The Journal of Veterinary Medical Science

FULL PAPER

Laboratory Animal Science

Hyperglycemia aggravates decreases of PEA-15 and its two phosphorylated forms in cerebral ischemia

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ABSTRACT. Diabetes is a metabolic health disorder and an important risk factor for stroke. Phosphoprotein enriched in astrocytes 15 (PEA-15) is a multifunctional protein modulating cell proliferation, survival, apoptosis and glucose metabolism. This study investigated whether diabetes modulates the expression of PEA-15 and two phosphorylated forms (Ser 104 and Ser 116) in middle cerebral artery occlusion (MCAO)-induced brain injury. Male Sprague-Dawley rats were administrated with streptozotocin (40 mg/kg) and were underwent right middle cerebral artery occlusion (MCAO) 4 weeks after streptozotocin injection. Brain tissues were collected 24 hr after MCAO and stained using triphenyltetrazolium chloride. Western blot analysis was performed to elucidate the expression of PEA-15 and two phosphorylated forms (Ser 104 and Ser 116) in right cerebral cortex. Infarct volume during MCAO injury was severely increased in diabetic animals compared to non-diabetic animals. We identified the decrease in PEA-15 in animals that underwent MCAO using proteomic approach. PEA-15 expression during MCAO was strongly decreased in diabetic animals compared to non-diabetic animals. Western blots analysis confirmed that diabetes exacerbated the decrease in PEA-15 expression after MCAO. Moreover, decrease in expression of phospho-PEA-15 (Ser 104 and Ser 116) was greater in diabetic than in non-diabetic animals. These results suggested that a diabetic condition may aggravate brain damage through decreasing expression of PEA-15 and phospho-PEA-15 (Ser 104 and Ser 116) in ischemic brain injury.

KEY WORDS: brain ischemia, diabetes, PEA-15

J. Vet. Med. Sci.
79(3): 654–660, 2017
doi: 10.1292/jvms.16-0437

Received: 25 August 2016
Accepted: 17 January 2017
Published online in J-STAGE: 17 February 2017

Stroke is a major cause of death and disability. Diabetes is a metabolic disorder associated with vascular disease that leads to three-fold higher risk of ischemic stroke than non-diabetes [19]. Patients with diabetes are reported to have higher mortality after stroke and higher risk of stroke compared to the non-diabetic population [22, 27, 30]. High blood glucose levels are associated with both degree of disability and infarct size in human and in rodent models of stroke [3, 17, 24]. Diabetes enhances the development of stroke and neuronal destruction by activating cortical apoptotic activity following cerebral ischemia [17, 34, 35]. Diabetes aggravates cerebral stroke and its poor outcomes after focal cerebral ischemia [17, 24]. Neuronal deficit after stroke is more serious in diabetic animals compared to non-diabetic animals [3, 12, 17, 24]. Thus, hyperglycemia is accepted as risk factors that appear to perform critical roles in the severity of stroke. Hyperglycemia aggravates cerebral damage, increases morbidity and mortality, and worse clinical outcomes [4, 20, 34, 35]. Inflammation response is one of the pathological mechanisms in diabetes mellitus. It increases the cerebral infarct volume, aggravates ischemic cerebrovascular disease and results in a higher disability rate in hyperglycemic condition than in normal condition. Thus, diabetes is a major risk factor for ischemic stroke [12]. Neuronal deficit after stroke is more serious in diabetic animals compared to non-diabetic animals [3, 12, 17, 24].

Phosphoprotein enriched in astrocytes 15 (PEA-15) is abundantly expressed in the central nervous system and is originally identified in astrocytes [26]. PEA-15 regulates functions including cell survival, death and signal integration [15, 23]. PEA-15 has an N-terminal death effector domain that interacts with death receptor-activated apoptosis proteins, such as tumor necrosis factor and the Fas-associated death domain [5, 7, 13]. Previous studies demonstrated that PEA-15 inhibits the death-inducing signaling complex formation, inactivates the caspase cascade and has anti-apoptotic functions [5, 7, 13]. PEA-15 regulates the fate of cells by modulating apoptosis and cell proliferation [15, 23]. Phosphorylation of PEA-15 is a critical step in determining the anti-apoptotic effects of PEA-15. PEA-15 has two phosphorylated serine residues, Ser 104 and Ser 116 [2, 6, 29]. Protein kinase B/Akt phosphorylates PEA-15 at Ser 116, and protein kinase C phosphorylates at Ser 104 [2, 6, 29]. We previously reported decreased

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PEA-15 expression in focal cerebral ischemia [14]. In this study, we hypothesized that diabetes regulates the expression of PEA-15 and its phosphorylated forms in ischemic brain injury. Thus, we investigated changes in PEA-15 and its phosphorylated forms in diabetic animals during focal cerebral ischemia.

