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Insulin expression in cultured astrocytes and the decrease by amyloid β

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ABSTRACT

Insulin resistance in brain has been reported in Alzheimer's diseases (AD). Insulin signaling is important for homeostasis in brain function and reported to be disturbed in neurons leading to tau phosphorylation and neurofibrillary tangles. Many investigations of insulin in neurons have been reported; however, it has not been reported whether astrocytes also produce insulin. In the present study, we assessed the expression of insulin in astrocytes cultured from rat embryonic brain and the effects of amyloid $\beta_{1\text{-}42}$ (Aβ) and lipopolysaccharide (LPS) on the expression. We found that astrocytes expressed preproinsulin mRNAs and insulin protein, and that Aβ or LPS decreased these expressions. Antioxidants, glutathione and *N*-acetylcysteine, restored the decreases in insulin mRNA expression by Aβ and by LPS. Insulin protein was detected in astrocyte conditioned medium. These results suggest that astrocytes express and secrete insulin. Oxidative stress might be involved in the decreased insulin expression by Aβ or LPS. The insulin decrease by Aβ in astrocytes could be a novel disturbing mechanism for brain insulin signaling in AD.

1. Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disease characterized by the progressive decline of memory, cognitive functions, and changes in behavior and personality (Kandimalla et al., 2016). AD is also characterized by deposition of amyloid β (Aβ) plaques and neurofibrillary tangles in the brain (Sevigny et al., 2016). Recently, there are many studies to show an essential link between diabetes mellitus and AD such as common features of insulin resistance (Liu et al., 2011; Pugazhenthi et al., 2016). In addition, innate immunity plays an important role in the occurrence and development of diabetes and AD, which increases the risk of developing type 2 diabetes and AD (Huang et al., 2017).

Insulin and its receptor are widely distributed in the brain (Havrankova et al., 1978a, 1978b). It has been reported that insulin exists also in cerebrospinal fluid, and that insulin elongates neuronal axon, potentiates protein synthesis in neurons, and increases synapse formation (Dickson, 2003; Kremerskothen et al., 2002; Laron, 2009; Song et al., 2003). Moreover, hypothalamic insulin inhibits feeding behavior (Obici et al., 2002; Vogt et al., 2014) and hippocampal insulin modulates memory and learning (McNay et al., 2010). These many reports suggest that insulin in brain is essential to maintain normal brain functions (Unger et al., 1991; Zhao et al., 1999).

It was reported that brain insulin was partly derived from pancreatic β cells to be permeated through blood-brain barrier (Banks, 2004) but brain homogenates contained high concentrations of insulin independently of peripheral insulin levels (Havrankova et al., 1979). Among brain cells in rat, neurons have been reported to have mRNA expressions of preproinsulin 1 (ins1),

Abbreviations: Aβ, amyloid-beta; ACM, astrocyte conditioned medium; AD, Alzheimer's disease; BCA, bicinchoninic acid; BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle medium; DMSO, dimethyl sulfoxide; FBS, fetal bovine serum; GSH, glutathione; GSK-3β, glycogen synthase kinase 3β; H_2DCF-DA, 2′,7′-dichlorodihydrofluorescein diacetate; HRP, horseradish peroxidase; IDE, insulin degrading enzyme; LDH, lactate dehydrogenase; LPS, lipopolysaccharide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NAC, *N*-acetyl-L-cysteine; ROS, reactive oxygen species; ThT, thioflavine T; TLR, toll-like receptor; TNF, tumor necrosis factor.

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which is an insulin precursor specific for rodents, and preproinsulin 2 (ins2), which is another insulin precursor comparable to human insulin, and also to express insulin protein (Nemoto et al., 2013; Schechter et al., 1996). On the other hand, it was suggested that insulin release from glial cells was much lower than that from neuronal cells (Clarke et al., 1986).

Aβ has been reported to decrease insulin receptors and impair insulin signaling in neurons, preventing phosphorylation of Akt and glycogen synthase kinase 3β (GSK-3β), downstream of insulin signaling, and to increase phosphorylation of tau protein causing neurofibrillary tangles (Tokutake et al., 2012; Zhao et al., 2008). Furthermore, it was reported that insulin concentration in cerebrospinal fluid decreased in severe AD patients (Craft et al., 1998) and that Aβ reduced insulin synthesis in cultured neurons (Nemoto et al., 2013). These results suggest that Aβ might decrease insulin levels in brain and impair insulin signaling to cause insulin resistance and AD pathology (Bedse et al., 2015).

