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Research Report

Diabetic Ketoacidosis Induces Tau Hyperphosphorylation in Rat Brain

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Abstract.
Background: Diabetes mellitus (DM) increases the risk for cognitive impairment and Alzheimer's disease (AD). Diabetic ketoacidosis (DKA), a serious complication of DM, may also cause brain damage and further AD, but the underlying molecular mechanisms remain unclear.
Objective: Our objective was to understand how DKA can promote neurodegeneration in AD.
Methods: We induced DKA in rats through intraperitoneal injection of streptozotocin, followed by starvation for 48 hours and investigated AD-related brain alterations focusing on tau phosphorylation.
Results: We found that DKA induced hyperphosphorylation of tau protein at multiple sites associated with AD. Studies of tau kinases and phosphatases suggest that the DKA-induced hyperphosphorylation of tau was mainly mediated through activation of c-Jun N-terminal kinase and downregulation of protein phosphatase 2A. Disruption of the mTOR-AKT (the mechanistic target of rapamycin−protein kinase B) signaling pathway and increased levels of synaptic proteins were also observed in the brains of rats with DKA.
Conclusions: These results shed some light on the mechanisms by which DKA may increase the risk for AD.

Keywords: Dementia, diabetic ketoacidosis, protein kinases, protein phosphatases, tau

INTRODUCTION

Diabetic ketoacidosis (DKA) is a serious complication of diabetes mellitus (DM) that can lead to diabetic coma. DKA is common in type 1 diabetes mellitus (T1DM) and is primarily caused by a severe lack of insulin, which results in high glucose levels and accumulation of ketones in the blood. In addition to numerous metabolic disorders, recurrent DKA in DM can lead to morphological and functional brain impairments that are associated with neurocognitive outcomes [1]. Substantial evidence suggests that DKA can cause different types of brain damage. Memory deficits are observed in children with T1DM plus DKA diagnosis as compared to those with only T1DM [2]. The cause of these deficits remains unclear, in part because T1DM often has other complications that may pose multiple risks for brain damage and cognitive impairment. Although the effects of T1DM on memory have been investigated previously [3–6], the underlying molecular mechanism is not understood.

DKA is also reported to increase the risk for Alzheimer's disease (AD) in patients with type 2

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diabetes mellitus (T2DM) [7]. It has been demonstrated in diabetic rats that DKA can induce deficits in learning and memory associated with astrogliosis [8, 9]. There is increasing evidence to support that DM is associated with neurological disorders. Patients with AD have shown a reduction of insulin in cerebrospinal fluid, impairment in insulin signaling, and reduction in glucose metabolism in the brain [10–15]. The impairment of brain insulin signaling appears to contribute to neurodegeneration and cognitive impairment by promoting abnormal hyperphosphorylation of tau, which may underlie neurodegeneration and cognitive deficits in AD and other tauopathies [16–20].

Under diabetic conditions, especially with DKA, hyperglycemia, free fatty acids, and cytokines produce an excess of reactive oxygen species [21], which in turn can induce impairment in synapses, synaptic plasticity, hippocampus-dependent learning and memory, and adult neurogenesis. The brain damage is associated with structural lesions, including axonal loss and neuronal death [21–23]. In addition, DKA is associated with brain changes that are also observed in hypoxic and ischemic brains, such as a decrease in cerebral blood flow, high levels of energy phosphates, and low pH [24–26]. Cognitive impairment with poorer performance on executive function was observed in older adults with T1DM with recurrent DKA [27]. Therefore, multiple mechanisms are likely involved in the increased risk for cognitive impairment and AD in DM, and DKA might add further insult to the brain.

In the present study, we induced DKA and investigated the subsequent brain alterations, focusing on tau phosphorylation and other changes involved in AD neurodegeneration and cognitive impairment in rats. We found that DKA resulted in hyperphosphorylation of tau and activation of several tau kinases. These findings provide some insights into the molecular mechanisms by which DKA may promote neurodegeneration and cognitive impairment.

MATERIALS AND METHODS

Animals and induction of DKA

This study was conducted according to the protocol approved by the Institutional Animal Care and Use Committee of the New York State Institute for Basic Research in Developmental Disabilities and was in accordance with the Guidelines by the NIH Office of Laboratory Animal Welfare. DKA was induced in female Wistar rats (weight approximately 300 g) by a single intraperitoneal injection of 150 mg/kg streptozotocin (STZ) followed by starvation for 48 h according to an established method previously described [28]. STZ was dissolved in a 100-mM citrate buffer, pH 4.5. Control rats were injected with a citrate buffer only. DKA in rats was confirmed by measuring ketoacids (acetoacetate) and pH in urine with Siemens Multistix 7 (Fisher Scientific, Santa Clara, CA) and tail blood glucose levels with a commercial glucometer. Only female rats were employed for this initial study due to the limited resources. Similar studies in male rats will be carried out in the future.

Rats were sacrificed by decapitation 48 h after STZ or control injection, and the hippocampi and the whole cerebral cortex tissue were dissected from the rat brains immediately and stored at –80°C until being used. The brain tissue was homogenized in cold homogenization buffer containing 50 mM Tris-HCl, pH 7.4, 8.5% sucrose, 2 mM EDTA, 2 mM EGTA, 10 mM β-mercaptoethanol, 5 mM benzamidine, 0.5 mM 4-(2-aminoethyl) benzenesulfonyl fluoride, 4 μg/ml pepstatin A, and 10 μg/ml each of aprotinin and leupeptin, 20 mM β-glycerolphosphate, 100 mM sodium fluoride, 1 mM sodium vanadate, and 100 nM okadaic acid. The homogenates 10% (w/v) were stored at –80°C in aliquots, and the protein concentrations of the homogenate samples were determined by using Pierce™ 660 nm Protein Assay Reagent (Thermo Scientific, Rockford, IL), according to the manufacturer's instructions.