MATERIALS AND METHODS

Experimental animals

Male Sprague-Dawley rats (200–220 g, *n*=60) were purchased from Samtako Co. (Animal Breeding Center, Osan, Korea) and randomly divided into four groups as follows: normal + sham, diabetic + sham, normal + middle cerebral artery occlusion (MCAO) and diabetic + MCAO (*n*=15 per group). Streptozotocin (40 mg/kg, Sigma, St. Louis, MO, U.S.A.) was dissolved in 0.1 mM citrate buffer (pH 4.2) and intraperitoneally injected to induce diabetes [8, 28]. Normal animals were injected with citrate buffer only as vehicle. Fasting blood glucose levels were examined by Accu-Chek sensor (Roche, Mannheim, Germany). Diabetes was defined as fasting blood glucose >300 mg/d*l* compared to normal fasting blood glucose <100 mg/d*l*. Animals were kept at standardized temperature (25°C) and light (12/12 light/dark cycle). All experimental procedures for animal use were in accordance with guidelines approved by the Institutional Animal Care and Use Committee of Gyeongsang National University.

Surgical process

Focal cerebral ischemia was induced by middle cerebral artery occlusion (MCAO) as previously described [18]. Body weight and blood glucose were measured before MCAO. MCAO was processed at 4 weeks after streptozotocin injection [28]. Rats were anesthetized with Zoletil (50 mg/kg; Virbac, Carros, France) through intramuscular injection. After neck incision, the right common carotid artery, external carotid artery and internal carotid artery were separated from surrounding tissue and carefully exposed. The occipital and suprathyroid arteries were permanently ligated and cut. The external carotid and pterygoid arteries were ligated permanently, and the right common carotid artery was temporarily ligated using microvascular clips. After cutting the external carotid artery, a 4/0 monofilament nylon with a tip rounded by heating was inserted from the external carotid artery into the internal carotid artery to block the origin of the middle cerebral artery. The external carotid artery and inserted nylon were ligated, and the clip on the right common carotid artery was removed. At 24 hr after onset of occlusion, animals were sacrificed, and brains were collected. Sham-operated animals had the same surgical process without insertion of nylon.

Triphenyltetrazolium chloride stain

Brain tissues were sectioned into 2 mm slices by a brain matrix, and brain slices were incubated in 2% triphenyltetrazolium chloride (TTC; Sigma) solution for 20 min at 37°C. The stained brain slices were scanned by Agfar ARCUS 1200™ (Agfar-Gevaert, Mortsel, Bekgium), and the infarct areas were measured using Image-ProPlus 4.0 software (Media Cybernetics, Silver Spring, MD, U.S.A.). The percentage of ischemic lesion area was calculated by the ratio of the infarction area to the whole slice area.

Two-dimensional gel electrophoresis

Proteomic analysis was performed according to a previously described method [25]. Right cerebral cortex tissues were homogenized in lysis buffer (8 M urea, 4% CHAPS, ampholytes and 40 mM Tris-HCl). Supernatant was collected after centrifugation at 16,000 × g for 30 min at 4°C, and proteins were precipitated with 10% trichloroacetic acid. Precipitated proteins were pelleted by centrifugation, washed with 1 M Tris-HCl (pH 7.6) and dried for 1 hr. Lysis buffer was added to dried pellets, and total protein concentrations were measured using Bradford protein assay kits (Bio-Rad, Hercules, CA, U.S.A.) according to the manufacturer's protocol. Immobilized pH gradient (IPG) gel strips (pH 4–7 and pH 6–9, 17 cm, Bio-Rad) were reacted in rehydration solution (8 M urea, 2% CHAPS, 20 mM DTT, 0.5% IPG buffer and bromophenol blue) for 13 hr at room temperature. Protein samples (50 μg) were loaded onto IPG strips using a sample cup, and isoelectric focusing was carried out using an Ettan IPGphor 3 (GE Healthcare, Uppsala, Sweden) at 250 V (15 min), 10,000 V (3 hr) and then 10,000 V-50,000 V. After the first dimension, strips were reacted with equilibration buffer (6 M urea, 30% glycerol, 2% sodium dodecyl sulfate, 50 mM Tris-HCl and bromophenol blue) with 1% DTT for 10 min. Strips were loaded on 7.5–17.5% gradient gels, and second-dimension electrophoresis was performed at 10°C using electrophoresis equipment (Protein-II XI, Bio-Rad) at 5 mA for 2 hr. Loading was continued at 10 mA at 10°C until the bromophenol blue dye reached the bottom of the gel.

