The Disturbance of Hippocampal CaMKII/PKA/PKC Phosphorylation in Early Experimental Diabetes Mellitus

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Introduction
Accumulating evidence indicates that the brain is susceptible to damage as a result of diabetes and that the impact of diabetes-related risk factors on brain structure and function is notably important to patients with diabetes [1–3]. Lower scores in global cognitive function have been recognized as a complication of diabetes mellitus in patients [4,5]. However, the precise in vivo intracellular molecular mechanisms underlying diabetes-mediated deficits in learning and memory have not been fully elucidated.

Protein kinases have a wide distribution in the brain and play a major role in learning and the formation of memory [6–8]. Ca2+/calmodulin-dependent protein kinase II (CaMKII) activity is essential for both long-term potentiation (LTP) induction and maintenance [9,10]. In addition, calcium/phospholipid-dependent kinase C (protein kinase C, PKC) [11] and cyclic AMP-dependent kinase A (protein kinase A, PKA) [12] play pivotal roles in hippocampal LTP induction. We recently determined that hippocampal LTP was impaired in olfactory bullectomized mice through N-methyl-D-aspartate (NMDA) receptor hypofunction with a concomitant decrease in CaMKII autophosphorylation and PKC phosphorylation, but without changes in PKA and ERK activity [7,8].

The present study addresses the relationship between diabetes mellitus and changes in cognition with a particular focus on how alterations in serine/threonine kinase signaling may function in the early phase of diabetes mellitus. Our study suggests that the disturbance of CaMKII/PKA/PKC phosphorylation in the hippocampus is an early change that might contribute to the development and progression of diabetes-related cognitive dysfunction.

Material and Methods
Animals
Male Sprague–Dawley (SD) rats weighing 200–230 g were obtained from Zhejiang Medical Animal Centre (Hangzhou,
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China). Rats were housed under climate-controlled conditions with a 12-h light/dark cycle and provided with standard food and water. An acclimation period of at least 1 week was provided before initiating experimental protocols. Type 1 diabetes was induced by a single injection of freshly prepared streptozotocin (STZ, 60 mg/kg; intraperitoneal; Sigma, St. Louis, MO, USA); control rats were injected only with vehicle (citrate buffer). Before diabetes induction, the animals were weighed and fasted for 12 h. The concentration of blood glucose was repeatedly monitored by test strips (Accu-Chek Active, Roche®), Indianapolis, IN, USA) every week after STZ injection. Blood glucose levels were measured. Only subjects with glucose levels >16.67 mmol/L were considered diabetic. Type 2 diabetic rats were fed the high-fat diet and treated as previously described [13]. Briefly, glucose and insulin levels were measured in the same way, and the insulin sensitivity index (ISI) was calculated as ln (FBG × FINS)−1. Diabetes was induced by multiple low-dose streptozotocin injections (30 mg/kg; Sigma) in rats with insulin resistance [13]. All of the animals from these groups were sacrificed by decapitation 4 weeks (type 1 diabetes) or 8 weeks (type 2 diabetes) after the induction of diabetes. The brains of all the animals were quickly removed and frozen. All experimental protocols and animal handling procedures were performed in accordance with the National Institutes of Health (NIH, USA) guidelines for the care and use of laboratory animals and were approved by the Committees for Animal Experiments in Zhejiang University in China.

