Differential activations of PKC/PKA related to microvasculopathy in diabetic GK rats

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DIABETIC MICROVASCULOPATHY is the most common complication for diabetes patients, which is often the leading cause of blindness, renal failure, and limb amputations. Diabetic microvasculopathy is involved in multiple organs (3, 8, 9) and exhibits different severity in different organs. Although diabetic microvasculopathy was also found in brain and liver by autopsies in some older individuals with diabetes (53, 8, 9) and exhibits different severity in different organs. Therefore, we were interested in investigating the mechanisms of diabetic microvasculopathy.

Impairment and dysfunction of endothelial cells are the key manifestations of diabetic microvascularopathy. Under diabetic conditions, microaneurysm and loss of pericytes have been observed in retinal microvascular but not in cerebral microvascularity (24, 14). It has been recognized that the increased formation of advanced glycation end products (AGEs), the activation of protein kinase C (PKC) isoforms, oxidative stress (4), and the activation of the polyol pathway are implicated in the etiological mechanisms of diabetes-induced microvasculopathy (59, 52, 13).

Formed by nonenzymatic glycation and oxidation of proteins and lipids, AGEs are critically involved in diabetic microvasculopathy. Much evidence links these molecular species to the complications of diabetes, largely via their major signal transduction receptor RAGE. The binding of AGEs to RAGE in endothelial cells activates PKC and subsequently leads to oxidative stress (49). Activation of the PKC pathway, especially the PKCB isof orm, has been extensively shown to be related to diabetic microvasculopathy (51, 55, 11). The PKCB isof orm-selective inhibitor ruboxistaurin has shown promising effects on delaying the progression of clinical diabetic nephropathy (51). Thus, PKC activation is possibly one key step in the pathogenesis of diabetic microvascularopathy.

In previous studies, it was reported that protein kinase A (PKA), an enzyme that plays an important role in the transduction of many external signals through the cAMP second messenger pathway, has inverse effects compared with PKC on some cellular functions (42, 43). For example, the differential activations of PKC/PKA were correlated with the function of the blood-brain barrier (45), regulation of TNF receptor expression (54), and genetic susceptibility to cerebral malaria (32). Recently, some studies have uncovered new insights on the possible involvement of PKA in diabetes. In pancreatic β-cell, activation of the PKA signal pathway mediated genistein-induced cell proliferation (16) and improved glucose-induced insulin secretion (60, 12). It has been reported that abnormal responsiveness of vascular relaxation in STZ-induced diabetic rats may be attributable to a lowered activity of PKA (33), whereas activation of PKA can improve the dysfunctions of vascular endothelial cells in diabetes (40, 47). Activation of the cAMP/PKA pathway attenuates oxidative stress-induced DNA damage, cellular senescence, and diabetes-induced attenuation of acetylcholine-induced endothelium-dependent relaxation (38, 47). Moreover, in isolated rat glomeruli, cilostazol, a phosphodiesterase inhibitor that activates PKA, reduces production of ROS in renal glomeruli stimulated with phorbol myristate acetate or TNF-α (26). These findings showed that activation of PKA has implications for the vascular protection in diabetes.

It has been reported that ROS can be induced on isolated rat glomeruli by PKC activation but inhibited by a phosphodiesterase inhibitor, which activates PKA and is reported to improve albuminuria in diabetic animals (26). Although the roles of PKC and PKA in diabetic vasculopathy have been recog-
Inhibitors interfere with the progression of diabetic nephropathy.

In our study, we found that significant microvasculopathy was found in kidney and retina but not in brain and liver in diabetic GK rats during a 6-mo duration, suggesting that the occurrence of diabetes microvasculopathy was not parallel in different organs. In the present study, we explored the possible mechanisms of organ difference in diabetic microvasculopathy. Specifically, the following questions were addressed: 1) does diabetic microvasculopathy present organ difference in diabetic GK rats; 2) are there differential activations of PKC/PKA in different organs in diabetic GK rats; 3) do PKA/PKC inhibitors interfere with the progression of diabetic nephropathy?

MATERIALS AND METHODS

Diabetic animal model. Four-week-old GK rats were purchased from Shanghai SLAC Laboratory Animal (Shanghai, China). They were maintained on a 12:12-h light-dark cycle with free access to standard rodent chow and water. All animal experiments were performed in accordance with the protocols and guidelines approved by the Animal Ethics Committee of China-Japan Friendship Hospital. GK rats over 16 wk old, with glycemia over 20 mM and urinary albumin excretion over 400 μg/24 h, were selected as the diabetic nephropathy group. Prediabetic GK rats with normal glycemia were used as the control group.