Silver staining, image analysis and protein identification

After electrophoresis, gels were incubated in fixation solution (12% acetic acid in 50% methanol) for 2 hr and washed two times with 50% ethanol for 20 min. Gels were immersed with 0.02% sodium thiosulfate for 1 min and rinsed with distilled water for 3 min. Gels were stained with silver stain solution (0.2% silver nitrate and 0.75 m*l*/*l* formaldehyde) for 20 min and washed with distilled water for 1 min. Gels were reacted in developing solution (2% sodium carbonate and 0.5 m*l*/*l* formaldehyde) until protein spots were visible. Stained gels were scanned using an Agfar ARCUS 1200™ (Agfar-Gevaert), and images were analyzed using PDQuest 2-DE analysis software (Bio-Rad) to detect differentially expressed protein spots among experimental groups. Protein spots were cut from stained gels and prepared for matrix associated laser desorption ionization-time of flight mass spectrometry (MALDI-TOF). Gel spots were dehydrated with 50 mM ammonium bicarbonate and acetonitrile. Protein particles were incubated with reduction solution at 56°C for 45 min and alkylation solution (55 mM iodoacetamide in 0.1 M ammonium bicarbonate) for

30 min. Dried gel spots were incubated with digestion solution (12.5 *n*g/m*l* trypsin and 0.1% octyl beta-D glycopyranside in 50 mM ammonium bicarbonate) at 37°C overnight. Proteins were collected by extraction buffer (1% trifluoroacetic acid in 66% acetonitrile) and dehydrated for 2 hr by vacuum centrifuge. Nitrocellulose (20 mg/m*l*) was dissolved into acetone, and isopropanol was added. Nitrocellulose solution and alpha-cyano-4-hydroxycinnamic acid solution were mixed at 1:4. Calibrants (angiotensin and neurotensin) were added to the matrix solution, and dried proteins were dissolved in matrix solution. Samples were loaded on MALDI-TOF plates. Voyager-DETM STR MALDI-TOF mass spectrometer (Applied Biosystem, Foster City, CA, U.S.A.) was used for MALDI-TOF, and peaks were analyzed by the MS-Fit and ProFound programs. SWISS-PROT and NCBI were the protein sequence databases.

Western blot analysis

Proteins were extracted from right cerebral cortices as previously described [10]. Tissues were homogenized in buffer (1% Triton X-100 and 1 mM EDTA in 1× PBS pH 7.4) containing 200 μM phenylmethylsulfonyl fluoride and 10 μM leupeptin. Homogenates were centrifuged at 15,000 × g for 20 min at 4°C. Protein concentrations of supernatants were measured using a bicinchoninic acid kit (Pierce, Rockford, IL, U.S.A.) according to manufacturer's protocols. Protein (30 μg) was mixed with gel-loading buffer, electrophoresed on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred from gel to polyvinylidene fluoride membranes (Millipore, Billerica, MA, U.S.A.). Membranes were blocked with 5% skim milk for 1 hr to block non-specific antibody binding and washed in Tris-buffered saline containing 0.1% Tween-20. Membranes were incubated at 4°C for 12 hr with the following antibodies: anti-PEA-15, anti-phospho-PEA-15 (Ser 104), anti-phospho-PEA-15 (Ser 116) (1:1,000, Cell Signaling Technology, Beverly, MA, U.S.A.) and anti-actin (1:1,000, Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.). Membranes were washed with TBST and reacted with horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse IgG (1:5,000, Cell Signaling Technology) for 2 hr. For detection of immunoreactive protein signals, an enhanced chemiluminescence system (GE Healthcare, Little Chalfont, Buckinghamshire, U.K.) was used according to the manufacturer's protocol, and membranes were exposed to X-ray film (Fujimedical X-ray film, FujiFilm Co., Tokyo, Japan).

