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Altered Expression and Localization of Insulin Receptor in Proximal Tubule Cells From Human and Rat Diabetic Kidney

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ABSTRACT

Diabetes is the major cause of end stage renal disease, and tubular alterations are now considered to participate in the development and progression of diabetic nephropathy (DN). Here, we report for the first time that expression of the insulin receptor (IR) in human kidney is altered during diabetes. We detected a strong expression in proximal and distal tubules from human renal cortex, and a significant reduction in type 2 diabetic patients. Moreover, isolated proximal tubules from type 1 diabetic rat kidney showed a similar response, supporting its use as an excellent model for in vitro study of human DN. IR protein down-regulation was paralleled in proximal and distal tubules from diabetic rats, but prominent in proximal tubules from diabetic patients. A target of renal insulin signaling, the gluconeogenic enzyme phosphoenolpyruvate carboxykinase (PEPCK), showed increased expression and activity, and localization in compartments near the apical membrane of proximal tubules, which was correlated with activation of the GSK3β kinase in this specific renal structure in the diabetic condition. Thus, expression of IR protein in proximal tubules from type 1 and type 2 diabetic kidney indicates that this is a common regulatory mechanism which is altered in DN, triggering enhanced gluconeogenesis regardless the etiology of the disease. J. Cell. Biochem. 114: 639–649, 2013.

KEY WORDS: INSULIN RECEPTOR; DIABETIC NEPHROPATHY; HUMAN KIDNEY; PROXIMAL TUBULE; GLUCONEOGENESIS

Diabetes affects over 25 million people and is characterized by hyperglycemia resulting from an absolute or partial lack of insulin production by the pancreas (type 1 diabetes) and/or reduced insulin sensitivity by peripheral tissues (type 2 diabetes). Diabetes is the major cause of end stage renal disease and ~30% of diabetic patients develop a devastating nephropathy after years of uncontrolled hyperglycemia [Vallon, 2011]. Despite the different etiology of type 1 and type 2 diabetes, diabetic nephropathy (DN) is a common syndrome in both forms of the disease [Ruggenenti and Remuzzi, 2000]. Glomerular dysfunction is considered as having a role in DN, but deterioration of renal function also correlates with tubular alterations [Singh et al., 2008]. One mechanism involved in uncontrolled hyperglycemia in type 1 and type 2 diabetes mellitus is the increase in renal gluconeogenesis [Gerich et al., 2001]. Studies

Abbreviations used: DN, diabetic nephropathy; GSK3, glycogen synthase kinase-3; FBPase, fructose 1,6-bisphosphatase; IR, insulin receptor; PEPCK, phosphoenolpyruvate carboxykinase; PT, proximal tubule; STZ, streptozotocin.

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Rodrigo Gatica and Romina Bertinat contributed equally to this work.

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by Meyer et al., [1998] have demonstrated that renal glucose production and release increases nearly 300% in diabetic patients, whereas the increment is just of 30% in the liver. Moreover, acidosis further increases renal gluconeogenesis but impairs hepatic gluconeogenesis [Gerich et al., 2001], indicating that the kidney may be the main responsible for increased glucose production during diabetic ketoacidosis. These data point out that kidneys are major sites of deregulated glucose production in diabetic conditions and stress the need to understand the different regulation between renal and hepatic glucose metabolism in humans.