Insulin expression in brain has been investigated mainly in neurons as described above; however, insulin synthesis in astrocytes and the effects of Aβ on it have not been reported for details. On the other hand, astrocytic activation is well-reported in AD brain, and lipopolysaccharide (LPS) is a well-known experimental activator of glial cells (Huang et al., 2017; Takano et al., 2011). In the present study, we investigated whether astrocytes expressed and produced insulin protein and Aβ affected astrocytic insulin synthesis. We assessed the expression of insulin both of mRNA and protein levels in cultured rat astrocytes, and the effects of Aβ and LPS on astrocytic insulin expressions.

2. Materials and methods

2.1. Reagents

Deoxyribonuclease I (DNase I), trypsin, polyethyleneimine, fetal bovine serum (FBS), lipopolysaccharide (LPS; from *Salmonella* serovar Enteritidis or *Escherichia coli*), anti-β-actin antibody, anti-Aβ antibody, and 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA) were purchased from Sigma (St. Louis, MO, USA). Dulbecco's modified Eagle medium (DMEM) and horse serum were from Gibco BRL (Grand Island, NY, USA). $Aβ_{1-42}$ was from Peptide Institute, Inc. (Osaka, Japan). FavorPrep RNA Purification Mini kit was from Favorgen (Ping-Tung, Taiwan). Omniscript Reverse Transcription kit and Taq PCR Master Mix kit were from Qiagen (Hilden, Germany). SYBR® Green Realtime PCR Master Mix was from Toyobo (Osaka, Japan). Insulin primer and 18S rRNA primer were obtained from Operon Biotechnologies (Tokyo, Japan). Pierce BCA protein assay kit was from Thermo Fisher Scientific (Kanagawa, Japan). Anti-insulin antibody was from Cell Signaling Technologies (Danvers, MA, USA). Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (H + L) antibody (secondary antibody) was from Bio-Rad Laboratories Inc. (Hercules, CA, USA). Ultra-sensitive mouse/rat insulin ELISA kit was obtained from Morinaga Institute of Biological Science (Kanagawa, Japan). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and lactate dehydrogenase (LDH) activity assay kit were from Wako Pure Chemical Industries (Osaka, Japan).

2.2. Preparation of aggregated amyloid β (Aβ)

2.2.1. Preparation of Aβ stock solution

$Aβ_{1-42}$ was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 1 mM as a stock solution and kept at −80 °C before using. The medium was added to Aβ stock solution at the concentration of 10 μM and incubated for 7 days at 37 °C, then used for "aggregated Aβ" stimulation.

2.2.2. Detection of Aβ aggregation

Stock solution of Aβ was diluted by fresh medium (DMEM containing 10% FBS) at a concentration of 10 μM and incubated at 37 °C for 0, 1, 7 days. Each Aβ-contained medium was added at a volume ratio of 4:1 to sample-buffer [50 mM Tris-HCl buffer (pH 6.8) containing 50% glycerol, 10% sodium dodecyl sulfate and 0.05% bromophenol blue] followed by mixing and boiling at 100 °C for 5 min. All samples were kept at −80 °C before analyzing.

Each sample was loaded on a 20% polyacrylamide gel for electrophoresis at a constant voltage of 120 V for 2 h at room temperature and subsequent blotting to a polyvinylidene fluoride membrane pretreated with 100% methanol. After blocking by 5% skimmed milk dissolved in 20 mM Tris-HCl buffer (pH 7.5) containing 137 mM NaCl and 0.05% Tween-20, the membrane was reacted with antibody against Aβ (1:1000) followed by a reaction with anti-mouse IgG antibody conjugated with HRP (1:10,000). Proteins reactive with antibody were detected using Immobilon™ Western chemiluminescent HRP substrate (Millipore, Billerica, MA., USA). Detection bands were assessed using a lumino-image analyzer (LAS-4000; Fujifilm, Tokyo, Japan). The bands of Aβ oligomer increased by incubation at 37 °C for 7 days (Fig. 1A).

2.2.3. Amyloidogenesis, thioflavine T (ThT) fluorescence assay

To measure amyloidogenesis of Aβ, ThT fluorescence assay was employed according to a previous report (Itakura et al., 2015). Briefly, a 10 μl sample was mixed with a 2 ml ThT solution [10 μM in 50 mM glycine-NaOH buffer (pH 8.0)], and fluorescence intensity was measured at wavelengths of 450 nm (excitation) and 482 nm (emission) using RF-1500 fluorescence spectrophotometer (Shimadzu, Kyoto, Japan). ThT fluorescence was not changed for 1 day incubation compared to 0 day, but significantly increased by incubation for 7 days (Fig. 1B). Henceforth (Fig. 2), "aggregated Aβ" was used for stimulation.