Western blots

The homogenate samples were resolved in 10 or 15% SDS-PAGE, followed by electro-transfer to PVDF membrane of 0.45 μm pore size. After initial blocking of the membrane with 5% skim milk, the blots were incubated with primary antibodies (Table 1) diluted in 5% skim milk at 4°C overnight. The blots were then probed with the corresponding anti-mouse or anti-rabbit (1 : 5000; Jackson ImmunoResearch, West Grove, PA) or anti-sheep HRP secondary antibodies (1 : 1000; Invitrogen, Waltham, MA), and finally detected by using enhanced chemiluminescence (ECL) reagents (Thermo Scientific). Multigauge V3 software (Fuji

Table 1
Primary antibodies used in this study

Antibody	Type	MW	Specificity	Source	Catalog #
CAMK II	Mouse	50KD	CaMKII	Santa Cruz	sc-5306
CAMK II α-Subunit	Mouse	50KD	p-CaMKII (Thr286)	Calbiochem	NB13
GSK-3β	Rabbit	46KD	GSK-3β	Cell signaling	27c10
P-GSK-3β	Rabbit	46KD	p-GSK-3β (S9)	Cell signaling	9336
MAPK (Erk1/2)	Rabbit	42/44	Total p44/42 MAP Kinase (Erk1/Erk2)	Cell signaling	9102
P-MAPK (Erk1/2) (197G2)	Rabbit	42/44	p-p44/p42 MAP Kinase (Thr202/Tyr204)	Cell signaling	4377
Cdk5 (DC17)	Mouse	34	Cdk5	Santa Cruz	sc-249
P-Cdk5 (C7)	Mouse	34/37	p-Cdk5 (Ser159)	Santa Cruz	sc-377558
p35 (4G11)	Mouse	35	Cdk5 regulatory subunit, p35	Santa Cruz	sc-293184
AMPK	Rabbit	62	Total AMPK	Cell signaling	2532
P-AMPK (40H9)	Rabbit	62	p-AMPK (Thr172)	Cell signaling	2535
AKT (pan) (11E7)	Rabbit	60	Total AKT	Cell signaling	4685
P-AKT (193H12)	Rabbit	60	p-AKT (Ser473)	Cell signaling	4058
JNK2 (56G8)	Rabbit	46/54	Total JNK2	Cell signaling	9258
P-SAPK/JNK	Rabbit	46/55	p-JNK (Thr183/Tyr185)	Cell signaling	9251
P13 Kinase p85	Rabbit	85	P13 Kinase p85 not p50 or p55	Cell signaling	4292
P-P13 kinase p85	Rabbit	85	p-p85/p55 (Tyr458/TYr199)	Cell signaling	4228
PDK	Rabbit	58/68	Total PDK	Cell signaling	3062
P-PDK	Rabbit	58/69	p-PDK (S241)	Cell signaling	3061
mTOR (7C10)	Rabbit	289	Total mTOR	Cell signaling	2983
P-mTOR (D9C2)	Rabbit	289	p-mTOR (Ser2448)	Cell signaling	5536
PP2A (46/PP2A catalytic α)	Mouse	36	PP2Ac	BD Trans. Lab	610555
Demethylated PP2A-C (4i57)	Mouse	36	Unmethylated PP2Ac subunit	Santa Cruz	sc-80990
Synpsin-1	Rabbit	80	Synpsin-1	Stressgen	VAP-SV060
PSD 95	Rabbit	95	PSD 95	Cell signaling	3450
NMDA-R2B	Rabbit	190	NMDA-R2B	Cell signaling	4207
R92e	Rabbit	50–75	pan tau	Pei et al. (1998)	
anti pS199	Rabbit	50–75	p-tau (S199)	Invitrogen	44734G
anti-pT205	Rabbit	50–75	p-tau (T205)	Invitrogen	44738G
anti-pT212	Rabbit	50–75	p-tau (T212)	Invitrogen	44740G
anti-pS214	Rabbit	50–75	p-tau (S214)	Invitrogen	44742G
anti-p262	Rabbit	50–75	p-tau (S262)	Invitrogen	44750G
PHF-1	Mouse	50–75	p-tau (S396/404)	Dr. P. Davis	
anti-PS409	Rabbit	50–75	p-tau (S409)	Biosource	44-760G
GAPDH	Rabbit	37	GAPDH	Sigma	G9545

Photo Film, Tokyo, Japan) was used to quantify the specific protein bands on western blots.

Immuno-dot blots

The cerebral cortical homogenates were centrifuged at 5,000 *g* at 4°C for 5 min, and the supernatants were collected and adjusted to the same protein concentration with the homogenate buffer, followed by dilution to 1.0 μg protein/μl with Tris-buffered saline (TBS). The amount of 5.0 μl each sample was doted in duplicates onto nitrocellulose paper. After drying at 37°C, the blots were blocked with 5% Carnation milk in TBS solution for 30 min. The blots were then probed with rabbit anti-4-HNE (Abcam, Cat# AB46545) or, as a loading control, rabbit anti-GAPDH (Sigma, Cat # G9545). The dot-blots were finally developed with ECL reagents (PierceThermal Scientific) and quantified by densitometry using ImageJ 1.54 g.