The human kidney contributes to glucose homeostasis through different processes: glucose filtration occurs in the glomeruli while glucose reabsorption and gluconeogenesis are carried out by proximal tubules (PT) [Yáñez et al., 2005; Eid et al., 2006]. Further glucose utilization is considered to occur in cortical collecting and distal tubules and the entire medulla [Yáñez et al., 2005; Mitrakou, 2011]. PTs are particularly sensitive to hyperglycemia because glucose uptake is insulin-independent; by contrast insulin is the main negative modulator of renal gluconeogenesis by down-regulating the expression of gluconeogenic genes [DeFronzo et al., 2012]. One of the main gluconeogenic genes responsible for enhanced endogenous glucose production encodes the cytosolic form of phosphoenolpyruvate carboxykinase (PEPCK) [Quinn and Yeagley, 2005]. In contrast to other metabolic enzymes, PEPCK activity is not regulated at the post-translation level, but is directly and proportionally modulated by the amount of PEPCK mRNA, which is primarily dictated by the rate of gene transcription [Quinn and Yeagley, 2005]. Normally, PEPCK expression is induced by glucagon and glucocorticoids during fasting, whereas insulin dominantly inhibits its transcription after feeding [Quinn and Yeagley, 2005]. However, the effect of diabetes over the expression and localization of PEPCK in rat and human kidney and its relationship with the expression of the main regulator of insulin pathway, the insulin receptor, remains unclear.

Insulin action is dependent on the interaction with the insulin receptor (IR). The IR gene encodes a 190 kDa pro-receptor protein, which is cleaved into α (containing the insulin-binding domain) and β (containing the tyrosine kinase domain) subunits [Gorden et al., 1989]. Upon ligand binding, β-subunits catalyze auto-phosphorylation of specific tyrosine residues, activating a cascade of intracellular signaling events, which regulate multiple biological processes. A key-signaling molecule in insulin pathway is GSK3β, a serine/threonine kinase which is normally active, and whose inactivation by insulin is considered essential for a normal insulin catabolic response. Indeed, several studies have implicated dysregulation of GSK3 in the pathogenesis of diabetes [Sakamaki et al., 2012]. One of the main consequences of insulin signaling is suppression of endogenous glucose production by kidney and liver [Pillot et al., 2009]. In parallel, inactivating mechanisms are also induced to limit IR activity and to prevent prolonged insulin action: protein tyrosine phosphatases reduce the IR activity by dephosphorylating important tyrosine residues on IR itself, whereas other proteins sterically block IR interaction with downstream signaling proteins [Ueki et al., 2004]. Another mechanism of negative control is down-regulation of IR level, that can be achieved by ligand-stimulated internalization and degradation or regulation of synthesis of new receptor [Knutson, 1991]. It is interesting that mRNA and protein levels of IR are increased in the liver from diabetic rats, compared with control individuals. By contrast, IR expression is decreased in the kidney from different rat models of diabetes [Tiwari et al., 2007]. Nevertheless, studies on the expression of IR in diabetic kidney have not been reported in human patients. This is an important point because murine models and humans do not always share the same molecular behavior. In this sense, in vivo human experiments indicate that infused insulin induces an almost 50% reduction in renal glucose production [Cersosimo et al., 1999], suggesting that regulation of renal gluconeogenesis can be associated to the expression of IR in the nephron.

Together these data open the question about the role of insulin signaling in the kidney and generate the need to further investigate and characterize the expression of IR in the different segments of the nephron in normal and diabetic conditions, in relation with one of its main targets, the expression of PEPCK. Since most studies are performed in murine models, care has to be taken when extrapolating data from one specie to another.

MATERIALS AND METHODS

HUMAN SAMPLES

Samples were obtained from the non-cancerous pole of surgically removed kidney from adult non-diabetic ($n = 4$) and type 2 diabetic ($n = 4$) patients with renal carcinoma, at the Urology Department of Hospital Clínic, Barcelona, Spain. The institutional review board at Hospital Clínic approved the study, and patients gave informed consent for biopsy collection and analysis. Diabetic patients were selected by fasting glycemia >140 mg/dl and glycosylated hemoglobin (HbA1c) >6.5%. Average age of non-diabetic patients was 69 ± 8 years-old ($n = 4$; all male patients), and that of diabetic patients was 67 ± 8 years-old ($n = 4$; three male and one female patients).

EXPERIMENTAL ANIMALS

Ten weeks-old male Sprague–Dawley rats (240–260 g) were maintained on a standard diet with free access to water. Type 1 diabetes was induced by a single intravenous injection of STZ (60 mg/kg body weight) (Sigma Chemical Co., St. Louis, MO) in 0.1 M citrate buffer, pH 4.5. Control rats received buffer alone. Control and diabetic rats were sacrificed by intraperitoneal injection of ketamine/xylazine (160 and 20 mg/kg body weight, respectively) 120 days after STZ treatment. All experiments were approved by the Institutional Animal Care and Use Committee of Universidad Austral de Chile.