Data analysis

All data are expressed as means ± S.E. The intensity analysis was carried out using SigmaGel 1.0 (Jandel Scientific, San Rafael, CA, U.S.A.) and SigmaPlot 4.0 (SPSS Inc., Point Richmond, CA, U.S.A.). The results for each group were compared by two-way analysis of variance (ANOVA) followed by *post-hoc* Scheffe's test. Differences in comparisons were considered significant at $P<0.05$.

RESULTS

We detected an increase in blood glucose and decrease in body weight in streptozotocin-induced diabetic animals. Blood glucose levels were 102.3 ± 3.5 mg/d*l* in normal and 325.7 ± 28.3 mg/d*l* in diabetic animals (Fig. 1A). Body weights were 322.7 ± 26.5 g in normal animals and 189.9 ± 24.5 g in diabetic animals (Fig. 1B). Increased infarct volume during MCAO injury was aggravated in diabetic + MCAO animals compared to normal + MCAO animals (Fig. 1C). Infarct volumes were 24.75 ± 2.65% in normal + MCAO animals and 37.85 ± 3.25% in diabetic + MCAO animals (Fig. 1D).

We identified a decrease in PEA-15 expression in MCAO-operated animals using a proteomic approach. Decreased PEA-15 expression was aggravated in diabetic + MCAO animals compared to normal + MCAO animals (Fig. 2A). However, PEA-15 expression was similar between normal + sham and diabetic + sham animals. PEA-15 levels, given as ratio to the intensity of normal + sham, were 0.56 ± 0.02 in normal + MCAO animals and 0.13 ± 0.03 in diabetic + MCAO animals (Fig. 2B). Western blots also showed that levels of PEA-15 protein decreased in the cerebral cortex of MCAO-operated animals. Diabetic animals had more severe decreases than normal animals during MCAO injury (Fig. 3A). PEA-15 levels were 0.48 ± 0.02 in normal + MCAO animals and 0.23 ± 0.03 in diabetic + MCAO animals (Fig. 3B). MCAO induced decreases in the two PEA-15 phosphorylated forms, phospho-PEA-15 (Ser 104) and phospho-PEA-15 (Ser 116). Diabetes aggravated the decrease of these phosphoproteins in ischemic brain injury (Fig. 4A). Phospho-PEA-15 (Ser 104) levels were 0.57 ± 0.01 in the cerebral cortices of normal + MCAO animals and 0.28 ± 0.03 in diabetic + MCAO animals (Fig. 4B). Phospho-PEA-15 (Ser 116) levels were 0.48 ± 0.03 in the cerebral cortices of normal + MCAO animals and 0.18 ± 0.03 in diabetic + MCAO animals (Fig. 4C). However, expression of phospho-PEA-15 (Ser 104) and phospho-PEA-15 (Ser 116) was similar between normal + sham and diabetic + sham animals.

DISCUSSION

Diabetes exacerbated brain damage and increased infarct volume from focal cerebral ischemia in rats. Diabetes exacerbates neuronal apoptosis and necrosis in the hippocampus and cerebral cortex of cerebral ischemic rats [24]. Neuronal damage from focal cerebral ischemia is more severe under diabetic than normal conditions. Diabetes intensifies brain damage through the activation of apoptotic signaling pathways [17, 24]. Our findings corresponded to results from previous studies showing that the combination of diabetes and cerebral ischemia leads to a worse outcome than cerebral ischemia alone [17, 24]. It is well accepted that streptozotocin is the most common causative agent for inducing hyperglycemia in MCAO rats. Several papers reported the streptozotocin-induced diabetes in an ischemic stroke animal model [1, 11]. Both type 1 and type 2 of diabetes equally worsen the outcome of ischemic stroke. It has been demonstrated that type 1 of diabetes caused by streptozotocin significantly increased