Western Blotting Analysis

Hippocampal samples were homogenized in buffer containing 50 mM Tris–HCl (pH 7.4), 0.5% Triton X-100, 4 mM EGTA, 10 mM EDTA, 1 mM Na3VO4, 30 mM sodium pyrophosphate, 50 mM NaF, 100 mM calycin A, 50 μg/ml leupeptin, 25 μg/ml pepstatin A, 50 μg/ml trypsin inhibitor, and 1 mmol/L dithiothreitol (DTT). Insoluble material was removed by a 10-min 13,000 g centrifugation. After determining supernatant protein concentration using Bradford’s solution, samples were boiled for 3 min in Laemmli’s sample buffer. Samples containing equivalent amounts of protein were subjected to SDS–polyacrylamide gel electrophoresis (PAGE). Proteins were transferred to an Immobilon PVDF membrane and then blocked with TTBS solution (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, and 0.1% Tween 20) containing 5% w/v nonfat dry milk for 1 h at room temperature, and membranes were incubated overnight at 4°C with anti-phospho-CaMKII (1:2000) and anti-CaMKII (1:1000) [14], anti-phospho-synapsin I (Ser 603) (1:1000; Thermo Scientific), anti-synapsin I [14], anti-phospho-GluR1 (Ser 831), and anti-phospho-synapsin I (Ser 603) (1:500; Millipore, Billerica, MA, USA), anti-phospho-GluR1 (Ser 845) (1:1000; Millipore, Billerica, MA, USA), anti-phospho-GluR1, anti-phospho-NMDAR1, and anti-GluR1 (1:1000; Sigma). Bound antibodies were visualized using the enhanced chemiluminescence detection system (Amersham Life Science, Buckinghamshire, UK) and analyzed semiquantitatively using the National Institutes of Health Image program.

Immunohistochemistry

Rats were anesthetized and transcardially perfusion-fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS). The serial 35-μm slice sections were prepared using a vibratome (Leica VT1000 A). For immunolabeling, slices were probed with anti-phospho-CaMKII (1:500) [14], anti-phospho-synapsin I (Ser 603) (1:500; Thermo Scientific), anti-phospho-NMDAR1 (Ser 896) (1:500; Millipore, Billerica, MA, USA), and anti-β-tubulin (1:500; Sigma) overnight at 4°C. After washing, the sections were incubated with Alexa 488 anti-rabbit IgG or Alexa 594 anti-mouse IgG (1:300; Molecular Probes, Eugene, OR, USA) in TNB buffer. Immunofluorescence was visualized using a Zeiss LSM 510 confocal microscope. The relative fluorescence intensity of phospho-CaMKII, phospho-synapsin I (Ser 603), and phospho-NMDAR1 (Ser 896) in hippocampus was quantified by using the software package MATLAB R2011b (Mathworks, USA). The 3D filled plots were processed by using ImageJ v1.45 with the accompanied “Interactive 3D Surface Plot” plug-in. The statistical and plotting software package Graphpad Prism v5.0 (GraphPad Software, Inc., La Jolla, CA, USA) was used to perform t-test.

Statistical Analysis

The significance of differences between different groups was made using the unpaired Student’s t-test. Differences were considered significant at *P* < 0.05. All data are expressed as the mean ± SEM.

Results

Decreased Phosphorylation of CaMKII in the Hippocampal Region in Type 1 and Type 2 Diabetic Rats

As shown in Figure 1A, the serum glucose levels remain significantly increased 30 days after streptozotocin (60 mg/kg) injection (Figure 1A). In addition, the T2DM rat model was verified by examining the insulin sensitivity index (ISI) (Figure 1B) and serum glucose levels (Figure 1C). In the present study, diabetic rats showed significant decreases in body weight compared with control rats (Figure S1). Cognitive impairment and hippocampal atrophy have been described in patients with type 1 and type 2 diabetes mellitus [4,5]. Multifunctional CaMKII is the most abundant kinase isoform in the brain and is particularly enriched in neurons [15]. Due to its unique properties regarding activation and Ca2+ sensitivity, CaMKII has long been considered a central player in memory functions [8,9]. Furthermore, we examined whether diabetes induced changes in the hippocampal phosphorylation of CaMKII. Constitutively active CaMKII was examined with a specific antibody (α-β-CaMK kinase II) [7,14] that recognizes the autophosphorylation of Thr 286/287. Both α (50-kDa) and β (60-kDa) isoforms of CaMKII are neuron specific and are highly abundant in the rat brain [16]. Our data demonstrated a significant reduction in CaMKII (Thr 286/287) phosphorylation in the hippocampal region of diabetic rats (Figure 1D,E). By contrast, the western blot analysis showed increased total CaMKII protein levels in type 1 (Figure 1D,E) and type 2 (Figure 2A,B) diabetic rats.
Subcellularly, CaMKII is localized to the dendrites and even more specifically to the postsynaptic densities of excitatory synapses [17]. Here, we employed immunohistochemistry to analyze the phospho-CaMKII (Thr 286/287) protein in hippocampal CA1 pyramidal neurons. Whereas the CA1 pyramidal neurons from nondiabetic control rats largely showed strongly labeled phospho-CaMKII (Thr 286/287)-immunoreactive neurons in the somata and dendrites of neurons, the CA1 pyramidal neurons from type 1 (Figure 3A,B) and type 2 (Figure 4A,B) diabetic rats showed weak staining compared with controls. No significant changes in β-tubulin- and DAPI-positive staining were observed in hippocampus between diabetic and control rats.