2.3. Preparation of astrocyte cultures

The present study was carried out in compliance with the Guide for Animal Experimentation at Osaka Prefecture University. Cultures of rat astrocyte were prepared as described previously (Takano et al., 2011). Briefly, brain cortices from 20-day-old embryos of Wistar rats were cleared of meninges, and treated with

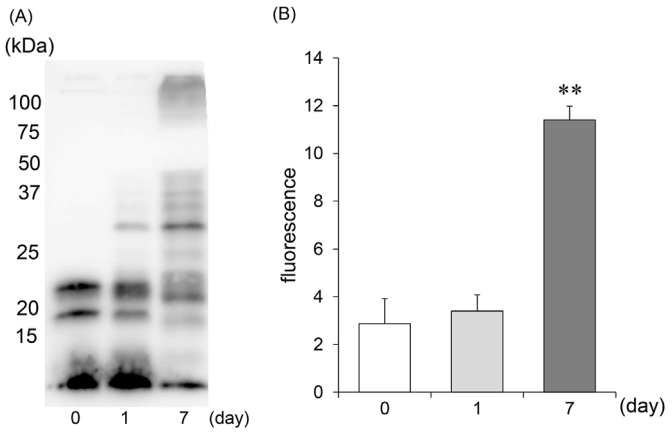

Fig. 1. Preparation of aggregated amyloid β (Aβ) and the detection of amyloidogenesis. (A) Stock solution of 1 mM amyloid $β_{1-42}$ in DMSO was diluted with DMEM containing 10% FBS as 10 μM, and incubated with 37 °C for 0, 1, 7 days. The Aβ aggregation was detected by western blotting as described in "Materials and methods". Representative photograph is shown. (B) The amyloidogenesis of Aβ was detected by ThT fluorescence assay. Data are mean ± SD of three samples from independent experiments of different cell preparations. $^{**}p < 0.01$, significantly different from 0 day.

0.25% trypsin in Ca^{2+}, Mg^{2+}-free phosphate-buffered saline containing 5.5 mM glucose for 15 min at 37 °C with gentle shaking. An equal volume of horse serum supplemented with 0.1 mg/ml of DNase I was added to the medium to inactivate the trypsin, and the tissues were centrifuged at 350 × *g* for 5 min. The tissue sediments were triturated through a yellow-tip-mounted pipette with DMEM containing 10% FBS, 100 mg/l streptomycin, and 5 × 10^4 unit/l penicillin. The cells were plated on polyethyleneimine-coated plastic dishes (100 mm diameter, Iwaki), and cultured at 37 °C in humidified atmosphere of 5% CO_2 and 95% air. The medium was changed every 3 days. After 1 week, astrocytes were replated to remove neurons. On day 14, they were replated onto appropriate plates for each experiment at a density of 1.1–1.9 × 10^5 cells/cm^2. They were stabilized for 1 day in DMEM containing 10% FBS before the experiments.

In our culture, neurons were removed by replating as described above. More than 90% of the cells were immunoreactively positive to an astrocyte marker, glial fibrillary acidic protein; and less than 10% of the cells were positive to a microglial marker, Iba-1 (Takano et al., 2011).

2.4. Reverse transcription-polymerase chain reaction (RT-PCR)

For RT-PCR assays, astrocytes were replated onto 12-well plates (Iwaki) and stimulated with 100 nM aggregated Aβ or 1 μg/ml LPS for 24 h. Total RNA from astrocytes was isolated using FavorPrep RNA Purification Mini kit. Complementary DNA was prepared using Omniscript Reverse Transcription kit according to the manufacturer's protocol. Qualitative PCR experiment was performed using Taq PCR Master Mix kit and quantitative PCR experiments were done using SYBR® Green Realtime PCR Master Mix. In the case of Taq PCR Master Mix kit, the PCR was carried out using the following cycling protocol: 30 cycles of 95 °C denaturation (45 s), 60 °C annealing (45 s), and 72 °C extension (60 s). All PCR products were resolved in agarose gels (1.8%), and stained with ethidium bromide. In the case of SYBR® Green Realtime PCR Master Mix, the PCR was carried out using the following cycling protocol: a 95 °C denaturation step for 1 min followed by 40 cycles of 95 °C denaturation (15 s), 60 °C annealing and extension (60 s). Detection of the fluorescent product was carried out at the end of the each 60 °C extension period. To compare mRNA levels among samples, mRNA for each gene of interest was normalized to the expression of a housekeeping gene, 18S rRNA, using the comparative Ct method. Insulin was known to have two preproinsulin mRNAs, ins1 is rodent specific insulin and ins2 is comparable to human insulin (Schechter et al., 1992, 1996).