ANTIBODIES

Rabbit anti-IR β subunit was from Santa Cruz Biotechnology, Inc. rabbit anti-tubulin was from Sigma, rabbit anti-total GSK3β was from Santa Cruz Biotechnology, Inc. rabbit anti-phosphorylated GSK3β (Ser 9) was from cell signaling, rabbit anti-hepatic FBPase and cytosolic PEPCK were prepared in our laboratory [Yáñez et al., 2003; Bertinat et al., 2012]. Secondary antibodies were donkey anti-rabbit IgG HRP (Jackson ImmunoResearch), Alexa Fluor 633-

conjugated goat anti-rabbit IgG (Molecular probes, Eugene, OR) and Universal ICQ LSAB plus kit, (DAKO Corporation, Carpenteria, CA).

ISOLATION OF PROXIMAL TUBULES

Rat proximal tubules were isolated using the procedure described in by [Kribben et al., 2003]. Basically, rats were anesthetized and right kidney was flushed with oxygenated solution A (in mM: 112 NaCl; 20 $NaHCO_3$; 5 KCl; 1.6 $CaCl_2$; 2 Na_2HPO_4; 1.2 $MgSO_4$; 5 glucose; 10 HEPES; 10 mannitol; 1 glutamine; 1 sodium butyrate; 1 sodium lactate; 2,000 I.E. heparin; pH 7.4), containing 0.17 mg/ml of collagenase (type A, 0.29 U/mg; lot 83827820/34) and 0.42 mg/ml of hyaluronidase (both from Boehringer-Mannheim, Mannheim, Germany). Renal cortex was minced and incubated in oxygenated solution A containing 0.35 mg/ml collagenase and 0.25 mg/ml hyaluronidase with gentle stirring for 30 min. Homogeneous population of nephron segments were separated by gradient Percoll centrifugation. Tubular fragments were resuspended in 50 ml of 45% Percoll (Phamacia, Uppsala, Sweden) and centrifuged at 45,700*g* and 4°C for 30 min. Proximal tubules (>95% of purity by FBPase immunofluorescence analysis) were removed from the lowest density band, washed three times in PBS and resuspended in serum-free culture media (DMEM/Ham's F12 with 10 ng/ml EGF, 5 mg/ml human transferrin, 5 mg/ml insulin, 0.05 mM hydrocortisone, 50 mM prostaglandin E1, 50 nM selenium, 5 pM tri-iodothyronine, 50 U/ml penicillin and 50 mg/ml streptomycin). For isolation of human proximal tubules the procedure was similar to that of rat, but starting material was a piece of the non-cancerous pole of the surgically removed kidney.

WESTERN BLOT

Cell homogenates were prepared with lyses buffer (20 mM HEPES pH 7.5, 10 mM EGTA, 2.5 mM $MgCl_2$) plus 1% NP-40 and protease inhibitors (Calbiochem, Darmstadt, Germany). Twenty micrograms of total proteins were fractionated in 4–12% SDS–PAGE, transferred to PVDF membranes and probed overnight with primary antibodies [Yáñez et al., 2007]. Following incubation with an HRP-conjugated secondary antibody, reaction was developed using the Pierce ECL Western Blotting Substrate (Pierce Biotechnology, Rockford, IL).

REAL-TIME RT-PCR

Total RNA was extracted from rat renal cortex and isolated proximal tubules, using SV Total RNA Isolation System (Promega). cDNA was synthesized from 2 μg of total RNA using the SuperScript III First-Strand Synthesis SuperMix (Invitrogen). For qPCR we used unlabeled specific primers for rat 18S (reference gene) and IR (Assay ID: Rn01637243_m1), FBPase (Rn00586801_m1) and PEPCK (Rn01529014_m1) genes, TaqMan Universal PCR Master Mix No AmpErase UNG and TaqMan ABI 7700 sequence Detection System (all from Applied Biosystems). Primers were annealed at 60°C and run for 40 cycles. Data were captured using Sequence Detector Software 2.2 (Applied Biosystems).