**Figure 1** Decreased phosphorylation of CaMKII in the hippocampal region in type 1 diabetic rats. (A) Changes in blood glucose 30 days after STZ injection in type 1 diabetic rats. (B) Measurement of insulin sensitivity index in type 2 diabetic rats. (C) Changes in blood glucose 60 days after STZ/high-fat treatment in type 2 diabetic rats. *P < 0.05 versus control animals. ***P < 0.001 versus control animals. Immunoblotting was carried out using antibodies recognizing phospho- or total protein. (D) The changes in CaMKII (Thr 286/287), synapsin I (Ser 603), and GluR1 (Ser 831) phosphorylation in the hippocampal region in type 1 diabetic rats were detected by western blots. (E) Quantitative analysis of relative CaMKII (Thr 286/287), synapsin I (Ser 603), and GluR1 (Ser 831) phosphorylation in type 1 diabetic rats was performed by densitometry. *P < 0.05; **P < 0.01 versus control rat (n = 5). (F) Representative western blots of PKC (Ser 657), N-methyl-D-aspartate 1 (NMDAR1) (Ser 896), and Myristoylated alanine-rich C-kinase substrate (MARCKS) (Ser 152) phosphorylation. (G) Quantitative analysis of relative PKC (Ser 657), NMDAR1 (Ser 896), and MARCKS (Ser 152) phosphorylation in type 1 diabetic rats performed by densitometry. *P < 0.05 versus control rat (n = 5). (H) The expression of phospho-PKA (Thr 197) and phospho-GluR1 (Ser 845) in control and type 1 diabetic rats. (I) The results of (H) were quantitated by densitometry. *P < 0.05 versus control rat (n = 5). β-Actin was used to ensure equal protein loading in each lane.
Increased Phosphorylation of GluR1 (Ser 831) and Synapsin I (Ser 603) in the Hippocampus in Type 1 and Type 2 Diabetic Rats

CaMKII serves as the synaptic tag that marks a synapse for long-term modification. After activation, CaMKII phosphorylates a variety of substrates, such as synapsin I, MAP-2, and several types of glutamate receptors [18,19]. Therefore, we evaluated synapsin I (Ser 603) and GluR1 (Ser 831) phosphorylation by immunoblotting with phospho-specific antibodies. Unexpectedly, in the present study, no decrease in synapsin I (Ser 603) and GluR1 (Ser 831) phosphorylation was observed in the same context in either type 1 or type 2 diabetes (Figures 1D,E and 2A,B). This result suggests that synapsin I (Ser 603) and GluR1 (Ser 831) phosphorylation may play a complementary response in the pathogenesis of diabetes. As shown in Figure 5A,B, immunoreactivity for the phosphorylation of synapsin I (Ser 603) was present in the presynapse of neurons (Figure 5A,B). Coinciding with the immunoblot data, our immunohistochemical staining demonstrated a significantly increased and intense immunolabeling of synapsin I (Ser 603) (Figure 5A) in hippocampal pyramidal neurons in type 1 diabetic rats. No significant changes in β-tubulin- and DAPI-positive staining were observed in the same context.