Isolation of glomeruli, brain microvessels and liver microvessels. At the end of experiments, all rats were euthanized with 0.5% pentobarbital sodium (0.5 ml/kg ip) under general anesthesia. Kidney, liver, and brain were harvested from all diabetic and nondiabetic control rats. Glomeruli were isolated from renal cortex by a sequential sieving technique with mesh sizes of 300 and 100 μm. Brain microvessels and liver microvessels were isolated by homogenization-centrifugation according to the method described previously (58). Then, the collected tissues were used for Western blot, immunohistochemistry, and real-time PCR.

ELISA for AGEs. Blood was collected from all rats at the time points of 0, 3, 6, and 9 mo. The serum levels of AGEs in diabetic rats and age-matched normal Wistar rats were measured by rat AGE ELISA kit (Uscn Life, China) according to the manufacturer’s protocols.

Real-time PCR. Total RNA was extracted from glomeruli, blood vessel-enriched liver, and brain tissues of all rats by RNease Mini Extraction Kit (Qiagen, Hilden, Germany). Reverse transcription of 2 μg of RNA was performed with the First Strand cDNA Synthesis Kit (Invitrogen) in accordance with the manufacturer’s protocol. Triplicate aliquots of the first-strand reactions were used as templates for real-time quantitative PCR by SYBR Green I dye (Toyobo, Japan) on an ABI 7300 Sequence Detection System (Applied Biosystems). The following primers were used (forward vs. reverse): GGAA-GGACTGAAGCTTGGAAGG vs. TCCGATAGCTGGAGAGGAG-GATT (RAGE), GAGACAGGAGAGGACCCCTCC vs. CAGCTTC-TACACACAGGCTCA (PKCβ), ACTTACTGACCCCCAGGT vs. TCATCCCTGAAGGCACACAG (PKA), and GACATCGTTAAA-GACCTCTTATGCC vs. AATAGAGCCACAACTCCACAGG (β-actin). Relative quantification of transcript levels was performed by the 2-ΔΔCT method using the Ct values obtained from the PCR amplification kinetics with the ABI PRISM SDS 2.1 software.

Western blot. Total protein was extracted from glomeruli, blood vessel-enriched liver, and brain tissues of all rats. The protein content was determined according to the Bradford method. Total tissue proteins were fractionated by 10% SDS-PAGE. Immunoblotting was performed with mouse anti-rat RAGE antibody (Sigma-Aldrich Chemical), mouse anti-rat PKCβII antibody (Sigma-Aldrich Chemical) or rabbit anti-rat PKA cat-α antibody (Abcam) as described previously. The detection method of enhanced chemiluminescence (Millipore) was employed.

Immunohistochemistry. All rats were injected with 0.5% pentobarbital sodium intraperitoneally and then fixed with a perfusion of 40 g/l 4% paraformaldehyde. The kidney, brain, and liver were excised, fixed in 4% paraformaldehyde, and processed for paraffin embedding. The tissue sections were immunostained with mouse anti-rat RAGE antibody, mouse anti-rat PKC antibody, rabbit anti-rat PKA antibody, and horseradish peroxidase-conjugated secondary antibodies. The results were observed under a light microscope (Olympus IX71, Japan).

Assay for PKC and PKA activity. The renal, liver, or brain tissues from normal Wistar rats and diabetic GK rats were homogenized in 5 ml of cold PKC extraction buffer or PKA extraction buffer. The lysates were centrifuged for 5 min at 4°C, 14,000 g, and the supernatants were collected for detecting kinase activity. PKC activity or PKA activity was measured using a PepTag Non-Radioactive Assay kit (Promega), and the activity of PKC or PKA was calculated according to the instructions. The experiment was performed in triplicate, and results are expressed as mean ± SD.