IMMUNOHISTOCHEMISTRY

Tissue was deparaffinized in xylene and rehydrated in graded ethanol and endogenous peroxidase activity was inhibited with 3% H_2O_2. Tissue was blocked with 3% BSA and permeabilized with 0.3% Triton X-100 for 20 min. Primary antibody was incubated overnight at 4°C and LSAB plus kit was incubated as described by the manufacturer, followed by washing in PBS [Yáñez et al., 2003]. The reaction was developed with DAB and nuclear counter-staining was performed with hematoxylin. For immunofluorescence, Alexa Fluor-secondary antibody was used and counter-staining was carried out with DAPI.

ENZYME ACTIVITY

FBPase and PEPCK activities were estimated spectrophotometrically by changes in the absorbance at 340 nm due to reduction or oxidation of NAD, respectively, in a coupled enzyme assay at 30°C in 0.5 ml final volume and 10 μl of dialyzed total kidney extract [Bertinat et al., 2012].

SEMIQUANTITATION OF RENAL ALTERATIONS

The anatomo-pathological analysis was performed in renal slices prestained with Masson's trichrome or PAS reagent, using the methodology described in Zoja et al. [2002]. Briefly, tubular changes (atrophy and dilatation) were graded from 0 to 4+ (0, no changes; 1+, changes affecting <25%; 2+, changes affecting 25–50%; 3+, changes affecting 50–75%; 4+, changes affecting 75–100% of the sample). On the other hand, at least 100 glomeruli were examined in each sample, and glomerular damage was expressed as the percentage of sclerotic glomeruli. Samples were analyzed by the same pathologist who was blind to experiment.

STATISTICAL ANALYSIS

Experiments were performed three times. Results are expressed as mean ± SD. Statistical analysis was performed with Student's *t*-test. Data were considered statistically significant for $P < 0.05$.

RESULTS

EXPRESSION OF GLUCONEOGENIC ENZYMES IN DIABETIC RAT KIDNEY

Compared with non-diabetic control animals, STZ-induced diabetic rats showed chronic hyperglycemia, glucosuria, and proteinuria (Table I). Renal alterations, including tubular damage and glomerular

TABLE I. Clinical Biochemistry of Control and STZ-Induced Diabetic Rats

	Control	Diabetic (4 month)	*P*
Body weight (g)	376.8 ± 41.9	165.4 ± 35.3	<0.01
Glycemia (mg/dl)	82.2 ± 10.7	409.8 ± 88.7	<0.01
Insulinemia (μU/ml)	10.8 ± 4.7	1.9 ± 1.2	<0.01
Triglyceridemia (mg/dl)	91.5 ± 20.1	379.0 ± 149.3	<0.05
Glucosuria (mg/dl)	0	961.2 ± 315.3	<0.01
Albuminuria/creatinuria	153.9 ± 43.0	769.1 ± 304.3	<0.05
Glomerullar lession (%)*	0	5.73	<0.05
Tubular damage (score)*	0.17 ± 0.2	2.73 ± 1.10	<0.01

The previous day rats were kept in metabolic cages to collect urine samples for glucose, albumin and creatinin determination. Body weight was checked immediately previous to sacrifice. After euthanasia, serum was collected and blood glucose, insulin and triglyceride levels were analyzed. After fixation, inclusion and Masson/PAS staining, tubular changes (atrophy and dilatation) and glomerullar alterations were analyzed.
*Semiquantitation of glomerular lession and tubular damage is described in Materials and Methods.
Values are expressed as mean ± SD and analyzed using Student's *t*-test. The number of animals used for detection of each parameter was n = 5.

