FULL PAPER *Anatomy*

Chronic type 2 diabetes reduces the integrity of the blood-brain barrier by reducing tight junction proteins in the hippocampus

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ABSTRACT. In the present study, we investigated the effects of type 2 diabetes-induced hyperglycemia on the integrity of the blood–brain barrier and tight junction markers in the rat hippocampus. Forty-week-old diabetic (Zucker diabetic fatty, ZDF) rats and littermate control (Zucker lean control, ZLC) rats were used in this study. We evaluated the integrity of the blood–brain barrier by measuring sodium fluorescein extravasation and blood vessel ultrastructure. In addition, tight junction markers, such as zona occludens-1, occludin and claudin-5, were quantified by western blot analysis. ZDF rats showed significantly increased sodium fluorescein leakage in the hippocampus. Tight junction markers, such as occludin and claudin-5, were significantly decreased in the hippocampi of ZDF rats compared to those of ZLC rats. In addition, ZDF rats showed ultrastructural changes with phagocytic findings in the blood vessels. These results suggest that chronic untreated diabetes impairs the permeability of the hippocampal blood–brain barrier by down-regulating occludin and claudin-5, indicating that chronic untreated diabetes may cause hippocampus-dependent dysfunction.
KEY WORDS: blood-brain barrier, hippocampus, tight junction, type 2 diabetes

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Chronic untreated diabetes mellitus (DM) is associated with complications at the microvascular level and increased incidence of cardiovascular disease [43]. In 2013, the number of people with diabetes in the world is 382 million, with type 2 DM (T2DM) making up about 90% of the cases, and this will be increased to 592 million by 2035 based on the reports from The International Diabetes Federation [17]. Prolonged hyperglycemic conditions, particularly in T2DM, elicit a progressive impairment of neuronal function in the brain [11, 25, 26]. T2DM is considered to be a predisposing factor for vascular dementia [4] and Alzheimer's disease (AD) [12]. The hippocampus is particularly vulnerable to damage from neurological syndromes including AD and other forms of dementia, and impaired hippocampal function has been associated with high-energy diets [13, 21, 28]. In addition, diabetes is associated with reduced hippocampal volume [27, 32].

The brain and spinal cord have distinctive structures including blood–brain barrier (BBB), which separates brain extracellular fluid from the circulating blood. The BBB consists of vascular endothelium lining the cerebral microvessels, with closely apposed astrocytic end-feet processes. Among tight junction proteins, claudin-5, occludin and Zona occludens-1 (ZO-1), play critical roles in maintaining BBB function [38]. Endothelial cells are connected by specific tight junction proteins, such as claudins, occludins and ZOs (ZO-1, ZO-2 and ZO-3) and exhibit specific transport mechanisms and pinocytic vesicles [44]. Diabetes-induced hyperglycemia aggravates BBB permeability, edema formation and neurological symptoms [1, 7, 10, 14, 20, 36]. Claudin-5 plays a key role in the earliest stage of CNS angiogenesis; occludin is the first tight junction protein identified [8]. Claudin and occludin interact with plasma membranes of adjacent cells forming the tight junction barrier [37], and ZO-1 is critical in forming and stabilizing tight junctions by binding occludin to the cytoarchitecture [19, 22].

Most studies have been conducted using a streptozotocin-induced type 1 DM (T1DM) model, not a T2DM model; however, magnetic resonance imaging following intravenous gadolinium administration identified increased BBB permeability in T2DM patients compared to controls [36]. In a previous study, we showed that a Zucker diabetic fatty (*fa/fa*, ZDF) rat model of T2DM demonstrated activation of microglia and subsequent increases in pro-inflammatory cytokines, such as interferon-γ and interleukin-1β (IL-1β),

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in the hippocampus at 30 weeks of age [15].

In the present study, therefore, we investigated the effects of chronic diabetes on the integrity of the BBB and specific tight junction markers including claudin-5, occludin and ZO-1 in the hippocampus based on biochemical and ultrastructural methods using the ZDF rat and littermate Zucker lean control (+/+, ZLC) rats at 40 weeks of age.

MATERIALS AND METHODS

Experimental animals: Male and female heterozygous type (*Lepr*$^{fa/+}$) of Zucker diabetic fatty (ZDF) rats were purchased from Genetic Models (Indianapolis, ME, U.S.A.) and mated to each other. They were housed under standard conditions with adequate temperature (22°C) and humidity (60%) control, a 12-hr light/12-hr dark cycle and free access to food and water. The handling and care of the animals conformed to the guidelines established to comply with current international laws and policies (NIH Guide for the Care and Use of Laboratory Animals, NIH Publication No. 85–23, 1985, revised 1996) and were approved by the Institutional Animal Care and Use Committee (IACUC) of Seoul National University (Approval number: SNU-120312-10). All of the experiments were conducted with an effort to minimize the number of animals used and the suffering caused by the procedures employed in the present study.