Statistical analysis

To analyze the densitometric data from Western blots, a Student's *t*-test was performed for the statistical analysis between the two groups. Correlation analysis was performed by the Spearman correlation test. All data were analyzed by employing Prisma 7 statistical software (GraphPad Software, Inc., Boston, MA). All data were expressed as means ± SEM. Differences between the means were treated as statistically significant if $p < 0.05$.

RESULTS

Verification of DKA in rats

To verify the success of DKA induction with STZ injection and starvation, we tested the levels of blood glucose, urine ketoacids (acetoacetate), and urine pH

Table 2
Rat blood glucose levels, urine ketone levels, and pH values

Rat #	Time (h) post STZ injection								
	Blood glucose (mg/dL)			Urine ketones* (mg/dL)			Urine pH value*		
	0	24	48	0	24	48	0	24	48
1	122	203	410	Neg	Neg	40	7	7	6
2	123	129	363	Neg	Neg	≥160	7	7	6
3	128	201	382	Neg	Neg	≥160	7	7	6
4	139	281	287	Neg	Neg	≥160	7	7	6
5	131	222	395	Neg	Neg	≥160	7	6	6
6	134	202	370	Neg	Neg	≥160	7	6	6
7	122	232	366	Neg	Neg	≥160	7	7	6

*Values were taken from Multistix as indicated by manufacturer's scale.

values at various time points post–STZ injection. The results (Table 2) confirmed that the rats had DKA 48 h post–STZ injection and starvation.

Tau hyperphosphorylation induced by DKA

Abnormal hyperphosphorylation of tau is believed to be the initial step of neurofibrillary pathology and is crucial to neurodegeneration [29]. We, therefore, studied whether DKA can induce hyperphosphorylation of tau at the phosphorylation sites observed in AD brain. Western blot analyses using several phosphorylation-dependent and site-specific tau antibodies indicated that tau in the brains of rats with DKA became markedly hyperphosphorylated at pT205, pT212, pS214, and pS262 in both the cerebral cortex and the hippocampus and at pS422 in the cerebral cortex (Fig. 1). All these sites are the abnormal hyperphosphorylation sites seen in AD brain. The total tau protein level, as determined by using the phosphorylation-independent tau antibody 92e, was not changed with DKA, because no significant differences were seen between the DKA and the control groups. These results indicate that DKA can induce hyperphosphorylation of tau at multiple phosphorylation sites relevant to neurofibrillary degeneration.

Alterations of tau kinases in the brains of rats with DKA

Hyperphosphorylation of tau at multiple sites in the brains of rats with DKA, as observed above, suggests dysregulation of tau protein kinases and/or phosphatases in the brain during DKA, because the phosphorylation level of tau at individual sites results from a balance of tau kinase and phosphatase activities acting at the site. Therefore, we investigated the levels and activities (evidenced by their phosphorylation status that regulates the kinase activity) by using quantitative western blots. We first determined the levels and the activity-determining phosphorylation of glycogen synthase kinase 3β (GSK3β) and cyclin-dependent kinase 5 (CDK5), the brain protein kinases implied in the regulation of tau phosphorylation at multiple sites [30]. GSK3β activity is mainly regulated negatively with its phosphorylation at S9 [31]. Surprisingly, we found marked phosphorylation of GSK3β at S9 in both the cerebral cortex and the hippocampus of rats during DKA (Fig. 2), suggesting downregulation of this kinase in the brains of rats with DKA as compared to controls. CDK5 activity requires both phosphorylation at its Ser159 and an activator p35. Whereas the CDK5 phosphorylation was found to be slightly increased, the p35 level was markedly decreased in the brains of rats with DKA as compared to the controls, suggesting no activation of this kinase in the brain during DKA.

The above results indicate that the alterations of GSK3β and CDK5 did not contribute to the hyperphosphorylation of tau observed in the brains of rats with DKA. Otherwise, decreased phosphorylation, instead of hyperphosphorylation, of tau would have been found. To learn what protein kinases contribute to tau hyperphosphorylation in the brain during DKA, we further studied c-Jun N-terminal kinase (JNK), calcium/calmodulin-dependent protein kinase II (CAMK-II), extracellular signal–regulated kinase (ERK), and AMP-activated protein kinase (AMPK), because these kinases are also reported to regulate tau phosphorylation in the brain [32]. Quantitative western blot analyses found increased phosphorylation/activation of JNK and CAMK-II in the rat brains with DKA as compared to controls, but the expression levels of these kinases were not altered with DKA (Fig. 2). No significant differences in the level and phosphorylation/activation of ERK or AMPK were seen between the DKA and the control groups. These