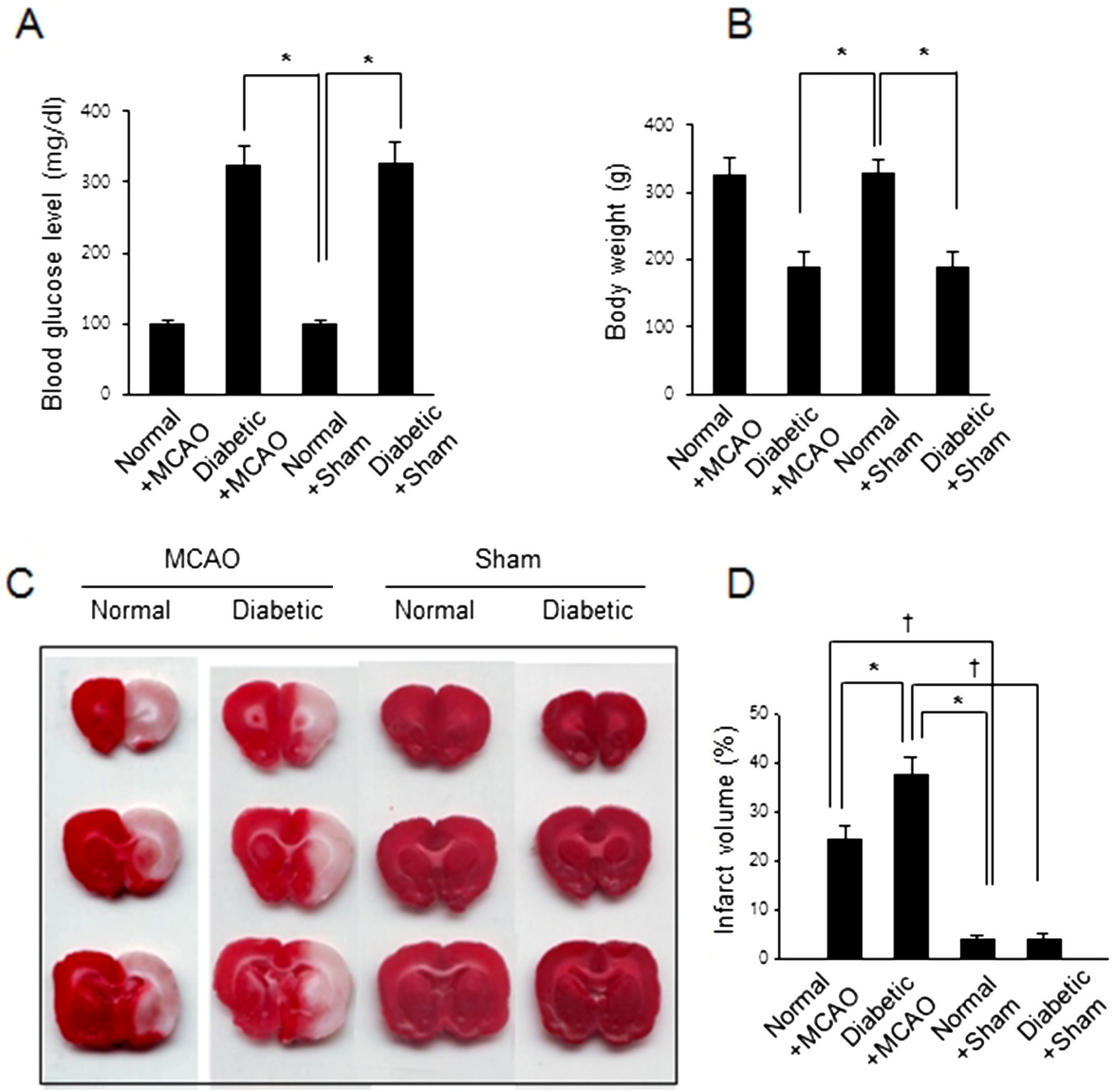

Fig. 1. Blood glucose (A), body weight (B), photographs of TTC staining (C) and infarct volume (D) in normal + middle cerebral artery occlusion (MCAO) animals, diabetic + MCAO animals, normal + sham animals and diabetic + sham animals. The ischemic area appeared white, while the intact area appeared red (C). The percentage of ischemic lesion area was calculated by the ratio of the infarction area to the whole slice area (D). Data (n=5) are shown as mean ± S.E. * P<0.05, † P<0.05.

vascular density, blood-brain barrier leakage and cerebral hemorrhagic transformation after stroke [32, 33]. Similarly, many inflammatory and apoptotic proteins are activated by streptozotocin-induced hyperglycemia in ischemic stroke [21, 33]. Moreover, streptozotocin-induced diabetic state markedly aggravated MCAO induced neurological deficits, infarction and apoptosis in the rat brain.