The sequences of the primers used in this study were as follows:

ins1; forward, 5′-CCA GTT GGT AGA GGG AGC AG-3′
reverse, 5′-GAC CCG CAA GTG CCA CAA CT -3′
ins2; forward, 5′-CAG CAC CTT TGT GGT TCT CA -3′
reverse, 5′-CAC TTG TGG GTC CTC CAC TT-3′;
18S rRNA; forward, 5′-GTA ACC CGT TGA ACC CCA -3′
reverse, 5′-CCA TCC AAT CGG TAG TAG CG-3′

2.5. Western blotting

Cells were homogenized in 20 mM Tris-HCl (pH 7.5) buffer containing 1 mM EDTA, 1 mM EGTA, 10 mM NaF, 10 mM sodium pyrophosphate, 10 mM β-glycerophosphoric acid, 1 mM sodium orthovanadate, and protease inhibitor cocktail (Sigma P8340). Each homogenate was added at a volume ratio of 4:1 to sample-buffer (for detection of insulin) or sample-buffer containing 25% 2-mercaptoethanol (for detection of β-actin), followed by mixing and boiling at 100 °C for 5 min. Each aliquot in a certain amount of protein was loaded onto a 20% polyacrylamide gel for electrophoresis at a constant voltage of 120 V for 2.5 h and subsequent blotting to a polyvinylidene fluoride membrane pretreated with 100% methanol. After blocking with 1% bovine serum albumin (BSA) dissolved in 20 mM Tris-HCl buffer (pH 7.5) containing 137 mM NaCl and 0.05% Tween-20, the membrane was reacted with antibodies against insulin (1:1000) or β-actin (1:100,000) followed by a reaction with anti-mouse IgG antibody conjugated with HRP (1:10,000). Proteins reactive with those antibodies were detected using Immobilon™ Western chemiluminescent HRP substrate (Millipore). Detection bands were assessed using a lumino-image analyzer (LAS-4000). The ratios of insulin/β-actin of detection bands were evaluated.

Protein concentrations were determined by the method using BCA protein assay kit (Thermo Fisher Scientific); according to the manufacturer's protocol, with BSA as the standard.

2.6. ELISA for detection of released insulin in the medium

To measure the amounts of insulin in the medium, astrocytes were replated onto 60 mm dish (Nunc) and stimulated with 100 nM Aβ or 1 μg/ml LPS in DMEM without FBS for 48 h. After the stimulation, culture medium was collected and centrifuged at 500 × *g* for 5 min. The supernatant was collected as astrocyte conditioned medium (ACM) and assayed using a commercial ELISA kit. All ELISA procedures were performed according to the manufacturer's protocol, and absorbance was measured at 450 nm using a microplate MultiReader (Wallac 1420 ARVO^{MX}; Perkin-Elmer, Life Science, Tokyo, Japan).

2.7. Mitochondrial activity

We measured total mitochondrial activity with the so-called MTT assay. Cultured astrocytes (6.0 × 10^4 cells/well) were replated onto 96-well plates (Sumilon) and stimulated with various concentrations of aggregated Aβ for 24 h. After the cells were stimulated, the medium was changed to a fresh medium and one-tenth volume of 5 mg/ml MTT solution was added. The cells were incubated for 1 h at 37 °C, and the formazan generated by total mitochondrial activity was dissolved in DMSO; then, the color development was measured at 585 nm with a microplate MultiReader.

2.8. Lactate dehydrogenase (LDH) assay

To evaluate whether Aβ caused cell death in cultured astrocytes, LDH activity in the medium was measured. After the cells had been stimulated, centrifuged at 350 × *g* for 3 min. The supernatant was collected and assayed using a commercial LDH assay kit. LDH assay procedures were performed according to the manufacturer's protocol, and absorbance was measured at 560 nm using a microplate reader (Versa Max, Molecular Devices, Sunnyvale, CA, USA). The absorbance in the medium from cell culture without Aβ was as control and the absorbance in the medium from cells stimulated with 0.2% Tween-20 for 24 h was as positive control. Cell death was assessed by calculation of the absorbance as percentage of (each sample − control)/(positive control − control).

2.9. Measurement of reactive oxygen species (ROS)

Intracellular ROS levels were evaluated using a cell-permeable fluorescent dye, H_2DCF-DA. After stimulation with 100 nM Aβ or 1 μg/ml LPS for 6 h, the cells were incubated with 10 μM H_2DCF-DA in serum-free DMEM for 30 min at 37 °C. The medium was changed

to Hepes-buffered saline containing 2 mM $CaCl_2$ and 2 mM $MgCl_2$, and then the level of intracellular production of ROS was evaluated with fluorescent intensity of dichlorofluorescein (DCF) by measurement with a microplate MultiReader (Excitation = 485 nm, Emission = 535 nm).