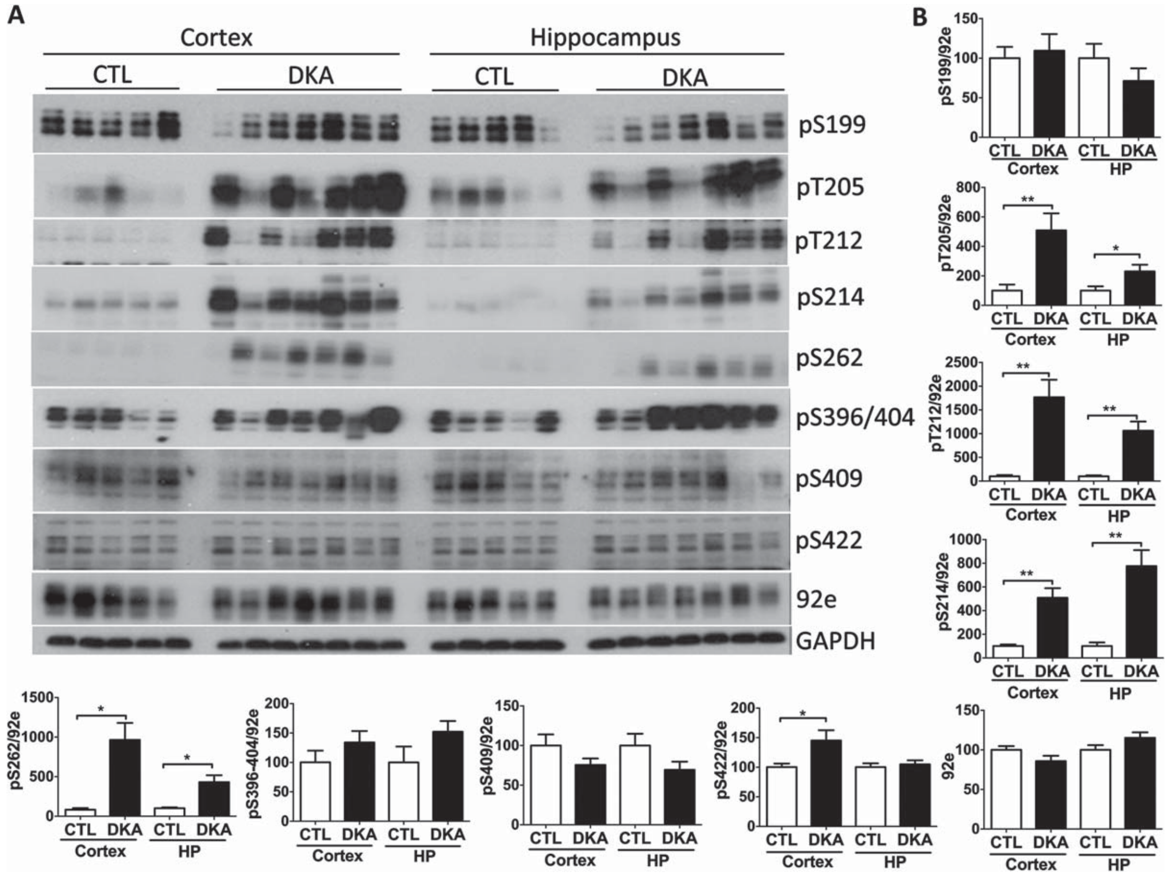

Fig. 1. Tau hyperphosphorylation in the brains of rats with DKA. Brain homogenates derived from the rat hippocampal and cortical homogenates were subjected to western blots developed with the indicated antibodies (A). The blots were then quantified by densitometry, and the relative levels of tau phosphorylation at individual phosphorylation sites were normalized with the total tau level (determined with 92e) (B). GAPDH blots were included as a loading control. Data shown in the graphs are presented as mean ± SEM ($n = 5-7$/group), where * and ** indicate $p < 0.05$ and 0.01, respectively.

results suggest that DKA may have led to activation of JNK and CAMK-II that in turn resulted in hyperphosphorylation of tau in the rat brains. To verify these possibilities, we performed a Spearman correlation between the phosphorylation of tau at the sites elevated during DKA and the phosphorylation/activation of JNK and CAMK-II. We found a positive correlation of tau phosphorylation at pT205, pT212, pS214, and pS262 with phosphorylation/activation of JNK but not of CAMK-II (Fig. 3). No significant correlation was found between tau phosphorylation at any site studied and phosphorylation/activation of CDK5, which was included as references. These results suggest that the increased phosphorylation of tau was mainly caused by activated JNK in the rat brains during DKA.

Because STZ injection could induce oxidative stress in the brain, which might in turn promote JNK activation. To learn whether there was significant oxidative stress that could explain the JNK activation we observed in the DKA rat brains, we determined the level of a commonly used oxidative stress marker, 4-hydroxy-2-nonenal (4-HNE), by using immune-dot blots. We found that the rat brain tissue of the DKA group had a reduced level of 4-HNE instead (Fig. 4). These results exclude the brain oxidative stress status at this time point, which could otherwise activate JNK.

Alterations of tau phosphatases in the brains of rats with DKA

Protein phosphatase 2A (PP2A) is the major phosphatase that regulates tau phosphorylation in the mammalian brain [33]. Thus, we also studied PP2A in the rat brains during DKA. Because PP2A activity

