNA using PrimeScript RT Reagent Kit (Takara Bio Inc.).

The PCR reaction used primer sets RA062454-F with the sequence 5′-TGCGGCCAACCCTATCATCTA-3′ and RA062454-R with the sequence 5′-ACC GGCTCTGCAAGGACAA-3′, which encodes hypocretin receptor 1 gene fragment (Hcrtr1; syn. orexin-A receptor), and RA015375-F with the sequence 5′-GGAGATTACTGCCCTGCTGGCTCCTA-3′ and RA015375-R with the sequence 5′-GACTCATCGTACTCCTGCTTGCTG-3′, which encodes β-actin gene fragment (Actb). For PCR, 10 µL of Premix Ex Tag (Takara Bio Inc.) and 0.4 µL of each of the forward and reverse primers (50 µM stock; Takara Bio Inc.) were added per PCR tube (0.2 mL; Thermo Fisher Scientific K.K., Tokyo, Japan). Finally, 2 µL of cDNA template and 7.2 µL of nuclease-free water were added to each tube for a final reaction volume of 20 µL.

PCR was performed using a standard thermocycler (SimpliAmp Thermal Cycler, Thermo Fisher Scientific K.K., Tokyo, Japan) and PCR conditions were as follows: Initial denaturation at 95°C for 1 min, followed by 35 cycles of denaturation (95°C for 30 sec), annealing (60°C for 30 sec) and extension (72°C for 30 sec), and a final extension at 72°C for 4 min. PCR products were removed from the thermocycler and maintained at room temperature for a few minutes before separation on an agarose gel.

Preparation of agarose gels and electrophoresis of RT-PCR products

Ready-to-use precast 4% agarose gels with 12 wells (Funakoshi Co., Ltd., Tokyo, Japan) were used for electrophoresis. The gel tray was placed in a submerge-mini electrophoresis system (ATTO Corp., Tokyo, Japan) and the electrophoresis chamber was filled with 1X Trisborate EDTA buffer until approximately 1 cm above the gel. Subsequently, 10 µL of each RT-PCR product was loaded into each well and electrophoresis was performed for 50 min. The power supply was set to 100 V when 4% agarose gels were run at room temperature. DNA size markers comprised 100 bp–200 bp DNA ladder marker (Takara Bio Inc.). After separation, electrophoresis gels were stained for 30 min with GelGreen nucleic acid gel stain (FUJIFILM Wako Pure Chemical Corp.) and RT-PCR products were visualized and photographed in an Ez-Capture MG machine (ATTO Corp.).

Statistical analyses

Data are expressed as mean ± S.E. Group comparisons used the two-tailed Student’s t-test. All statistical analyses were performed on the Pharmaco Basic software (Scientist Press Co., Ltd., Tokyo, Japan). A p-value < 0.05 indicated statistical significance.

RESULTS

Metabolic parameters in obese diabetic OLETF rats

At 5 weeks of age, both the OLET and LETO rats had similar body weights and blood glucose levels. At 58 weeks of age, body weight and blood glucose levels increased as expected and were higher in obese diabetic OLETF rats than non-obese diabetic normal LETO rats (Fig. 1A and 1B).

Plasma orexin-A levels in obese diabetic OLETF rats

The levels of plasma orexin-A in obese diabetic rats that were overweight (Fig. 1A) and had high blood glucose levels tended to be higher in obese diabetic OLETF rats than non-obese diabetic normal LETO rats (Fig. 1A and 1B).

Orexin-A receptor expression in the obese diabetic OLETF rat brain

Expression of hypocretin receptor 1 (orexin-A receptor) was evident in the brain of obese diabetic OLETF rats (Fig. 3A). The obtained RT-PCR product of 137 bp corresponded to the predicted size of the orexin-A receptor and obese diabetes OLETF rats exhibited greater orexin-A receptor gene expression in the brain compared to non-obese diabetic LETO rat brain (Fig. 3A). The gene expression of β-actin in the brain was shown as an internal standard (Fig. 3B).