2.10. Data analysis

For statistical analysis of the data, one-way ANOVA followed by Tukey-Kramer test or Student's *t*-test was used.

3. Results

3.1. Detection of insulin mRNA in cultured astrocytes and the decreases by Aβ and LPS

To assess whether insulin mRNA was expressed in astrocyte and changed with Aβ or LPS stimulation, we detected and quantified insulin mRNA using RT-PCR techniques. Under the non-stimulated (control) conditions, the PCR products, both of ins1 and ins2, were detected in qualitative PCR as bands (data not shown). In quantitative real-time PCR, stimulation with 100 nM Aβ or with 1 μg/ml LPS for 24 h decreased significantly both of ins1 and ins2 mRNAs compared with control (Fig. 2).

The expression level of ins2 mRNA normalized to that of 18S rRNA was much higher than that of ins1, more than 10-times based on the law data of ΔCt (data not shown).

3.2. Detection of insulin protein in cultured astrocytes and the effects of Aβ and LPS

To assess whether insulin protein was expressed in astrocyte and changed with Aβ or LPS stimulation, we detected and quantified insulin protein by western blotting. Cultured astrocytes without stimulation expressed insulin protein. Aβ (100 nM) stimulation for 48 h significantly decreased insulin protein. On the other hand, 1 μg/ml LPS stimulation tended to decrease insulin protein, but not significantly (Fig. 3A and B).

To measure insulin release from astrocytes to medium, cultured astrocytes were incubated for 48 h with Aβ or LPS in serum-free DMEM and the medium was assessed using insulin ELISA kit. Under the non-stimulated conditions, insulin protein could be detected in the medium. Neither Aβ nor LPS stimulation changed the amounts of insulin in the medium (Fig. 3C).

3.3. Effects of Aβ on total mitochondrial activity and cell death in cultured astrocytes

It was reported that Aβ impaired mitochondrial activity and increased intracellular reactive oxygen species (ROS) (Faizi et al., 2016). To assess the effects of Aβ on mitochondrial function in astrocytes, we measured cellular mitochondrial activity by MTT assay. When cultured astrocytes were stimulated with various concentrations of Aβ for 24 h, 10–100 nM Aβ decreased cellular mitochondrial activity (Fig. 4A).

At the same time, the activity of LDH released into medium was assayed to estimate the percentage of cell death. All concentrations of Aβ used in the present study did not significantly affect LDH activity released to the medium (Fig. 4B).

3.4. Effects of Aβ on intracellular reactive oxygen species (ROS) in cultured astrocytes

It was reported in pancreatic β cells that intracellular ROS production decreased insulin synthesis (Chang-Chen et al., 2008). To elucidate the mechanisms that Aβ and LPS decreased insulin expression in cultured astrocytes, we examined intracellular ROS production. After stimulation of cultured astrocytes with 100 nM Aβ or with 1 μg/ml LPS for 6 h, intracellular ROS increased assessed by DCF fluorescence (Fig. 5A). When the cells were stimulated for 24 h, intracellular ROS tended to increase but not significantly (data not shown).

To assess whether ROS production was involved in Aβ- or LPS-decreased insulin expression, effects of antioxidants, *N*-acetyl-L-cysteine (NAC) and glutathione (GSH), on insulin expression were examined. We evaluated the expression level of ins2 mRNA, which was 10-times higher than that of ins1 in our astrocyte culture (see Sec. 3.1), as a major type of mRNA for insulin. Cultured astrocytes were stimulated with 100 nM Aβ (Fig. 5B) or 1 μg/ml LPS (Fig. 5C) with or without 5 mM NAC and GSH for 24 h, and ins2 mRNA was