Immunofluorescence double staining. To confirm the expression of PKC and PKA in endothelial cells, immunofluorescence double staining was performed in kidney, liver, and brain of diabetic rats and nondiabetic controls, respectively. Briefly, the freshly dissected kidney, liver, and brain were embedded in Tissue-Tek (Miles, Elkhart, IN) and frozen in liquid methylbutane prequillibrated with liquid nitrogen and stored at −80°C. Serial frozen sections were sliced with a cryostat at 4-μm intervals. Tissue sections were fixed with precooled acetone for 10 min and then incubated with phosphate-buffered saline.
(PBS) plus 0.5% bovine serum albumin (BSA; Sigma-Aldrich Chemical) for 15 min to block nonspecific binding. The sections were separately stained with mouse anti-rat PKCβ II antibody (Sigma-Aldrich Chemical) or rabbit anti-rat PKA antibody (Abcam) and rabbit anti-rat vWF antibody (Santa Cruz Biotechnology), and then they were incubated with the relevant FITC-conjugated second antibody (R&D Systems) and rhodamine-conjugated second antibody separately (Sigma-Aldrich Chemical). The slides were examined under a fluorescence microscope (Nikon).

Culture of rat glomerular endothelial cells with AGE. To investigate the effect of AGEs on glomerular microvascular endothelial cells, further in vitro experiments were performed by treating endothelial cells with AGEs. Rat glomerular microvascular endothelial cells (RGECs) were purchased from Pricells and more than 95% RGEC cells were used for further experiments.

Effects of PKC and PKA on AGE-induced ROS production. ROS were analyzed according to the method as previously described (10). RGECs were plated in 96-well plates and preincubated with PKC inhibitor (Sigma-Aldrich Chemical) H-89, or PKA inhibitor (Sigma-Aldrich Chemical) or PKA agonist 8-bromo-adenosine 3′,5′-cyclic monophosphate (8-Br-cAMP; Sigma-Aldrich Chemical) for 30 min and then treated with AGEs for 12 h. After washing twice with PBS, the cells were stained with DCFH-DA (5 μM) in PBS at 37°C for 10 min. The intensity of fluorescence was assayed with a fluorescence plate reader with excitation at 488 nm and emission at 535 nm.

Effects of PKC and PKA on AGE-induced NO production. RGECs were plated in six-well plates and preincubated with PKCβ inhibitor LY-333531, PKC agonist PMA, PKA inhibitor H-89, or PKA agonist 8-Br-cAMP for 30 min and then treated with AGEs for 24 h. Detection of NO production was performed by measurement of NO2- and NO3- content (NOx) in the samples with nitrite as a standard.

Effects of PKC and PKA on AGE-induced iNOS. To investigate the effects of PKC and PKA on AGE-induced oxidative stress, endothelial cells were loaded on a six-well plate overnight. RGECs were treated with PKCβ inhibitor, PKA agonist, and AGE as described above. Total RNA was extracted and reversed as described above.

The expression levels of RAGE, iNOS (inducible NO synthase), were studied by real-time PCR, and the following primers were used (forward vs. reverse): GGAAGACTGAACGTGGAAGG vs. TCCGATACGCTGGAAGGAGT (RAGE), GTCTGGC-GAAGAACATCC vs. TCCCGAAAAGCTACATT (iNOS), and GACATCCGAAAAGCTCATGAGC vs. AATAGAGCCA- CCAATCCACAGAG (β-actin).

The total cellular protein was extracted. Immunoblot was performed with rabbit anti-rat iNOS antibody (Abcam) as described above.

Effects of agonist and antagonist of PKA on diabetic nephropathy. PKA inhibitor H-89, dihydrochloride salt (LC Laboratories) and PKA activator 8-Br-cAMP (Sigma, Shanghai, China) were dissolved in 0.9% sterile saline for intraperitoneal injection (10 mg/ml). The rats with diabetic nephropathy were selected and randomly assigned to diabetic controls. Diabetes, rats with a diabetic course of 6 mo and with nephropathy; diabetic rats and nondiabetic controls. Diabetes, rats with a diabetic course of >6 mo and with nephropathy; Control, age-matched Wistar rats.