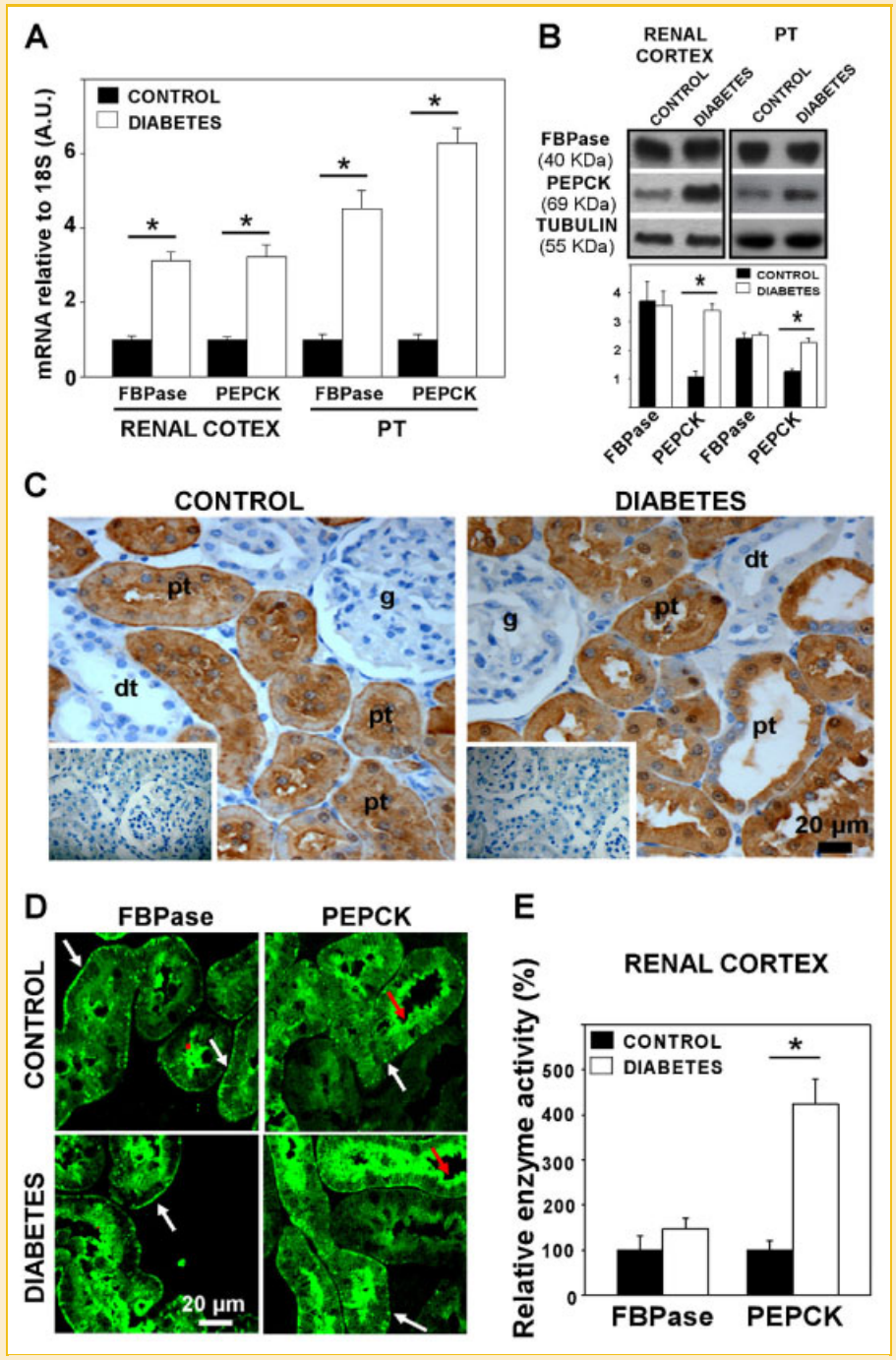


Fig. 1. Expression of gluconeogenic enzymes in diabetic rat kidney. A: qRT-PCR analysis of PEPCK and FBPase in renal cortex and isolated PT from control (black bars) and diabetic (white bars) rats. B: Western blots of FBPase, PEPCK and tubulin in renal cortex and isolated PT from control and diabetic rats. C: Immunohistochemistry of PEPCK in control and diabetic rat kidney slices—g, glomerulus; dt, distal tubule; pt, proximal tubule; Inner images, negative control. D: Immunofluorescence of FBPase and PEPCK in kidney from control and diabetic rats. Red arrow: apical distribution; white arrow: basolateral distribution. E: Relative FBPase and PEPCK activity in cortex from control (black bars) and diabetic (white bars) rat kidney; $^{*}P < 0.05$; n = 5 for each group.

sclerosis, were homogeneously established 4 months after diabetic induction (Table I). In this group of diabetic animals, enhanced mRNA expression and protein level of PEPCK in renal cortex (Fig. 1A,B) were observed. Moreover, another key gluconeogenic enzyme, FBPase, was only over-expressed at the mRNA level (Fig. 1A), but protein content did not evidence variations (Fig. 1B). The expression of PEPCK and FBPase in isolated PT was similar to that observed in total kidney (Fig. 1A,B), in agreement with the exclusive expression of gluconeogenic enzymes in this particular renal epithelium (Fig. 1C,D). This result indicates that freshly isolated PTs are an