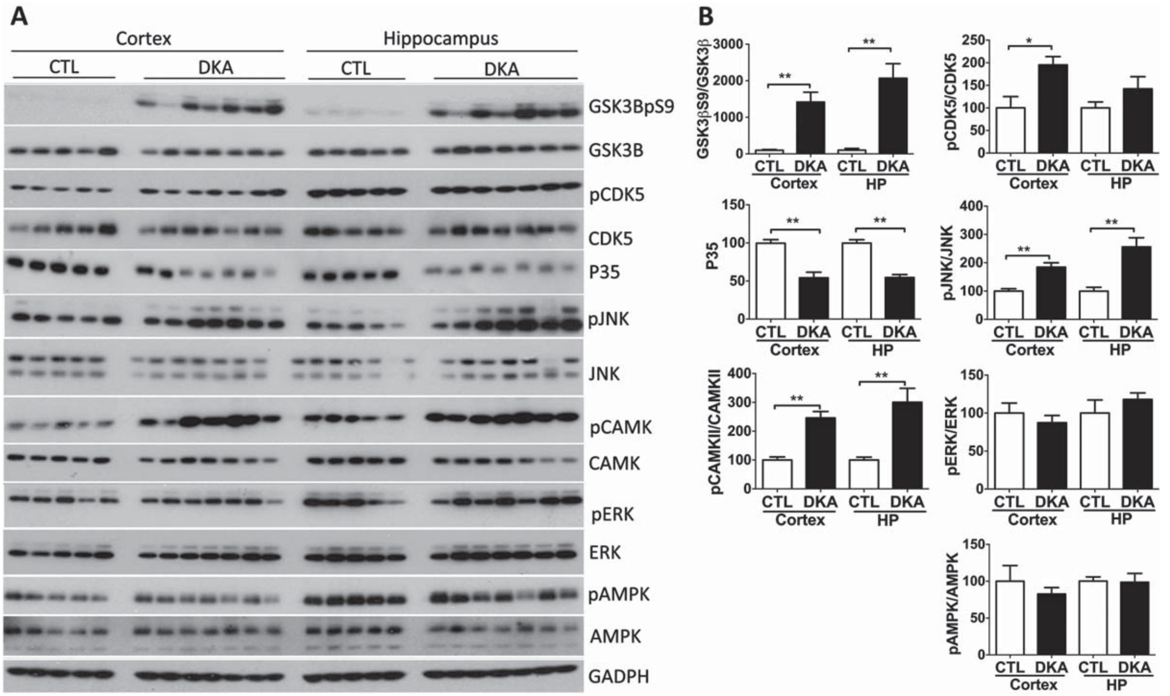

Fig. 2. Western blots of tau protein kinases in the brains of rats with DKA. Brain homogenates derived from the rat hippocampal and cortical tissue were subjected to western blots developed with the indicated antibodies (A). The blots were then quantified by densitometry, and the relative levels of phosphorylated kinase were normalized with the total kinase level (B). GAPDH blots were included as a loading control. Data shown in the graphs are presented as mean ± SEM (n = 5−7/group), where * and ** indicate $p < 0.05$ and 0.01, respectively.

is promoted through methylation of the C-terminus of the PP2A catalytic subunit (PP2Ac), and its demethylation inhibits phosphatase activity [34], we determined the levels of total PP2A and demethylated PP2A (dPP2A) in the rat brains by using western blots. We found that while the total level of PP2A was not altered, the dPP2A level was significantly increased in the hippocampus of rats with DKA as compared to the controls (Fig. 5). These results suggest a downregulation of PP2A through its demethylation at least in the hippocampus of rats with DKA, which can also contribute to the hyperphosphorylation of tau that we found in the brains of rats with DKA.

Disruption of brain AKT signaling in rats with DKA

AKT (protein kinase B) signaling is the key molecular signal mediating insulin's function and is also involved in neurofibrillary degeneration [14, 35]. We thus investigated AKT and its upstream signaling molecules, phosphatidylinositol 3-kinase (PI3K), phosphoinositide-dependent kinase-1 (PDK1), and the mechanistic target of rapamycin (mTOR). These signaling molecules are protein kinases in nature, and their kinase activities are initiated/promoted by phosphorylation. Therefore, we determined the phosphorylation of these kinases in the brains of rats with DKA by western blots developed with the specific phosphorylation-dependent antibodies. We found a marked increase in the phosphorylation of AKT at S473 in the rat brains with DKA, suggesting activation of this kinase in the brain during DKA (Fig. 6). AKT is mainly activated via phosphorylation at T308 by PI3K and PDK1 or at S473 by mTOR [36, 37]. Our quantitative analysis of western blots indicated significant activation/phosphorylation of mTOR in the cerebral cortex but no significant alterations of PI3K or PDK1 in the brains of rats with DKA (Fig. 6). These results revealed disruption of mTOR-AKT signaling in the brains of rats with DKA.

Increase in neuronal plasticity markers at the early stage of DKA

Neuronal synapses connecting and regulating neural networks in the brain are responsible for learning, memory, and cognition. This mechanism is regulated by excitatory synaptic transmission when