Genotyping for the *fa* gene and experimental design: Genotype of *fa* gene herein was determined with the strategy described in our previous study [16]. In order to investigate the effects of diabetes on integrity of BBB and specific tight junction markers, 40-week-old male ZLC and ZDF rats were used in this study. The timing points were chosen, because the body weight was significantly decreased in ZDF rats and the microglia as well as pro-inflammatory cytokines were significantly increased at 30 weeks of age [15, 41].

Blood glucose and HbA1c levels analysis: Animals (n=8 in each group) were anesthetized with 2 g/kg urethane (Sigma, St. Louis, MO, U.S.A.), and blood was sampled by cardiac puncture (at 9–11 a.m.) using a 22 G needle for analyzing blood glucose levels. Fasting glucose levels were analyzed using a blood glucose monitor (Ascensia Elite XL Blood Glucose Meter, Bayer, Toronto, ON, Canada). In addition, the HbA1c levels in blood samples were quantified with the use of an HbA1c analyzer (Tosoh, Ayase, Japan) according to the protocol provided by the manufacturer.

Thereafter, animals were perfused transcardially with 0.1 M phosphate-buffered saline (PBS, pH 7.4) to wash out the remained blood, and the brains were removed. The brain was cut to 500 μm thickness using a vibratome (Leica Microsystems, GmbH, Heidelberg, Germany) between 3.00–4.08 mm posterior to the bregma in reference to the rat atlas [30]. Hippocampal tissues (n=5 in each group) were used for western blot analysis, and other tissues (n=3 in each group) were used for transmission electron microscopy (TEM).

Ultrastructural analysis using TEM: Hippocampal tissues were cut into 1-mm pieces and laid on a glass slice. The isolated tissues were immersed in mixture of 4% paraformaldehyde and 1% glutaraldehyde for 2 hr and washed twice with PBS. The tissues were immersed in 1% osmium tetroxide for 2 hr and washed with PBS for 5 min. The specimens were then dehydrated with serial gradient ethanol (10 min each) and then embedded in Lowicryl HM20 (PolySciences, Niles, TL, U.S.A.) at UV lamp for 48 hr. The ultrathin sections (50–60 nm) were cut and picked up on 200 mesh nickel grids. After repeated washing, the grids were double-stained with uranyl acetate and lead citrate. The stained grids were examined with an electron microscope (JEM1010, JEOL, Tokyo, Japan).

Western blot analysis: Hippocampal tissues were homogenized in 50 mM PBS (pH 7.4) containing 0.1 mM ethylene glycol-bis (2-aminoethyl ether)*N,N,N',N'*-tetraacetic acid (EGTA) (pH 8.0), 0.2% nonidet P-40, 10 mM ethylendiamine tetraacetic acid (EDTA) (pH 8.0), 15 mM sodium pyrophosphate, 100 mM β-glycerophosphate, 50 mM NaF, 150 mM NaCl, 2 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1 mM dithiothreitol (DTT). After centrifugation, the protein level was determined in the supernatants using a Micro BCA protein assay kit with bovine serum albumin as the standard (Pierce Chemical, Rockford, IL, U.S.A.). Aliquots containing 20 μg of total protein were boiled in loading buffer containing 150 mM Tris (pH 6.8), 3 mM DTT, 6% SDS, 0.3% bromophenol blue and 30% glycerol. Then, each aliquot was loaded onto polyacrylamide gel. After electrophoresis, the gels were transferred to nitrocellulose transfer membranes (Pall Crop, East Hills, NY, U.S.A.). To reduce background staining, the membranes were incubated with 5% non-fat dry milk in PBS containing 0.1% Tween 20 for 45 min, followed by incubation with rabbit anti-ZO-1 (1:500, SantaCruz Biotechnology, Santa Cruz, CA, U.S.A.), claudin-5 (1:500, SantaCruz Biotechnology) and occludin (1:500, SantaCruz Biotechnology). Thereafter, the membranes were reacted with peroxidase-conjugated goat anti-rabbit IgG (Sigma) and an enhanced luminol-based chemiluminescent (ECL) kit (Pierce Chemical). The blot was densitometrically scanned for the quantification of relative optical density (ROD) of each band using Scion Image software (Scion Corp., Frederick, MD, U.S.A.). These data were normalized against β-actin (1:1,000, SantaCruz Biotechnology), and ROD was demonstrated as the percentage value of ZLC group.