PEA-15 is involved in apoptosis and cell proliferation [2, 17]. PEA-15 has anti-apoptotic functions through inhibiting formation of death-inducing signaling complexes and preventing activation of the caspase cascade. We identified decreased PEA-15 expression in focal cerebral ischemia using a proteomic approach. PEA-15 decrease was aggravated in diabetic animals with MCAO injury. These results were confirmed by Western blots. PEA-15 is originally detected in astrocytes [6]. The localization of PEA-15 in astrocyte and neuron was clearly elucidated [26]. Although we did not show the cellular localization of PEA-15 between diabetic and non-diabetic animals with MCAO injury, the existence of PEA-15 in astrocyte and neuron is clear. We previously demonstrated a significant reduction in PEA-15 expression in MCAO-induced injury and glutamate-induced cell death [14]. This study further showed the decrease of PEA-15 expression during cerebral ischemic injury was exacerbated by a combination of diabetes.

Phosphorylation of PEA-15 is important for the activation of its anti-apoptotic functions. PEA-15 has two phosphorylation sites in the C-terminus at Ser 104 and Ser 116 [2, 6, 29]. PEA-15 phosphorylated forms are significantly reduced in MCAO-

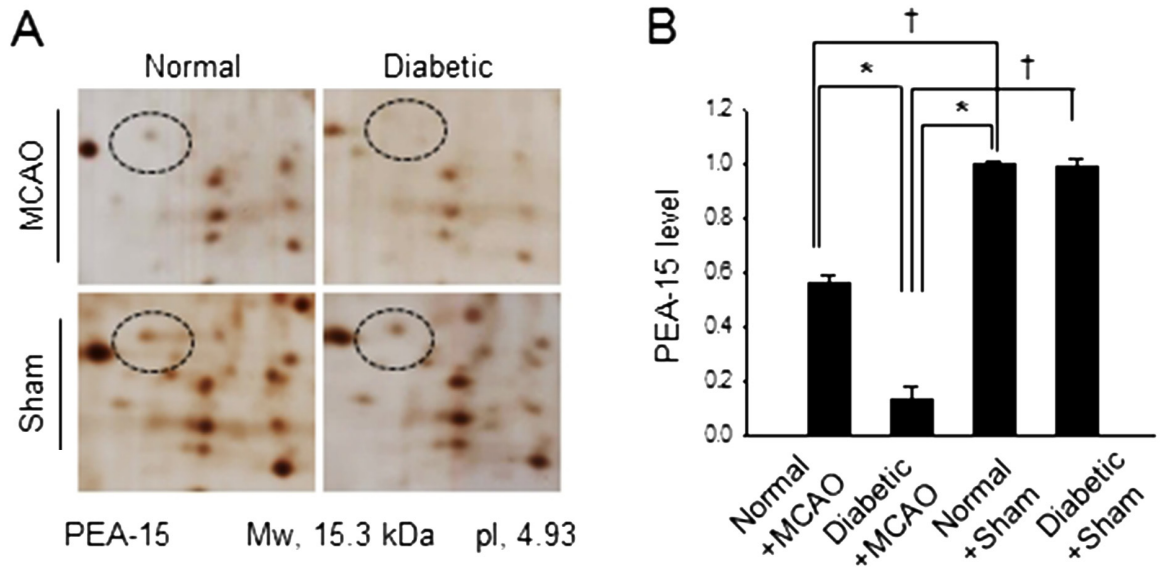

Fig. 2. A proteomic analysis of PEA-15 in the cerebral cortex from normal + middle cerebral artery occlusion (MCAO) animals, diabetic + MCAO animals, normal + sham animals and diabetic + sham animals. Circles indicate the protein spots. Mw and pI indicate molecular weight and isoelectrical point, respectively. Spot intensities were measured by PDQuest software. The spot intensities are reported as a ratio relative to normal + sham animals. Each lane represents an individual experimental animal. Data (n=5) are shown as mean ± S.E. * P<0.05, † P<0.05.