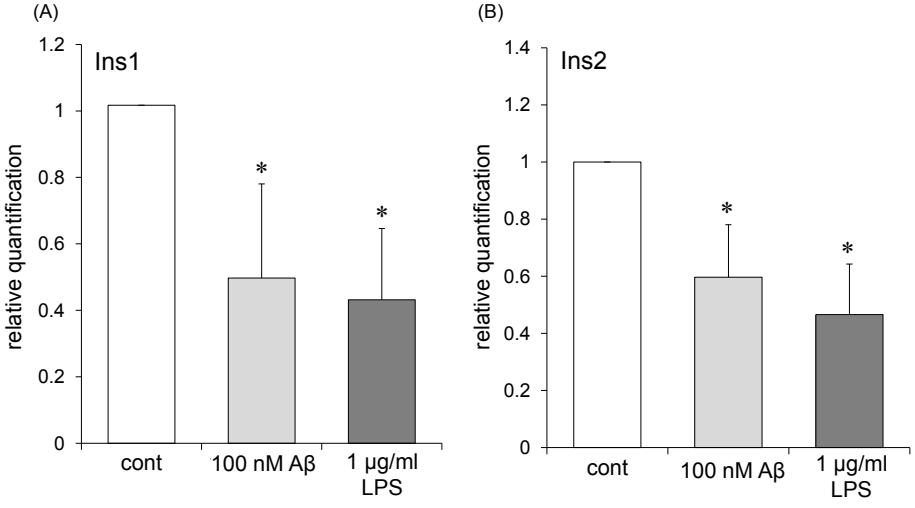

Fig. 2. Effects of Aβ and LPS on preproinsulin mRNA. Astrocytes were stimulated with 100 nM Aβ or 1 μg/ml LPS for 24 h. After the stimulation, preproinsulin mRNAs (ins1; A and ins2; B) were determined by real-time PCR. The expression of ins1 or ins2 was normalized to 18S rRNA as a housekeeping gene and the relative quantification with control as 1 was shown in the graph according to the comparative Ct method in real-time PCR. Data are mean ± SD of three samples from independent experiments of different cell preparations. *$p < 0.05$, significantly different from control (cont).

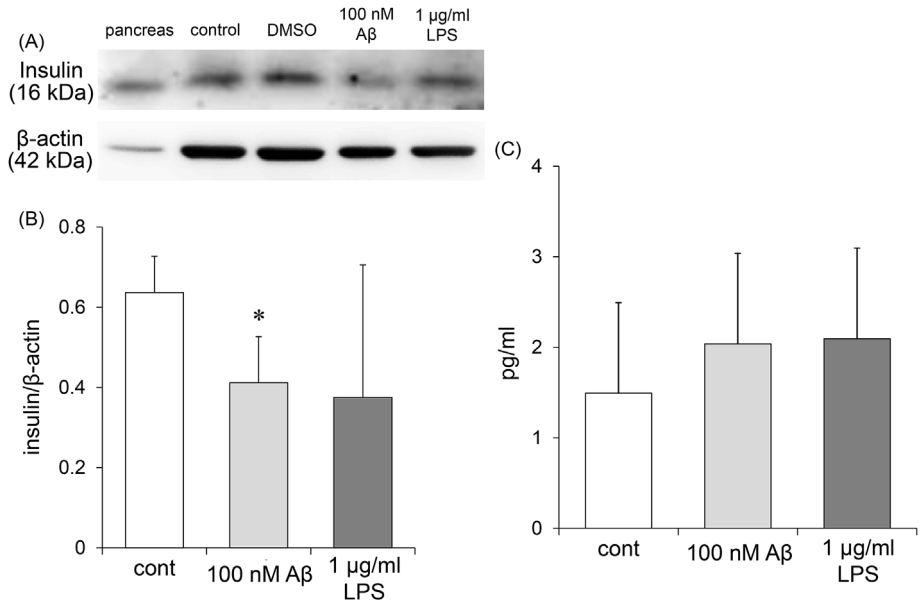

Fig. 3. Effects of Aβ and LPS on insulin protein. (A, B) Astrocytes were stimulated with 100 nM Aβ or 1 μg/ml LPS for 48 h. After the stimulation, insulin protein was detected by western blotting. Typical bands are shown in A. Pancreas tissue homogenate was used as a positive control marker for the detection of insulin protein in western blotting. DMSO is vehicle for Aβ. The intensity of detective bands for insulin protein was quantified, and standardized with that of β-actin. The quantified result is shown in B. (C) Astrocytes were stimulated with 100 nM Aβ or 1 μg/ml LPS for 48 h in serum-free DMEM. After the stimulation, the amount of insulin protein in the medium was measured by ELISA procedures. Data are mean ± SD of three samples from independent experiments of different cell preparations. $^{*}p < 0.05$, significantly different from control (cont).