Measurement of sodium fluorescein (NaF) extravasation: NaF assay was carried out as previously described [9]. Animals (n=4 in each group) received intravenous treatment of 10% NaF (Sigma) via the tail vein. At 30 min after treatment, animals were anesthetized with 2 g/kg urethane and sacrificed to confirm the extravasation of NaF. After sacrificing them and removing the tissues, the hippocampal tissues were cut to 500 μm thickness in a vibratome (Leica), homogenized in 3 m*l* of cold 7.5% trichloroacetic acid (Sigma) and centrifuged at 10,000 × g for 10 min. NaF concentrations in supernatants were analyzed using a fluorescent microplate reader (excitation, 485 nm; emission, 535 nm, Tecan GENios, Männedorf, Switzerland). The results were expressed as μg/mg of brain tissue against standard curves.

Statistical analysis: The data shown here represent the means and standard errors of means (SEM) of experi-

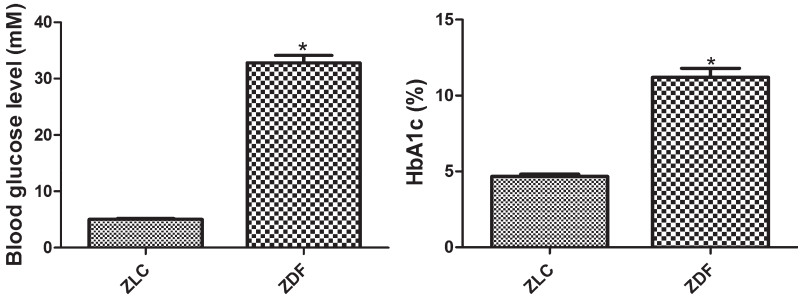

Fig. 1. Blood glucose and HbA1c levels of Zucker lean control (ZLC) and Zucker diabetic fatty (ZDF) rats (n=8 per group; *P<0.05, significant difference between the ZLC and ZDF groups). The data represent mean ± standard error of the mean (SEM).

ments performed for each experimental area. The data were analyzed by using GraphPad Prizm 5.0 software (GraphPad Software, Inc., La Jolla, CA, U.S.A.), and differences among the means were statistically analyzed by Student t-test in order to elucidate differences between ZLC and ZDF groups.

RESULTS

Effects of diabetes on blood glucose and HbA1c levels: In the ZLC rats, blood glucose and HbA1c levels were 5.07 ± 0.29 mM and 4.67 ± 0.42%, respectively. In the ZDF rats, the blood glucose and HbA1c levels were significantly higher than those in the ZLC rats and were 647.7% and 240.0% of the levels in the ZLC group, respectively (Fig. 1).

Effects of diabetes on BBB permeability: In the ZLC rats, the amount of NaF in the hippocampal homogenates was 583.55 ± 74.46 μg/g protein. In the ZDF rats, NaF in the hippocampus was significantly higher than that in the ZLC rats and was 171.6% of the level in the ZLC group (Fig. 2).

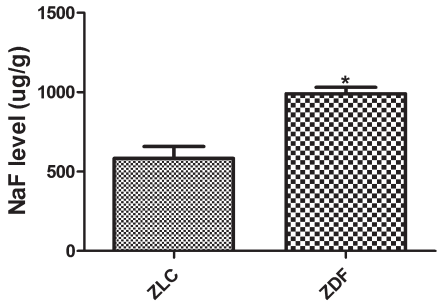

Fig. 2. Diabetes-induced increase in extravasation of sodium fluorescein (NaF) in the hippocampus of Zucker lean control (ZLC) and Zucker diabetic fatty (ZDF) rats (n=4 per group; *P<0.05, significant difference between ZLC and ZDF groups). The data represent mean ± standard error of the mean (SEM).

Effects of diabetes on tight junction proteins: ZO-1 levels in hippocampal homogenates were similar between ZLC and ZDF rats. However, occludin and claudin-5 protein levels in hippocampal homogenates were significantly lower in ZDF rats than in ZLC rats. In the ZDF rats, occludin and claudin-5 protein levels were 37.7% and 63.6%, respectively, of the levels in the ZLC group (Fig. 3).

Effects of diabetes on ultrastructural changes in microvessels: In the ZLC rats, pericyte and astrocyte end feet were intact in microvessels (Fig. 4A). In the ZDF rats, connective tissues were observed in the perivascular area, and some inclusions were also seen in this area. In addition, endothelial cells displayed a plasma membrane profile with protrusions and abundant cytoplasmic transport vesicles (Fig. 4B).