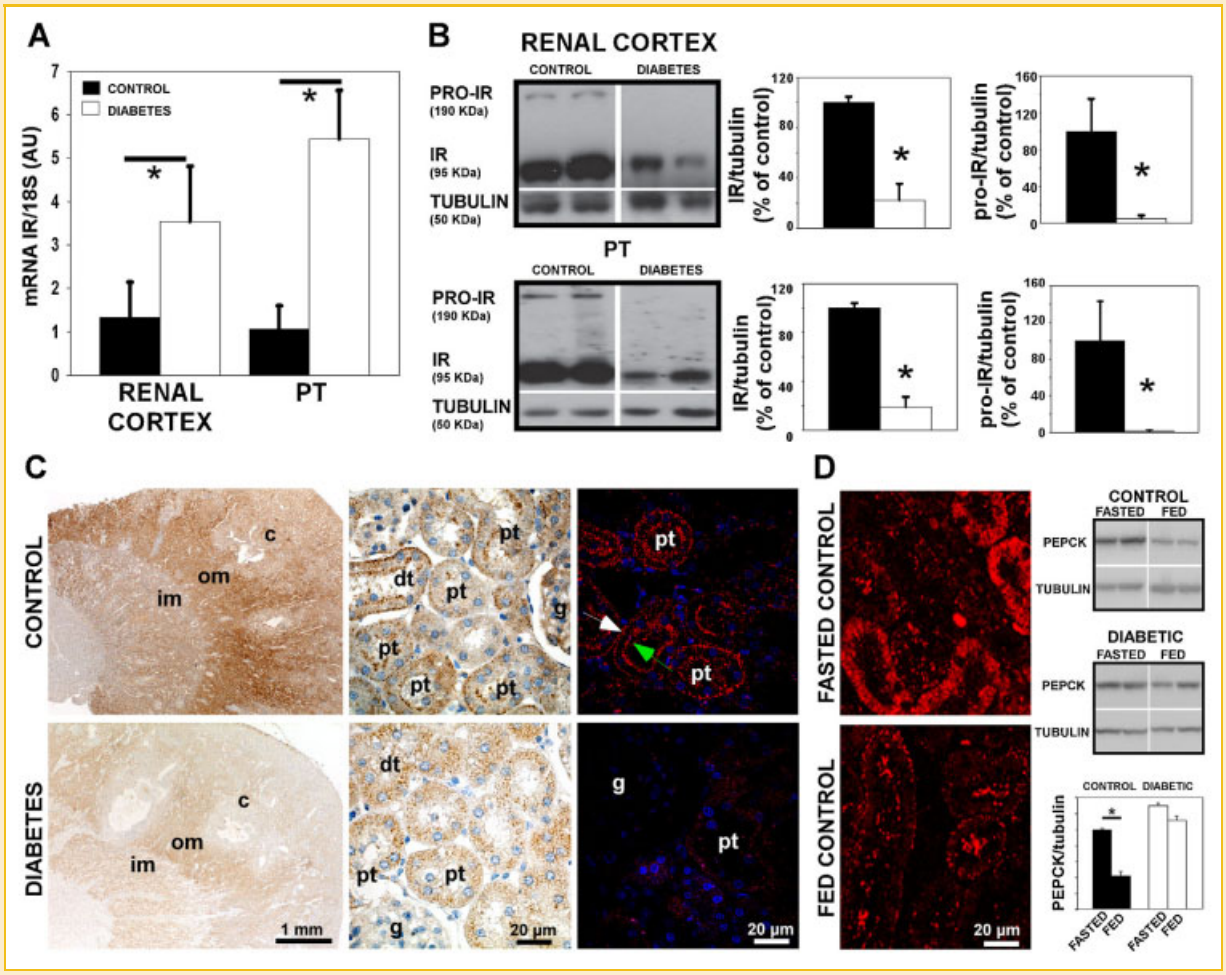


Fig. 2. Expression of IR in diabetic rat kidney. A: qRT-PCR of IR in renal cortex and PT from control (black bars) and diabetic (white bars) rats. B: Western blots of IR, pro-IR and tubulin in renal cortex and isolated PT from two representative control and diabetic rats. C: Immunohistochemistry and immunofluorescence of IR in rat kidney slices from control and diabetic rats. c, cortex; mi, inner medulla; om, outer medulla; g, glomerulus; dt, distal tubule; pt, proximal tubule. D: Immunofluorescence of IR and Western blot analysis of PEPCK expression in kidney from fasted/refed control rats. $^{*}P < 0.05$; n = 5 for each group.

excellent model for analysis of tubular alterations. Immunohistochemical analysis of PEPCK confirmed the morphological changes, specially atrophy and dilatation, induced by diabetes in PT (Fig. 1C). At the subcellular level, both PEPCK and FBPase are detected in compartments close to the basolateral and apical membranes of the PT from control rat kidney, but an enhanced localization to the apical membrane compartment was evidenced in PT from diabetic rat kidney (Fig. 1D). Interestingly, the analysis of enzymatic activity revealed that only PEPCK, but not FBPase, presented an enhanced activity in diabetic versus control rat kidney, with a fourfold increase (Fig. 1E).

EXPRESSION OF IR IN DIABETIC RAT KIDNEY