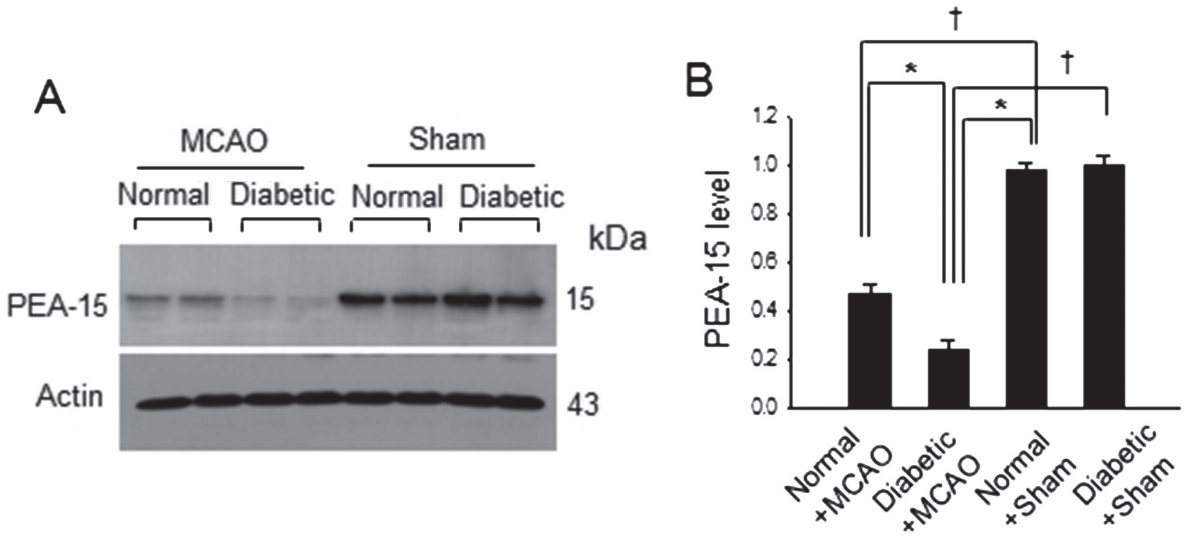

Fig. 3. Western blot analysis of PEA-15 in the cerebral cortex from normal + middle cerebral artery occlusion (MCAO) animals, diabetic + MCAO animals, normal + sham animals and diabetic + sham animals. Densitometric analysis of Western blot is represented as a ratio of PEA-15 protein intensity to actin intensity. Each lane represents an individual experimental animal. Molecular weight markers (kDa) are depicted at right. Data (n=5) are shown as mean ± S.E. * P<0.05, † P<0.05.

induced cerebral ischemia and glutamate-induced neuronal cell damage [14]. Our study clearly showed serious decrease of these phosphorylated forms in cerebral ischemia with diabetes. However, only diabetic condition did not alter the expression of PEA-15 and its phosphorylated forms in the cerebral cortex of rats. This study demonstrated that hyperglycemia aggravated the decrease in PEA-15 phosphorylated forms in ischemic brain injury. The decrease in PEA-15 and its phosphorylation forms is closely related to apoptosis in neuronal cells and is implicated with serious brain damage. Interestingly, we found the significant decrease of phospho-PEA-15 (Ser 116) in cerebral ischemia with diabetes than that of phospho-PEA-15 (Ser 104). The phosphorylation of PEA-15 by Akt regulates the anti-apoptotic function of PEA-15 through controlling its stability [29]. Protein kinase B/Akt and protein kinase C phosphorylate PEA-15 at Ser 116 and Ser 104, respectively [9, 16, 31]. The PI3K/Akt signaling pathway performs an important role in insulin signal transduction [16]. Akt activation regulates various physiological features, such as