**Fig. 2.** Comparison of serum levels of advanced glycation end products (AGE) and the expression of AGE receptor (RAGE) in glomeruli, liver, and brain vessels isolated from diabetic rats and nondiabetic controls. A: comparison of serum levels of AGE between diabetic rats and matched age-matched Wistar rats (*P < 0.05, **P < 0.01 vs. control group) B–D: comparison of expression of RAGE in glomeruli, liver, and brain vessels isolated from diabetic rats with nephropathy and nondiabetic controls. Diabetes, rats with a diabetic course of >6 mo and with nephropathy; Control, age-matched Wistar rats. B: real-time PCR for RAGE expression in glomeruli, liver, and brain vessels of diabetic GK rats and nondiabetic controls. Results are expressed as means ± SD of 3 independent experiments (*P < 0.01). C: Western blot for RAGE expression in glomeruli, liver, and brain vessels of diabetic GK rats and nondiabetic controls. Results are expressed as means ± SD of 3 independent experiments (*P < 0.01). D: Immunohistochemistry for RAGE.
three groups (n = 6 each) and treated as follows: normal group and diabetic group treated with 0.9% sterile saline, 8-Br-cAMP group treated with 5 mg/kg 8-Br-cAMP, and H-89 group treated with 5 mg/kg H-89. All treatments were performed by intraperitoneal injection twice a day for 2 wk. For urine collection, the rats were placed in individual rat metabolic cages with an ad libitum supply of water and food. At the indicated time points, glycemia was measured from tail vein blood sampling. The excretion level of urinary albumin was detected by the AssayMax Rat Albumin ELISA Kit (Assaypro) and urinary protein measured with a BCA Protein Assay Kit (Beyotime, China) according to the instructions.

Statistics. The results are expressed as means ± SD, and the nonparametric Mann-Whitney test was used to evaluate the intergroup difference. P < 0.05 and P < 0.01 were taken to denote statistical significance.

RESULTS

Detection of diabetic microvasculopathy. To detect organ susceptibility to diabetic microvasculopathy, the histopathological changes of kidney, liver, and brain isolated from diabetic rats and nondiabetic controls were compared. By periodic acid-Schiff staining, the increased thickness of glomerular basement membrane and decreased interspace in glomerular capsule were observed in diabetic rats. There was no detectable change in cerebral or sinusoidal microvasculature in diabetic rats (Fig. 1).

Increased serum levels of AGEs in diabetic rats. To investigate the involvement of AGEs in diabetic microvasculopathy, the levels of serum AGEs in diabetic rats were examined at different stages. ELISA results showed that the levels of serum AGEs gradually increased in diabetic rats under diabetic conditions. The levels of serum AGEs in diabetic rats with a 9-mo course increased onefold over that of age-matched Wistar rats (Fig. 2A).

Elevated expression of RAGE in glomeruli of diabetic rats. Since the effect of AGEs was dependent on binding to RAGE, we then investigated the expression of RAGE in glomeruli, liver, and brain blood vessels of diabetic rats. As demonstrated by real-time PCR (Fig. 2B) and Western blotting (Fig. 2C), the mRNA and protein levels of RAGE were upregulated in glomeruli but were comparable in liver or brain blood vessels of diabetic rats compared with those of nondiabetic rats. Immunohistochemical results showed that the expression of RAGE was elevated in glomeruli but not in cerebral or sinusoidal endothelial cells in diabetic rats (Fig. 2D).

Elevated expression of PKC in glomeruli of diabetic rats. It has been well documented that activation of PKC is involved in diabetic microvasculopathy. As demonstrated by the results of real-time PCR (Fig. 3A) and Western blot (Fig. 3B), the mRNA and protein levels of PKC were elevated in glomeruli (Fig. 3C). The enzyme activity of PKC, which was determined by addition of a specific PKC substrate, was estimated by measuring the amount of phosphorylated peptide (Fig. 3D).

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Fig. 3. Comparison of expression and activity of PKC in glomeruli, liver, and brain vessels isolated from diabetic GK rats and nondiabetic controls. A: real-time PCR for PKC expression in glomeruli, liver, and brain vessels of diabetic and nondiabetic rats. Results are expressed as means ± SD of 3 independent experiments (*P < 0.01). B: Western blot for PKC expression in glomeruli, liver, and brain vessels of diabetic and nondiabetic controls. Top: bands of Western blotting; β-actin blot is same as previously presented in Fig. 2C. Bottom: quantitative analysis of bands signal intensity. Data from quantitative analysis of 3 independent experiments are shown as means ± SD. *P < 0.05 vs. control. C: immunohistochemistry for PKC. D: measurement of PKC activity. Enzyme activity of PKC was evaluated by addition of a specific PKC substrate, and PKC activity was estimated by measuring the amount of phosphorylated peptide. Top: bands of phosphorylated peptides on an agarose gel. Bottom: PKC activity (nmol·min⁻¹·ml⁻¹) determined at 570 nm. *P < 0.01 vs. control.
but not in liver or brain vessels of diabetic rats. These results were confirmed by immunohistochemistry (Fig. 3C). Furthermore, the enzyme activity of PKC in renal cortical, liver, and brain of normal Wistar rats and diabetic GK rats was detected. As shown in Fig. 3D, PKC activity increased in glomerule, but was comparable in liver and brain of diabetic GK rats compared with the normal Wistar rats.