DISCUSSION

Long-term uncontrolled hyperglycemia affects microvascular conditions by lowering perfusion rates, thickening capillary walls and causing abnormal proliferation of endothelial cells with increased vascular permeability [18, 29, 33, 42]. The ZDF rats exhibit hyperglycemia at 7–10 weeks of age [31] and an increase in pro-inflammatory cytokines in the hippocampus at 30 weeks of age [15, 41]. In the present study, we observed the chronic effects of uncontrolled diabetes on hyperglycemia and microvasculature in the hippocampus at 40 weeks of age. The ZDF rats had significantly higher blood glucose (6.5-fold) and HbA1c (2.4-fold) levels than the ZLC rats. This result suggests that ZDF rats at 40 weeks of age exhibit chronic diabetes and potentially increased neurological symptoms. In human studies, the duration of diabetes is associated with impaired cognition in patients with higher HbA1c levels [39], and patients with higher HbA1c levels have increased behavioral and psychological symptoms, such as apathy, overeating and excessive daytime sleeping and impaired activities of daily living [34].

There are conflicting reports about BBB integrity in diabetes resulting in increased barrier permeability [1, 10, 14, 36]. In streptozotocin-induced T1DM rats, albumin extravasation and [^{3}H]inulin did not show any significant changes in the hippocampus 90 days after streptozotocin treatment [14]. However, in streptozotocin-induced T1DM rats, [^{14}C] sucrose significantly increased [10, 14]. In the present study, we investigated BBB integrity by measuring NaF leakage in T2DM rats at 40 weeks of age. NaF concentration in the

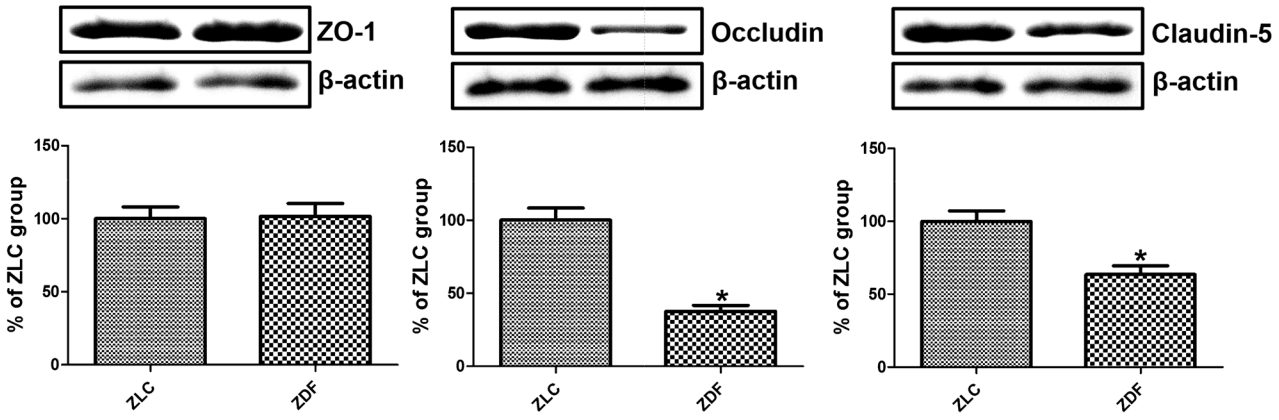

Fig. 3. Western blot analysis of zona occludens 1 (ZO-1), occludin and claudin-5 in hippocampal homogenates of Zucker lean control (ZLC) and Zucker diabetic fatty (ZDF) rats (n=5 per group; *P<0.05, significant difference between ZLC and ZDF groups). The data represent mean ± standard error of the mean (SEM).