Effect of Diabetes on the Phosphorylation of PKC Signaling in the Hippocampal Region

As shown previously, the activation of PKC leads to the translocation of conventional isoforms to the plasma membrane and primarily serves to phosphorylate potential substrates [11]. Immunoblot analysis showed that neither the levels of phosphorylated PKC nor those of total PKC were affected in the hippocampus of either type 1 (Figure 3F,G) or type 2 (Figure 2C,D) diabetic rats compared with controls. High-affinity substrates of PKC include Myristoylated alanine-rich C-kinase substrate (MARCKS) (Ser 152) and NMDAR1 (Ser 896), which show regional specificity within the mammalian brain [7]. To evaluate the physiological relevance of PKC activation following diabetes further, we next assessed the phosphorylation of NMDAR1 (Ser 896) and MARCKS (Ser 152) by immunoblotting with phospho-specific antibodies. We found that phosphorylation of NMDAR1 (Ser 896) was significantly increased in both type 1 and type 2 diabetes, whereas PKC (Ser 657) phosphorylation remained unchanged in the hippocampal region (Figures 3F,G and 2C,D). Consistently, the increased immunostaining of phosphorylated NMDAR1 (Ser 896) in the cytoplasm of CA1 pyramidal neurons of T1DM rats was also observed when compared with control rats (Figure 5C,D). In addition, no significant changes were observed in either total MARCKS protein or phospho-MARCKS (Ser 152) in the same context (Figures 1F and 2C).

Changes in the Phosphorylation of PKA in the Hippocampus in Diabetic Rats

Protein kinase A-dependent phosphorylation of the GluR1 (Ser 845) subunit plays an essential role in the regulation of α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptors. PKA catalyzes GluR1 phosphorylation at Ser 845,
Figure 3 Changes in immunostaining of phospho-CaMKII in hippocampus of type 1 diabetic rats. (A) Representative image of hippocampal CA1 pyramidal neurons stained with anti-phospho-CaMKII and β-tubulin. Markedly decreased phospho-CaMKII staining was observed by confocal microscopy in the hippocampus of type 1 diabetic rats. DAPI counterstaining indicates nuclear localization (blue). Scale bar = 50 μm. (B) Visualization of grayscale intensities of phospho-CaMKII through 3D mesh plots (Left). The integrated optical density (IOD) determined by Graphpad Prism v5.0 according to the staining intensity of phospho-CaMKII (Right). ***P < 0.001 versus control (n = 6 slices from three animals for each group). T1DM, type 1 diabetes mellitus.

Figure 4 Changes in immunohistochemical expression of phospho-CaMKII in the hippocampus of type 2 diabetic rats. (A) Immunohistochemical localization of phospho-CaMKII was examined in the hippocampal CA1 region. Markedly decreased phospho-CaMKII staining was observed by confocal microscopy in the hippocampus of type 2 diabetic rat. DAPI counterstaining indicates nuclear localization (blue). Scale bar = 50 μm. (B) The representative 3D filled surface plots (Left) indicate the grayscale intensities of phospho-CaMKII in control and T2DM rats. The integrated optical density (IOD) determined by Graphpad Prism v5.0 according to the staining intensity of phospho-CaMKII (Right). ***P < 0.001 versus control (n = 6 slices from three animals for each group). T2DM, type 2 diabetes mellitus.
thereby increasing AMPA channel conductance [20]. Our study shows that diabetes had no effects on total PKA protein expression (Figure 1H,I). Moreover, in the type 1 diabetic rat hippocampal region, no significant change was observed in the expression of PKA phosphorylation (Thr 197) compared to controls (Figure 1H, I). Conversely, in the type 2 diabetic rats’ hippocampal regions, we found a significant increase in the expression of PKA phosphorylation (Thr 197) (Figure 2E,F). The phosphorylation of GluR1 (Ser 845) was measured in the same tissue extracts used for the determination of phosphorylated proteins in this study. Notably, we found that the phosphorylation of GluR1 (Ser 845) was dramatically increased in the hippocampi of both type 1 (Figure 1H,I; 158.73 ± 19.70% of controls, \( P < 0.05 \)) and type 2 (Figure 2E,F; 266.33 ± 20.73% of controls, \( P < 0.01 \)) diabetic rats. These results suggest a link between the increased phosphorylation of both PKA (Thr 197) and GluR1 (Ser 845) in the hippocampus in the early diabetic state.