Lowered expression of PKA in glomeruli but elevated expression in liver and brain of diabetic rats. To investigate the possible involvement of PKA in diabetic microvasculopathy, the expressions of PKA in glomeruli, liver, and brain vessels of diabetic rats were analyzed by real-time PCR (Fig. 4A), Western blot (Fig. 4B), and immunohistochemistry, respectively (Fig. 4C). The mRNA and protein levels of PKA were markedly lowered in glomeruli but elevated in brain of diabetic rats compared with those of control rats. The enzyme activity of PKC in renal cortical, liver, and brain of normal Wistar rats and diabetic GK rats was also detected. As shown in Fig. 4D, PKA activity was decreased in glomerule but was comparable in liver and brain of diabetic GK rats compared with the normal Wistar rats.

To investigate the expressions of PKC and PKA in microvascular endothelial cells of glomeruli, liver, and brain isolated from diabetic and nondiabetic rats, the samples were simultaneously immunolabeled for vWF and PKC or PKA. As shown in Fig. 5, the expression of PKC was upregulated in glomeruli but was comparable in liver or in brain of diabetic rats, whereas expression of PKA was down-regulated in glomeruli, but was comparable in liver and in brain of diabetic rats compared with non-diabetic controls. This result was consistent with the result of immunohistochemistry.

In vitro and in vivo effects of PKA agonist and antagonist on microvasculopathy. Since abnormal organ-specific expressions of PKC and PKA were found in diabetic rats, their possible involvements in diabetic microvascular pathologies were further investigated with rat glomerular microvascular endothelial cells in vitro.

Effects of PKC and PKA on AGE-induced RAGE expression and oxidative stress in RGECs. Effects of PKC and PKA on AGE-induced RAGE expression. As demonstrated by the results of real-time PCR (Fig. 6A) and Western blot (Fig. 6B), the mRNA and protein levels of RAGE in RGEC became elevated after a 3-day treatment of AGEs. This result suggested that RAGE could be upregulated by AGEs in RGEC in vitro.

Effects of PKC and PKA on AGE-induced production of ROS. It has been recognized that oxidative stress is implicated in the pathogenesis of diabetic microvasculopathy. Thus, the possible roles of PKC and PKA were studied in AGE-induced ROS production. As demonstrated by Fig. 7A, ROS production in RGECs was significantly enhanced by AGE treatment compared with control. Furthermore, the AGE-induced production of ROS was inhibited by PKCβ inhibitor and PKA agonist; but such an effect was absent in endothelial cells treated with PKC agonist or PKA inhibitor.

![Fig. 4. Comparison of expression and activity of PKA in glomeruli, liver, and brain vessels isolated from diabetic GK rats and nondiabetic controls. A: real-time PCR for PKA expression in glomeruli, liver, and brain vessels of diabetic and nondiabetic rats. The mRNA and protein levels of PKA were determined at 570 nm. *P < 0.05 vs. control. B: Western blot for PKA Cat-α expression in glomeruli, liver, and brain vessels of diabetic and nondiabetic rats. Top: bands of Western blotting; β-actin blot is same as previously presented in Fig. 2C. Bottom: quantitative analysis of bands' signal intensity. Data from quantitative analysis of 3 independent experiments are shown as means ± SD. *P < 0.05 vs. control. C: immunohistochemistry for PKA. D: Measurement of PKA activity. Enzyme activity of PKA was evaluated by addition of a specific PKA substrate, and PKA activity was estimated by measuring the amount of phosphorylated peptide. Top: bands of phosphorylated peptides on an agarose gel. Bottom: PKA activity (nmol·min⁻¹·m⁻¹) determined at 570 nm. *P < 0.05 vs. control.](http://ajpendo.physiology.org/doi/10.1152/ajpendo.00184.2011)
EFFECTS OF PKC AND PKA ON AGE-INDUCED PRODUCTION OF NO. Then, the possible involvements of PKC and PKA on the AGE-induced production of NO were investigated. The production of NO in RGECs was significantly boosted by the treatment of AGEs. Moreover, the AGE-induced production of NO was inhibited by a PKC inhibitor and a PKA agonist (Fig. 7B) but was not affected by PKC agonist PMA or PKA inhibitor H-89.