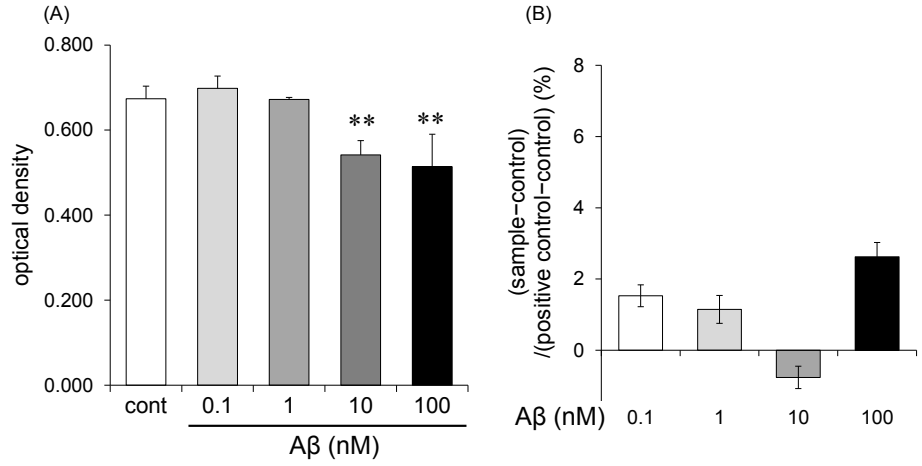

Fig. 4. Effects of Aβ on cellular mitochondrial activity and cell death. Astrocytes were stimulated with various concentrations of Aβ for 24 h. (A) The cellular mitochondrial activity was assessed using MTT assay. (B) Cell death was assessed by LDH assay of the medium. The result was calculated as percentage of (each sample − control)/(positive control − control). Data are mean ± SD of three samples of independent experiments. $^{**}p < 0.01$, significantly different from control (cont).

evaluated by real-time PCR. NAC and GSH prevented Aβ- and LPS-decreased ins2 mRNA (Fig. 5B and C).

4. Discussion

4.1. Insulin expression and effects of Aβ- and LPS-stimulations in cultured astrocytes

It has been reported that insulin signaling is important in normal neuronal functions and that neurons produce and release insulin by exocytotic mechanism and use it by themselves (Nemoto et al., 2013). On the other hand, insulin expression and synthesis in glial cells is not well-investigated, though insulin signaling in astrocytes was reported (García-Cáceres et al., 2016). In the present study, we found in cultured astrocytes that both of preproinsulin mRNAs and insulin protein were expressed and that insulin was released to extracellular medium. Furthermore, the preproinsulin mRNAs were significantly decreased by both Aβ- and LPS-stimulation, and insulin protein was decreased by Aβ-stimulation; LPS tended to decrease insulin protein but not significantly. At present, we could not clearly explain the reason why LPS did not significantly decrease insulin protein; time point of measurement or other factors might be associated.

In neurons, it was reported that Aβ impaired insulin synthesis and its secretion (Nemoto et al., 2013). Therefore, it was suggested that Aβ might impair insulin synthesis in astrocytes as well as neurons to cause reduction of insulin signaling in AD brain. Insulin in the medium of cultured astrocytes was not changed by Aβ- and LPS-stimulation. Astrocytes are known to release insulin degrading enzyme (IDE) and activated astrocytes are known to produce