**Disturbance of PP1 and PP2A Proteins in the Hippocampal Region in Diabetic Rats**

We were particularly interested in the levels of phosphoprotein phosphatase 1 (PP1) and protein phosphatase 2A (PP2A), which belong to a class of phosphatases known as protein serine/threonine phosphatases [21,22]. Thus, we sought to confirm PP1x and PP2A hippocampal expression in both type 1 and type 2 diabetic rats by immunoblotting. As shown in Figure 6, we found a significant decrease in PP1x (62.82 ± 4.24% of controls, \( P < 0.01 \)) and PP2A (70.84 ± 7.61% of controls, \( P < 0.05 \)) protein levels in the hippocampus of type 1 diabetic rats compared to controls (Figure 6A-B). In addition, a significant increase in serum alanine transaminase (ALT) and blood urea nitrogen (BUN) was observed in high-fat-fed type 2 diabetic rats.

**Figure 5** The STZ treatment induced aberrant immunostaining of phospho-synapsin I (Ser 603) and phospho-NMDAR1 (Ser 896) in type 1 diabetic rats. Immunohistochemical localization of phospho-synapsin I (Ser 603) (A, B) and phospho-NMDAR1 (Ser 896) (C, D) was examined in the hippocampus CA1 region. Markedly increased phospho-synapsin I (Ser 603) (A) and phospho-NMDAR1 (Ser 896) (C) stainings were observed by confocal microscopy in the hippocampus of type 1 diabetic rats. DAPI counterstaining indicates nuclear localization (blue). Scale bar = 50 μm. The 3D filled surface plots (Upper) indicate the grayscale intensities of phospho-synapsin I (Ser 603) (B) and phospho-NMDAR1 (Ser 896) (D). The integrated optical density (IOD) determined by Graphpad Prism v5.0 according to the staining intensity of proteins (Lower). **\( P < 0.001 \); ***\( P < 0.001 \) versus control \((n = 6\) slices from three animals for each group). T1DM, type 1 diabetes mellitus.

**Figure 6** Changes in expression of hippocampal PP1x and PP2A in type 1 diabetic rats. (A) Representative western blots of PP1x and PP2A in the hippocampal region in type 1 diabetic rats. (B) Quantitative analysis of relative PP1x and PP2A in type 1 diabetic rats was performed by densitometry. Immunoblotting with an anti-β-actin antibody shows equal amounts of loaded protein in each lane. Data are expressed as percentage of values of control animals (mean ± SEM, \( n = 5 \)). *\( P < 0.05 \); **\( P < 0.01 \) versus control rat. T1DM, type 1 diabetes mellitus.
Defining the impact of diabetes and related risk factors on brain structure and function is critically important for patients with diabetes [1–3]. However, few studies have been conducted on the effects of diabetes on serine/threonine kinase signaling in the brain in the early phases of diabetes mellitus. We investigated the biochemical alterations in phosphorylation of CaMKII, PKC, and PKA signaling in the hippocampi of type 1 and type 2 diabetic rats. Our study suggests that disturbances of CaMKII/PKA/PKC phosphorylation in the hippocampus are early changes that may contribute to the development and progression of diabetes-related cognitive dysfunction.

Both type 1 and type 2 diabetes mellitus are associated with derangements in the regulation of intracellular Ca²⁺ [23]. Biochemical and biophysical research has revealed that the induction and maintenance of LTP require the activation of protein kinases [6–8]. In this study, we investigated alterations in the phosphorylation of CaMKII in the hippocampi of type 1 and type 2 diabetic rats. Consistent with previous reports [24], we also found that total CaMKII protein levels increased. Furthermore, our data showed an evident and significant reduction in the phosphorylation of CaMKII (Thr 286/287) in the hippocampi of both type 1 and type 2 diabetic rats. Both the AMPA receptor and synaptic I phosphorylation sites are known to be preferentially phosphorylated by CaMKII [25,26]. CaMKII (Thr 286/287) autophosphorylation and GluR1 phosphorylation function as molecular switches underlying LTP and explicit memory [27]. In hippocampal LTP, CaMKII activation is closely associated with stably increased phosphorylation of the GluR1 and synapsin I [9]. Unexpectedly, we observed a significant increase in the phosphorylation of synapsin I (Ser 603) and GluR1 (Ser 831), which are substrates of CaMKII in presynaptic and postsynaptic regions, respectively, of the hippocampus. The reasons for the different effects of diabetes on the protein expressions of CaMKII and its potential substrates in the hippocampus are presently unknown. It is likely that the increase in phosphorylation of synapsin I (Ser 603) and GluR1 (Ser 831) in type 1 diabetic rats was secondary to PP1α and PP2A inactivation, as the PP1α and PP2A protein levels were significantly decreased in the type 1 diabetic rats.