EFFECTS OF PKC AND PKA ON AGE-INDUCED INOS EXPRESSION. Since the expression of iNOS might be induced due to early endothelial alterations under diabetic conditions and thereby contribute to an elevated production of endothelial NO, the possible involvement of iNOS was studied in AGE-induced endothelial injury. Our finding demonstrated that the mRNA and protein expression of iNOS in RGECs, as measured by real-time PCR and Western blotting, were markedly elevated by the treatment of AGEs. Moreover, the AGE-induced expression of iNOS was inhibited by PKC inhibitor and PKA agonist (Fig. 7C).

Effects of 8-Br-cAMP and H-89 on the excretion rate of urinary albumin. Since there was a lowered expression of PKA in glomeruli of diabetic rats, the possible effects of PKA agonist 8-Br-cAMP and PKA antagonist H-89 on diabetic nephropathy in GK rats were analyzed. The results showed that hyperglycemia in diabetic GK rats was not affected by 8-Br-cAMP or H-89 (Fig. 8A). Urinary protein increased about threefold more in diabetic GK rats than in normal controls. There was a significant decline after treatment of 8-Br-cAMP, and the difference was quite significant at 2 wk compared with diabetic group (P < 0.01; Fig. 8B). H-89 had no significant effect on urinary protein (Fig. 8B). The excretion rate of urinary albumin increased over 50-fold in diabetic GK rats compared with normal rats. Compared with the diabetic group, the levels of urinary albumin were lowered by 8-Br-cAMP (P < 0.05) but boosted by H-89 (P < 0.01). And the difference was significant at week 2 (P < 0.05; Fig. 8C). The results indicated that PKA agonist might protect, whereas the PKA antagonist might impair, the microvascular functions in kidney.

DISCUSSION

Diabetic microvasculopathy is a major complication of diabetes. It is the leading cause of blindness, renal failure, and limb amputation in patients with diabetes by impairing retinal, glomerular, and peripheral microvasculature. Diabetic microvasculopathy is involved in multiple organs and exhibits different severity in different organs. Diabetic microvasculopathy was more significant and severe in kidney and retina than in brain and liver in diabetic GK rats, suggesting that the occur-