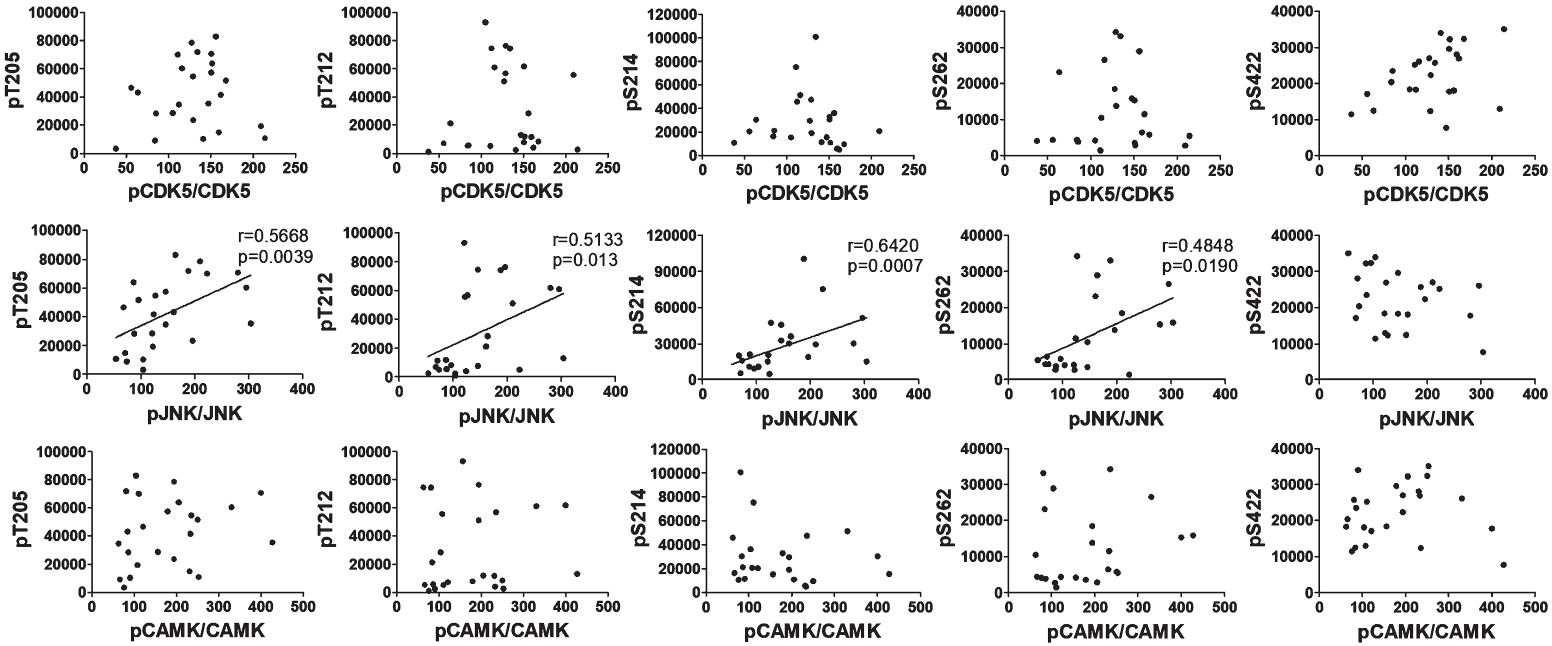

Fig. 3. Correlation analysis between phosphorylation of tau and phosphorylation/activation of CDK5, JNK, and CAMK-II. The phosphorylation levels of tau at the indicated sites of brain samples were plotted against the phosphorylation/activation of CDK5, JNK, and CAMK-II and analyzed using Spearman correlation. The linear correlation lines, correlation coefficients (*r* values), and *p* values are included in the graphs only if they are statistically significant.

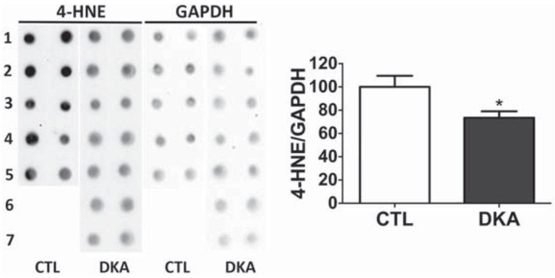

Fig. 4. Immuno-dot blots of 4-HNE in cerebral cortical tissue of rats with DKA. Crute extracts of the cerebral cortical homogenates were subjected to immuno-dot blot assays developed with anti-4-HNE against oxidated protein adducts. The blots were then quantified by densitometry, and the relative levels of 4-HNE are shown the graph (mean ± SEM, $n = 5-7$/group).

pre-synapses release neurotransmitters that activate receptors on the postsynaptic neuron. In this study, we investigated the alteration of neuronal plasticity markers in the hippocampus and cerebral cortex of rats under DKA conditions. The levels of synapsin 1 and PSD95, a pre- and post-synaptic protein, respectively, were found to be significantly higher in rats with DKA than in the control group. Likewise, the same response was found in NMDA receptors, which can trigger lethal injury, inducing calcium influx and subsequently cellular calcium overload (Fig. 7).

DISCUSSION

DKA is a serious acute complication of DM that occurs in patients with uncontrolled hyperglycemia or other complications. Clinical studies have demonstrated that DKA can increase the risk of, or cause, long-term cognitive impairment. Significant memory impairment was observed in children with T1DM complicated with a history of DKA as compared to those without DKA, and the memory impairment is associated with subtle cerebral injury [2]. Increased white matter volume with decreased grey matter and poor mental score have been observed in

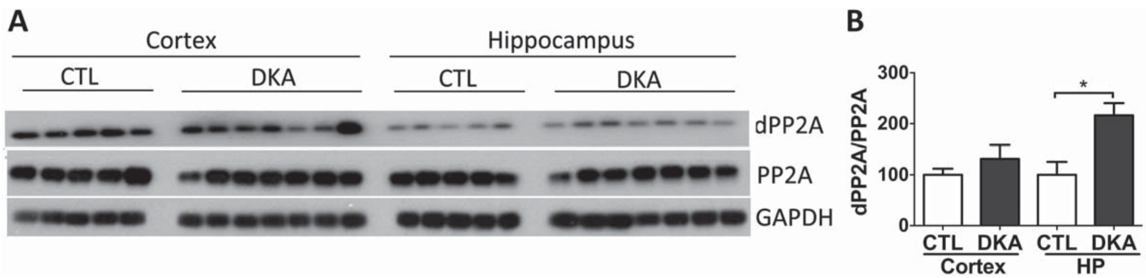

Fig. 5. Western blots of PP2A in the brains of rats with DKA. Brain homogenates derived from the rat hippocampal and cortical homogenates were subjected to western blots developed with antibodies against the catalytic subunit of PP2A or demethylated PP2A (dPP2A) (A). The blots were then quantified by densitometry, and the relative levels of dPP2A/PP2A are shown in (B) (mean ± SEM, $n = 5-7$/group).