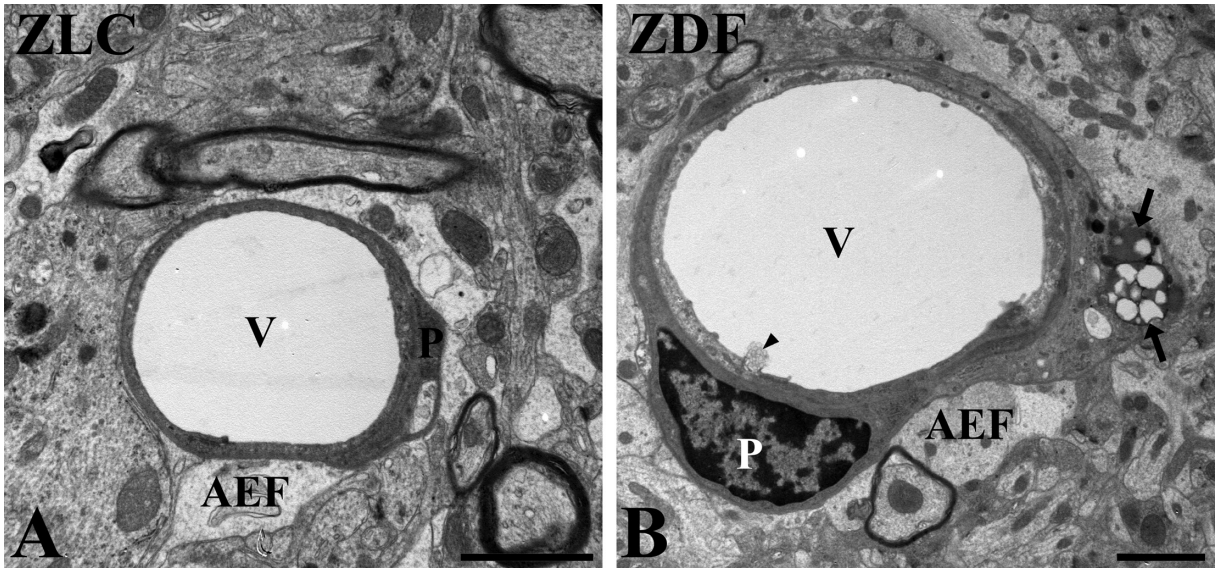

Fig. 4. Electron micrographs of the hippocampus in (A) Zucker lean control (ZLC) and (B) Zucker diabetic fatty (ZDF) rats (n=3 per group). In the ZLC rats, microvessels (V) are intact with astrocytic end feet (AEF) and pericyte (P). In the ZDF rats, vacuolar inclusions are observed (arrows), and abundant cytoplasmic transport vesicles (arrowhead) are observed in the perivascular area. Scale bar=2 μm.

tissue was significantly increased in ZDF rats compared to that in ZLC rats. This result suggests that BBB permeability increased in the hippocampi of T2DM rats. This result was supported by previous studies that reported significantly increased BBB permeability in patients with diabetes over that in controls based on magnetic resonance imaging following intravenous gadolinium administration [36], in the hippocampi of T2DM KKA^{y} mice [24] and in the cerebral cortex of diabetic-hypercholesterolemic pigs [1].

We also observed changes in tight junction proteins in the hippocampi of T2DM rats. The ZDF rats showed a significant reduction in occludin and claudin-5 in the hippocampus, while ZO-1 levels were similar in the ZLC and ZDF rats. These results suggest that BBB leakage may be associated with a reduction in occludin and claudin-5 expression in the BBB. This result was supported by previous studies that showed that the expression of occludin decreased in streptozotocin-induced T1DM rats [3, 10]. Streptozotocin-induced T1DM rats did not exhibit any changes in ZO-1 expression [3]. However, in another study, Hawkins *et al.* observed a reduction in ZO-1 expression after streptozotocin treatment [10]. In the present study, we also investigated the ultrastructure of microvessels in the hippocampus of ZLC and ZDF rats. ZDF rats showed protruding endothelial cells and cytoplasmic transport vesicles. In addition, some inclusions were also detected in the perivascular area. This

result suggests that chronic diabetes significantly induces morphological changes of endothelial cells with perivascular structures which may result in decrease of the BBB integrity in the hippocampus.

In the present study, we did not elucidate the pathogenesis of BBB dysfunction in diabetes. However, several lines of evidence demonstrate that inflammation plays a critical role in the pathogenesis of vascular injury and BBB dysfunction [5, 6, 20, 23]. In a previous study, we demonstrated that chronic diabetes activated microglia and subsequently increased tumor necrosis factor alpha (TNF-α), IL-6 and IL-1β levels in the hippocampus [15, 40, 41]. Diabetes increases TNF-α levels [2], and IL-1β release has been proposed to lead to a decreased concentration or altered localization of the tight junction protein occludin, and thus increased BBB permeability [35]. In addition, TNF-α increases BBB permeability by direct action on the endothelium [5] and indirectly via endothelin-1 production and IL-1β release from astrocytes [6].

In conclusion, chronic uncontrolled diabetes changes the ultrastructural morphology of cytoplasmic transport vesicles as well as BBB permeability by reducing occludin and claudin-5 expression. These results suggest that chronic uncontrolled diabetes affects hippocampus-dependent function and may facilitate neurodegenerative disease, such as AD.

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