Fig. 5. Immunofluorescence double staining for vWF and PKC or PKA in glomeruli, liver, and brain tissue derived from diabetic and control rats. Tissues were double-immunostained for vWF and PKC/PKA. PKC and PKA were labeled with green fluorescence; vWF was labeled with red fluorescence.
RAGE protein expression in RGEC. AGEs in the microvasculature of these organs. Thus, it may
regulations of RAGE expression were different in response to
observed in the glomeruli of diabetic rats. It indicated that the
expression of its ligands (59). Our results showed that the level
of serum AGEs gradually increased under diabetic conditions,
expression of RAGE is often observed in parallel with an elevated
Microvascular lesions correlate with the accumulation of
AGEs, as demonstrated in diabetic retinopathy or renal glomerulosclerosis. RAGE is constitutively expressed at a low
level in microvascular endothelial cells. In diabetes, upregulation
of RAGE is often observed in parallel with an elevated expression of its ligands (59). Our results showed that the level of serum AGEs gradually increased under diabetic conditions, while a markedly elevated expression of RAGE was only observed in the glomeruli of diabetic rats. It indicated that the regulations of RAGE expression were different in response to AGEs in the microvasculature of these organs. Thus, it may
The activation of PKC and the generation of ROS are the
major ways through which AGEs exert their effects via RAGE. Our results showed that the expression of PKCβ was elevated in glomeruli of diabetic rats vs. those of the control rats. This agreed with the findings of previous studies (22, 39) showing that PKC activity was heightened in the pathogenesis of diabetic nephropathy. However, an elevated expression of PKCβ was not observed in liver or brain of diabetic rats in the present study. Previous studies have reported that the activity of PKC was heightened in retina, glomeruli, and aorta under diabetic conditions, but there was no alteration in brain (20, 30, 48). The activation of PKC has
been considered a leading cause in the pathogenesis of diabetic microvasculopathy. Recent studies indicated that different PKC isoforms played specific roles in diabetic microvasculopathy. The activation of PKCα boosted the expression of VEGF and lowered the expression of nephrin in glomeruli of diabetic animals. Ultimately, it led to an increased permeability of glomerular capillaries and albuminuria (20, 30). And the activation of PKCβ, a major isoform of PKC in the vasculature, induced the diabetic microvasculopathy. PKCβ activation has diverse vascular signaling effects that promote diabetes-related vascular pathologies via oxidant stress (4), inflammation (28), and endothelial dysfunction (44). In endothelial cells, PKCβ activation of NAD(P)H oxidase resulting in ROS generation (25), modulation of endothelial (e)NOS (36), and induction of adhesion molecules (28) leads to endothelial dysfunction. Therefore, an inhibitor of PKC was indicated for the prevention of diabetic microvasculopathy (34, 2).
Intriguingly, our study also found that the expression of
PKA catalytic subunit Cat-α was significantly lowered in
glomeruli of the diabetic rats but was elevated in brain and
liver compared with the nondiabetic controls. It was demonstrated that PKA plays a different role from PKC in the
regulation of endothelial cell functions, including TNF receptor
expression (46), endothelial barrier function (62, 17, 23), and vascular relaxation (29, 61, 56). Although the mechanisms for differential expression of PKA remain unclear in different
organs of diabetic rats, metabolic and hormonal alterations
might be involved. The previous studies showed that the
expression of PKA subunit was regulated by several hormones (31, 41). And the releases of glucagon and adrenocorticotrophic hormone became markedly heightened under diabetic conditions (1, 7). A lowered secretion might contribute to a reduced expression of PKA Cat-α subunit (15).
Several mechanisms likely contribute to the protective effect of PKA in diabetic microvasculopathy. First, PKA may suppress the expression of the p22phox subunit of NADPH oxidase to reduce oxidative stress (47, 57). Second, the activation of PKA stimulates the expression and activity of eNOS through inhibiting Rho kinase and activating Akt (35, 50); it consequently improves the vascular endothelial dysfunctions. Third, the activation of PKA may prevent the apoptosis of microvascular endothelial cells (38). Fourth, the activation of PKA could attenuate the hyperpermeability of endothelial cells under diabetic conditions (21).
It was speculated that diabetes-induced reduction in the expressions of PKA Cat-α found in glomeruli might impair the protec-
The role of PKA so as to lead to diabetic nephropathy. And the overexpression of PKA in diabetic rats might exert the protective effects for the development of diabetic microvasculopathy.

Oxidative stress plays a key role in the pathogenesis of diabetic microvasculopathy (4). Nishikawa et al. (37) demonstrated that normalization of mitochondrial ROS levels prevented the glucose-induced activation of PKC, the formation of AGEs, and the accumulation of sorbitol. The expression of iNOS in glomeruli and peritubular capillaries contributed to the elevated endothelial NO production during diabetic nephropathy (27).

In the present study, the crucial roles of PKC/PKA in oxidative stress under diabetic conditions were evaluated in RGEs. Our results showed that AGEs increased the ROS and NO productions in RGEs. However, the generations of AGE-induced ROS or NO were significantly reduced by either an inhibitor of PKC or an activator of PKA. It suggested that the generations of AGE-induced ROS or NO were at least partially promoted by PKC and inhibited by PKA. Therefore, PKC and PKA were both involved in the mitochondrial metabolism in AGE-induced oxidative stress. Thus, it underscored the important role in the pathogenesis of diabetic microvasculopathy.

Furthermore, the protective role of PKA in the development of diabetic nephropathy was investigated in diabetic rats with nephropathy. As shown in RESULTS, albuminuria in diabetic rats was significantly reduced by the PKA activator 8-Br-cAMP but...
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13. Increased by the PKA inhibitor H-89, indicating that PKA could be used as a new target for the prevention and treatment of diabetic nephropathy.

In conclusion, the regulations of RAGE expression in microvascular endothelial cells of different organs are different in response to an elevated level of AGEs. Furthermore, the differential organ-specific activations of PKC/PKA expression in diabetic animals may be one crucial step in organ susceptibility to diabetic microvasculopathy. Thus, PKA activation should be a potential strategy for the therapeutic interventions of diabetic microvasculopathy.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS
Author contributions: H.W., W.-J.Z., and S.-Q.X. performed experiments; H.W. prepared figures; H.W. drafted manuscript; Y.-W.J., S.-Q.X., and H.-N.L. conceived and design of research; J.-N.L. approved final version of manuscript.

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