Because impaired LTP in the hippocampus is correlated with reduced PKC activities, these observations led us to evaluate whether changes in the phosphorylation of PKC, which is required for the activation of PKC kinase, are associated with the pathological process of diabetes [7]. In our study, STZ (type 1 diabetes) or STZ/high-fat (type 2 diabetes) treatment did not alter hippocampal PKC phosphorylation until after at least 4 weeks in type 1 diabetic rats and 8 weeks in type 2 diabetic rats. Postsynaptically, PKC may act instructively by directly phosphorylating AMPA receptors in the hippocampus [11]. This effect could be enhanced through the liberation of calmodulin from neurogranin [28]. Unexpectedly, our study demonstrated that NMDAR1 (Ser 896) phosphorylation dramatically increased both STZ (60 mg/kg) and STZ/high-fat-treated animals. Whether a complementary response loop exists between kinases/phosphatases and increased phosphorylation of NMDAR1 (Ser 869) in the hippocampus of diabetic rats remains to be observed. Because experimental animals in this study are in the early phases of diabetes mellitus, we postulate that a significant complementary response arises against diabetes-related risk factors.

Although PKA does not appear to alter the conductance of the AMPAR [29], substantial evidence indicates that the cAMP pathway acts through CaMKII to facilitate LTP [30]. In this study, we suggest that PKA signaling is involved in the hippocampal pathological process in diabetic rats. Enhancement of synaptic transmission during LTP can result from several mechanisms that are regulated by the phosphorylation of GluR1 (Ser 845) [20]. Unexpectedly, a significant increase in GluR1 (Ser 845) phosphorylation was observed in the hippocampi of type 1 and type 2 diabetic rats, suggesting a complementary response of PKA signaling during the early diabetic state.

Cellular serine/threonine phosphatases are involved in the regulation of cell homeostasis through the negative regulation of signaling pathways initiated by protein kinases. Our data highlight a difference in the expression patterns of PP1α and PP2A in the hippocampi of type 1 and type 2 diabetic rats. It appears likely that PP1α and PP2A may be associated with the aberrant increase in synapsin I (Ser 603) and GluR1 (Ser 831) phosphorylation in type 1 diabetic rats because PP1α and PP2A levels are down-regulated in diabetic rats. This finding is consistent with the fact that PP1α, PP2A, and other intracellular factors are also potential upstream kinases for GluR1 and NMDA phosphorylation [22,31]. Future studies using pharmacological and genetic approaches should further confirm the interrelationships between GluR1/synapsin I and PP1α/PP2A. Although we cannot determine the precise role of increased PP1α and PP2A levels in type 2 diabetic rats, this effect could be partly related to the dephosphorylation of CaMKII [32].

In conclusion, we suggest that alternations in hippocampal CaMKII/PKC/PKA pathways in early experimental diabetes mellitus could be, at least in part, responsible for the detrimental postdiabetic outcomes associated with cognitive dysfunction.
in diabetic models. The abnormal increase in synapsin I (Ser 603), GluR1 (Ser 831), and GluR1 (Ser 845) phosphorylation may be due to the complementary response of pyramidal neurons against the progression of injury due to diabetes mellitus. Nevertheless, the overall picture regarding the role of serine/threonine kinases in the diabetic brain remains incomplete. Further studies are needed to define the extent to which changes in each of these enzymes lead to cellular dysfunction in relation to learning and memory. It is critically important to investigate further the effect of STZ or STZ/high fat on NMDA and AMPA ion channels in the various pathological stages of diabetic rats.

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Conflict of Interest

The authors declare no conflict of interest.

References


Supporting Information

The following supplementary material is available for this article:

Figure S1. Changes of body weight in type 1 (A) and type 2 (B) diabetic rats.

Figure S2. Serum biochemical measurements of ALT and BUN in type 2 diabetic rat.