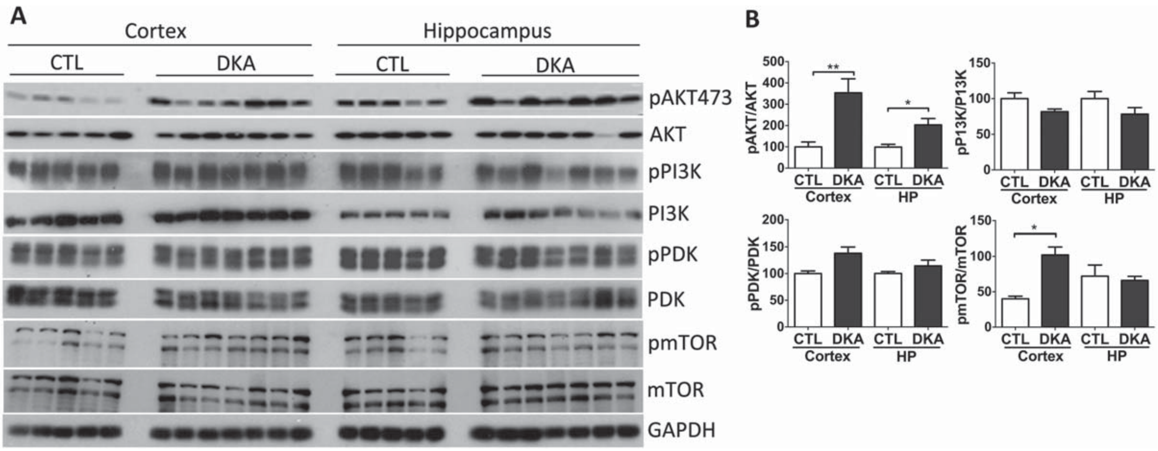

Fig. 6. Western blots of AKT, PI3K, PDK1, and mTOR from brains of rats with DKA. Brain homogenates derived from the rat hippocampal and cortical homogenates were subjected to western blot developed with the antibodies indicated at the right side of (A). The blots were then quantified by densitometry, and the net phosphorylation levels of each kinase were plotted (mean ± SEM, $n = 5-7$/group) (B).

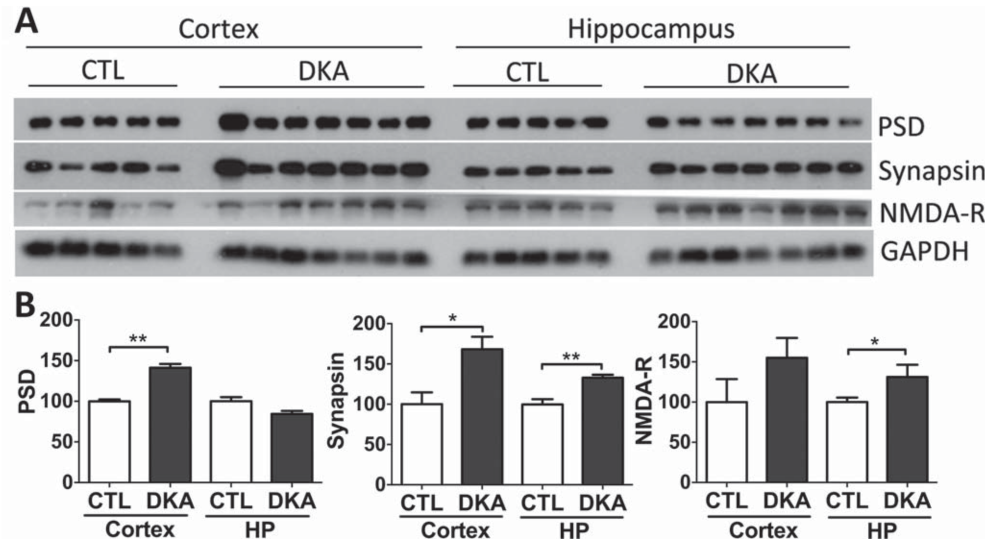

Fig. 7. Western blots of synaptic proteins in the brains of rats with DKA. Brain homogenates derived from the rat hippocampal and cortical homogenates were subjected to western blots developed with antibodies indicated at the right side of (A). The blots were then quantified by densitometry, and the levels, after being normalized with GAPDH levels, are shown (mean ± SEM, $n = 5-7$/group) (B).

children with T1DM with recurrent DKA [1, 38]. A longevity study found that older adults with T1DM, specifically those with recurrent DKA, had lower global cognitive function and reduced scores on psychomotor activity and executive functions [27]. In a large population study that evaluated the relationship between T2DM-DKA and the risk of dementia in adults, DKA was found to increase the risk for AD by 1.86-fold [7]. However, how DKA promotes cognitive impairment and AD remains unclear. Several possible mechanisms, such as hyperglycemic state associated with oxidative stress [39], blood-brain barrier disruption [40, 41], neuroinflammation [42], and brain insulin deficiency [43], have been proposed. In the present study, we found that DKA can induce AD-like hyperphosphorylation of tau, which is crucial to neurofibrillary degeneration and the consequent cognitive impairment in AD. It should be noted that DKA itself is an acute, short-time condition/complication of the chronic disease diabetes, and it is life-threatening if not corrected timely. Our results of the substantial changes in the brains of rats with DKA indicate that the brain is a sensitive organ responding to insults. Our findings suggest that recurrent DKA can probably increase the risk for AD by promoting abnormal hyperphosphorylation of tau in the brain. However, it is unlikely that young diabetes patients with a DKA episode could develop long-lasting tau pathology in the brain, but repeated DKA attacks can induce recurrent abnormal hyperphosphorylation of tau in the brain, which in turn could promote neurodegeneration and thus increase the risk for AD.