DISCUSSION

Here, we have evaluated plasma orexin-A levels in OLETF rats that were old, overweight, and had high...
blood glucose levels, and show that obese diabetic rats exhibited higher plasma orexin-A levels compared to non-obese diabetic LETO rats. Orexin-A was originally identified as a factor that enhanced feeding behavior and available research suggests that orexin can elicit polyphagia and obesity (Sakurai et al., 1998, 2010; DeLecea, 2012). We evaluated OLETF rats developed by Kawano et al. (1991, 1992)—these polyphagia-induced OLETF rats lack functional receptors for cholecystokinin-A, which is associated with satiety control.

Orexin receptors are expressed throughout the brain, and we focused on hypocretin-1 receptor (syn. orexin-A receptor) gene expression. We assessed cerebral orexin-A receptor gene expression by RT-PCR and the PCR product obtained from OLETF and LETO rat whole brain tissue was of expected size. As enhanced orexin-A receptor expression was seen in the brain of obese OLETF rats, our results suggest that orexin-A receptor is susceptible to upregulation in the diabetic OLETF rat brain.

Orexin-A has been suggested to control obesity and be involved in the pathogenesis of Alzheimer's disease (Karuppagounder et al., 2009; Lu'o'ng et al., 2011; Gibson et al., 2013, 2020; Liu et al., 2017; Wang et al., 2017). Moreover, orexin is reported to be involved in the oxidative stress response (Li et al., 2020), and higher plasma orexin-A levels and orexin-A receptor expression in the rat brain might play important roles in oxidative stress-induced injury in OLETF rats. The effect of cerebral oxidative stress and associated upregulation of orexin-A receptor expression in the obese diabetic brain require further investigation.

It has been demonstrated that metabolic diseases like obesity, diabetes, and insulin resistance are associated with alterations in the central nervous system, that they
are linked with the development of cognitive and memory impairment, and that they present higher risk of developing dementia and Alzheimer’s disease (Boles et al., 2017; Jiang et al., 2017; Pugazhenthi et al., 2017; Arnold et al., 2018; Tumminia et al., 2018; Ebrahimpour et al., 2020). In fact, type 3 diabetes is the new label given to Alzheimer’s disease (Kandimalla et al., 2017; Nguyen et al., 2020). Thus, the threat of premature aging represents another reason to effectively control insulin resistance. Further, aging itself is associated with progressive worsening of glucose tolerance and a reduction in the ability of pancreatic β-cells to secrete insulin in response to a glucose challenge (Kulstad et al., 2006; Chiu et al., 2008). Obesity is related to a decline in the activities of daily living in the elderly and is related to a high prevalence of disorders, such as diabetes, hyperlipidaemia, and hypertension (Ogden et al., 2003; DeWinter et al., 2012; Bozkurt et al., 2016). It has been demonstrated that older adults with metabolic syndrome were 20% more likely to suffer from cognitive decline on a memory test than those who did not suffer from the metabolic syndrome (Raffaitin et al., 2011).

The rising prevalence of diabetes, together with its increasing earlier onset, suggests that diabetes-related cognitive dysfunction will increase in the near future. A reduction in insulin secretion or action, dysregulation of glucose homeostasis, impairment of the hypothalamic–pituitary–adrenal axis, and obesity, may act to disrupt neuronal homeostasis and cause diabetes-associated cognitive decline (Stranahan et al., 2008; Gaspar et al., 2016). Multiple studies have shown that orexin-A is involved in the pathogenesis of Alzheimer’s disease and available evidence indicates that a gene linked to the development of type 2 diabetes may also be a factor in the pathogenesis of Alzheimer’s disease (Karuppagounder et al., 2009; Lu'o'ong et al., 2011; Gibson et al., 2013, 2020; Liu et al., 2017; Wang et al., 2017). In the past few years, key strategies involving neuroprotective and antioxidant drugs have emerged as promising therapies for brain disease, which remains preventable or treatable (Kikuchi et al., 2014; Feng et al., 2019; Singh et al., 2019).

We hope that fundamental studies in the OLETF rat, such as focus on orexin-A, will lead to a treatment breakthrough and the development of preventive measures against obesity and brain toxicity in obese diabetics.

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Conflict of interest— The authors declare that there is no conflict of interest.

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Vol. 8 No. 7


Orexin-A receptor expression in the obese diabetic OLETF rat brain