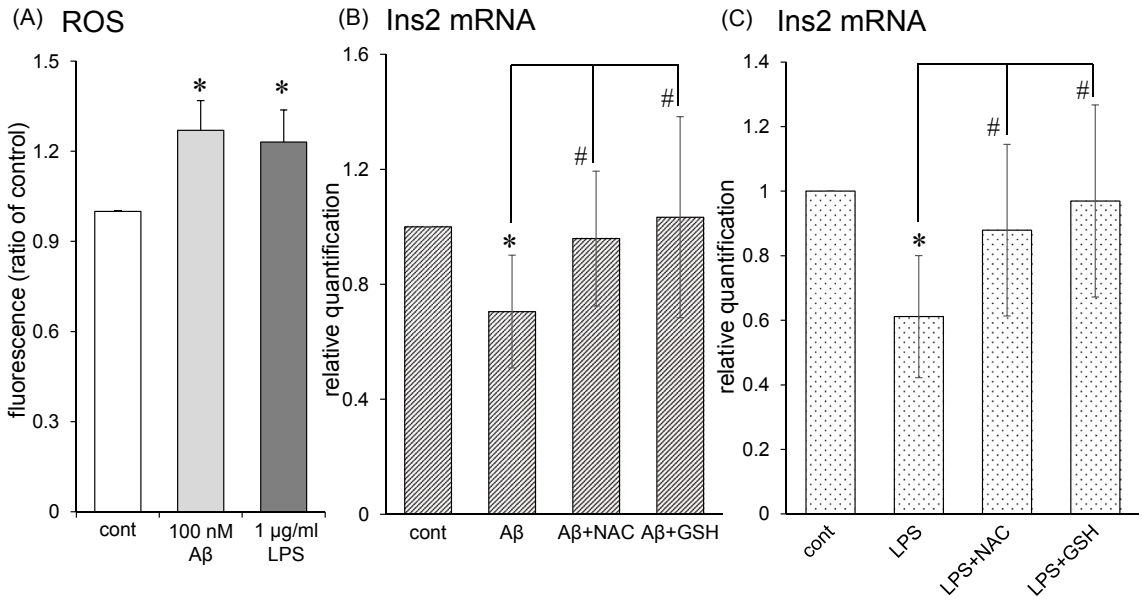

Fig. 5. Effects of Aβ on intracellular ROS production and effects of antioxidants on Aβ-decreased insulin mRNA. (A) Astrocytes were stimulated with 100 nM Aβ or 1 μg/ml LPS for 6 h. After the stimulation, intracellular ROS production was determined by DCF fluorescence. (B, C) Astrocytes were stimulated by 100 nM Aβ (B) or 1 μg/ml LPS (C) with or without antioxidants (5 mM *N*-acetyl-L-cysteine, NAC; and 5 mM glutathione, GSH) for 24 h. After the stimulation, preproinsulin mRNA (ins2) was determined by real-time PCR. Data are mean ± SD of three (A) or eight (B, C) samples from independent experiments of different cell preparations. $^{*}p < 0.05$, significantly different from control (cont). $^{\#}p < 0.05$, significantly different from Aβ or LPS.

proinflammatory cytokines such as tumor necrosis factor (TNF)-α (Dorfman et al., 2010; Sawada et al., 1989). It was reported that TNF-α decreased IDE production (Wei et al., 2014) and that IDE degraded not only insulin but also Aβ (Dorfman et al., 2010); suggesting that insulin protein in ACM might be affected by both changes in insulin expression and in degradation by IDE. Further investigation is needed for the details.

4.2. Mechanisms of Aβ-decreased insulin expression

It was reported that intracellular ROS elevation impaired insulin synthesis in pancreatic β cells (Chang-Chen et al., 2008). Therefore, we assessed ROS involvement in Aβ- and LPS-decreased insulin expression in cultured astrocytes. In the present study, stimulation with Aβ and LPS for 6 h significantly increased intracellular ROS levels, and antioxidants, NAC and GSH, prevented Aβ- and LPS-decreased insulin expression. These results suggest that ROS production might be involved in Aβ- and LPS-decreased insulin expression.

It was reported that Aβ impaired mitochondrial activity in rat brain and increased intracellular ROS (Faizi et al., 2016). In the present study, Aβ also decreased cellular mitochondrial activity in cultured astrocytes. However, LDH release was not significantly changed by Aβ stimulation even in the concentration of which decreased cellular mitochondrial activity. Therefore, Aβ-decreased insulin expression would not be due to cell death.

It has been reported that both Aβ and LPS bind to toll-like receptor (TLR) and activate glial cells (Glass et al., 2010). TLR4 is a main receptor for LPS; and it is expressed in microglia and astrocytes, but very low or undetectable levels in neurons (Glass et al., 2010). LPS stimulation has been used to activate glial cells and it is well-known that LPS induced inflammatory changes in glial cells such as the production of NO and proinflammatory cytokines (Takano et al., 2011). It was reported that TLR4 played important roles in both diabetes and AD, and that its signaling pathway might be a potential link between diabetes and AD (Huang et al., 2017). Aβ was reported to bind various molecules on the cell membrane such as TLR2 and receptor for advanced glycation end-products (RAGE) as well as TLR4, and to activate glial cells similar to LPS (Glass et al., 2010). In the present study, we used LPS as an experimental activator of cultured astrocytes to compare with Aβ, and the results showed that Aβ and LPS similarly changed the expression of insulin. Further investigation is needed to evaluate the differences between them.

4.3. Released insulin from astrocytes

In the present study, we could detect insulin protein by ELISA in ACM, although significant changes by Aβ or LPS were not observed. The insulin levels detected in Fig. 3C (pg/ml order) were rather low. In practice, it was reported in cultured astrocytes that 3 pM (about 18 pg/ml) insulin significantly decreased LPS-induced iNOS expression (Li et al., 2013). Local concentration of insulin around astrocytes in brain tissue might be higher than the concentration in ACM detected in the present study. In addition, cell population of astrocytes in brain is much higher than neurons. We consider that insulin produced by astrocytes might have significant effects in CNS.

5. Conclusion

In the present study, we demonstrated that astrocytes expressed and produced insulin and that Aβ impaired the insulin synthesis in astrocytes. Insulin production from astrocytes and impairment of it might be involved in insulin signaling impairment in brain which is a risk factor for AD.

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