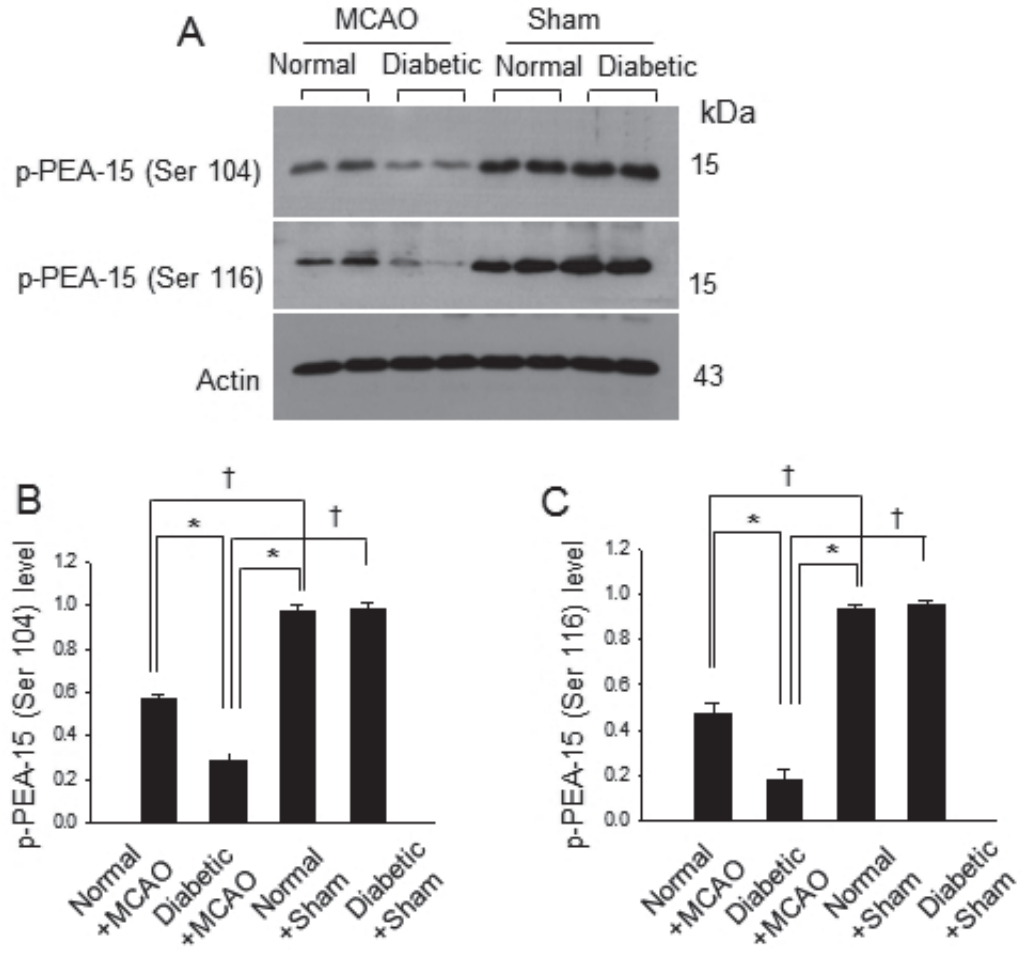

Fig. 4. Western blot analysis of phospho-PEA-15 (Ser 104) (A, C) and phospho-PEA-15 (Ser 116) (B, C) in the cerebral cortex from normal + middle cerebral artery occlusion (MCAO) animals, diabetic + MCAO animals, normal + sham animals and diabetic + sham animals. Each lane represents an individual animal. Densitometric analysis is represented as a ratio of these proteins intensity to actin intensity. Molecular weight markers (kDa) are depicted at right. Data (n=5) are shown as mean ± S.E. * P<0.05, † P<0.05.

cell proliferation, cell growth and cell death. Moreover, phospho-Akt inhibits glycogen synthase kinase 3 function and activates insulin signal transduction [9]. Diabetes leads to down-regulation of phospho-Akt, and anti-diabetic agents regulates hypoglycemic mechanism through the PI3K/Akt signaling pathway [24, 29]. Moreover, we previously demonstrated the ischemic-injury induced the decline of Akt [23]. Inactivation of phospho-Akt induces serious decrease of phospho-PEA-15 (Ser 116) and consequently leads to neuronal cell damage. The mechanisms of diabetes mellitus are complex and remain unclear. Although further investigation is required on the relation between diabetes and PEA-15, this study showed more severe reduction in PEA-15 and its phosphorylated forms in diabetic animals combined with MCAO injury. PEA-15 has an apoptotic effect, and phosphorylation of PEA-15 is crucial for its anti-apoptotic function. Thus, our findings propose that decreased PEA-15 and its phosphorylation forms exacerbate brain damage in cerebral ischemic injury combined with diabetes.

ACKNOWLEDGMENT. This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (NRF- 2015R1D1A1A01058270).

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