A common pathological feature of AD and other tauopathies is the formation of intraneuronal aggregation of tau protein as neurofibrillary tangles. Abnormal hyperphosphorylation of tau is believed to be crucial to neurofibrillary degeneration in these neurodegenerative diseases that all lead to memory loss and dementia [44]. To learn whether DKA promotes neurodegeneration and cognitive impairment through inducing AD-like hyperphosphorylation of tau in the brain, we determined the level of tau phosphorylation at several sites that are seen in AD brain and found that DKA induced significant hyperphosphorylation of tau at T205, T212, S214, S262, S396/404, and S422 in rat brains. Considering that abnormal tau hyperphosphorylation is an early and crucial event of neurofibrillary degeneration, our results suggest that repeated DKA may promote or induce cognitive impairment by promoting abnormal hyperphosphorylation of tau and, consequently, neurofibrillary degeneration in individuals with DM.

Abnormal hyperphosphorylation is an early stage of tau pathology that eventually leads to the formation of neurofibrillary tangles in AD brain. Acute tau phosphorylation does not necessarily indicate a path toward tauopathies such as AD. This initial stage is reversible and can be reversed through inhibition of tau kinases and/or activation of tau phosphatases [29]. In addition, brain insulin signaling is downregulated in AD brain [14], which may also contribute to tau-mediated neurofibrillary degeneration in AD [15]. We previously found that restoration of brain insulin signaling through either insulin sensitizers or intranasal insulin administration can reverse the AD-like tau hyperphosphorylation and improve cognitive function in mice [45–47]. Thus, timely management of DKA for diabetes patients may help prevent the DKA-induced increase in AD risk in these individuals.

The level of tau phosphorylation is a net outcome of the activities of tau protein kinases and phosphatases. To determine which tau kinases mediate hyperphosphorylation of tau in DKA, we investigated the major known tau kinases, including GSK3β, CDK5, JNK, CAMK-II, ERK, and AMPK [30]. Surprisingly, we found downregulation of both GSK3β and probably CDK5 activities, as evidenced by increased S9 phosphorylation and decreased CDK5 activator p35, respectively, in the brains of rats with DKA. Downregulation of these two kinases would induce decreased phosphorylation, rather than hyperphosphorylation, of tau if other modulators were not involved. Therefore, the fact that tau was hyperphosphorylated in the brains of rats during DKA indicates that tau hyperphosphorylation was not mediated by these two protein kinases under this condition. Significant alterations of S9 phosphorylation of GSK3β are not seen in human AD brain either [14]. Further studies of other tau kinases suggested activation of JNK that appears to contribute to tau hyperphosphorylation during DKA, which was further supported by a positive correlation between tau phosphorylation at multiple sites and phosphorylation/activation of JNK. Thus, it appears that JNK in the brain may be activated as a stress response to DKA, which in turn induces hyperphosphorylation of tau during DKA. The type of stress that activates JNK under this condition is unlikely to be oxidative stress because the total oxidative stress level in the rat brains at the time point of investigation was lower with DKA, as determined by our immuno-dot blot assay.

Under both biological and pathological conditions, tau phosphorylation is usually regulated by the combined actions of several tau kinases and phosphatases. In addition to JNK activation, we found that the major tau phosphate in the mammalian brain, PP2A [33], was downregulated through increased demethylation of the PP2A catalytic subunit. Downregulation of PP2A also occurs in the AD brain and may contribute to abnormal hyperphosphorylation of tau in AD [33, 48–50]. We thus speculate that during DKA, activation of JNK and downregulation of PP2A collectively induce tau phosphorylation, which outweighs the counteraction of downregulation of GSK3β and probably also of CDK5, resulting in hyperphosphorylation of tau in the rat brains. A similar phenomenon was previously reported in the brains of starved mice [51].

AKT is a key mediator of both insulin signaling and mTOR signaling, as well as the major upstream protein kinase of GSK3β [52]. We found that mTOR-AKT signaling was activated in rat brains during DKA. These results are consistent with the downregulation of GSK3β activity through its phosphorylation at S9 that we found because the S9 phosphorylation of GSK3β is catalyzed by AKT. The activation of mTOR-AKT signaling appears to be an acute response to DKA, which may contribute to the increased level of synaptic proteins, such as synapsin I, PSD95, and NMDA-R, that we found in the brains of rats during DKA.

In conclusion, we found that DKA induced hyperphosphorylation of tau protein at multiple phosphorylation sites associated with female AD and disruption of the mTOR-AKT signaling and increased levels of synaptic proteins in rat brains. The DKA-induced hyperphosphorylation of tau was mainly mediated through activation of JNK and downregulation of PP2A. Future studies will need to confirm whether these findings can be replicated in male animals. Nevertheless, the present study shed some light on how DKA may increase the risk for AD.

AUTHOR CONTRIBUTIONS

Gustavo Basurto-Islas (Conceptualization; Data curation; Formal analysis; Funding acquisition; Investigation; Methodology; Validation; Visualization; Writing – original draft; Writing – review & editing); Yunn Chyn Tung (Data curation; Methodology; Writing – original draft); Chun-ling Dai (Data curation; Investigation; Methodology; Writing – review & editing); Khalid Iqbal (Conceptualization; Investigation; Project administration; Resources; Supervision; Writing – review & editing); Cheng-Xin Gong (Conceptualization; Formal analysis; Funding acquisition; Investigation; Project administration; Resources; Supervision; Writing – review & editing).

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CONFLICT OF INTEREST

KI and CXG are Editorial Board Members of this journal but were not involved in the peer-review process for this article and did not have access to any information regarding its peer review. KI is cofounder and Chief Scientific Officer of Phanes Biotech, Inc. This company is in the process of developing biomarkers and therapeutics of Alzheimer's disease and related disorders. CXG serves in the Scientific Advisory Board of Alectos Therapeutics.

DATA AVAILABILITY

The data supporting the findings of this study are available within the article.

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