Whereas IR mRNA was significantly elevated in renal cortex from diabetic rats (Fig. 2A), protein levels were drastically reduced (Fig. 2B). Isolated PT from diabetic rat kidney also showed a significant increment on IR mRNA (Fig. 2A) and also a significant reduction in IR protein levels (Fig. 2B), indicating that IR is strongly expressed in this epithelium and that overall changes in IR regulation are maintained after isolation. By contrast, IR mRNA levels and protein number were both enhanced in the diabetic rat liver, compared with the respective control (data not shown). Interestingly, protein levels of IR precursor (Pro-IR) are strongly reduced in the diabetic condition, in both total renal cortex and isolated PT. In the context of the renal tissue, IR showed a higher immunoreactivity in the outer medulla (enriched in segment 3 - straight-PT) than in the cortex and inner medulla of kidney (Fig. 2C), and a polarized distribution to the apical and basolateral membrane compartments was observed in PT and distal tubules (Fig. 2C). Notably, IR expression and subcellular localization was altered in diabetes, being mainly detected in the cytoplasm of tubular epithelial cells, with reduced immunoreactivity in all tubules (Fig. 2C). In kidneys from control rats, IR displayed cytoplasmic accumulation during fasting and membrane accumulation after refeeding, suggesting a physiological regulatory function for the subcellular compartmentalized distribution (Fig. 2D). In the diabetic rat kidney, re-feeding did not stimulate IR membrane accumulation (data not shown). A clear correlation between membrane accumu-

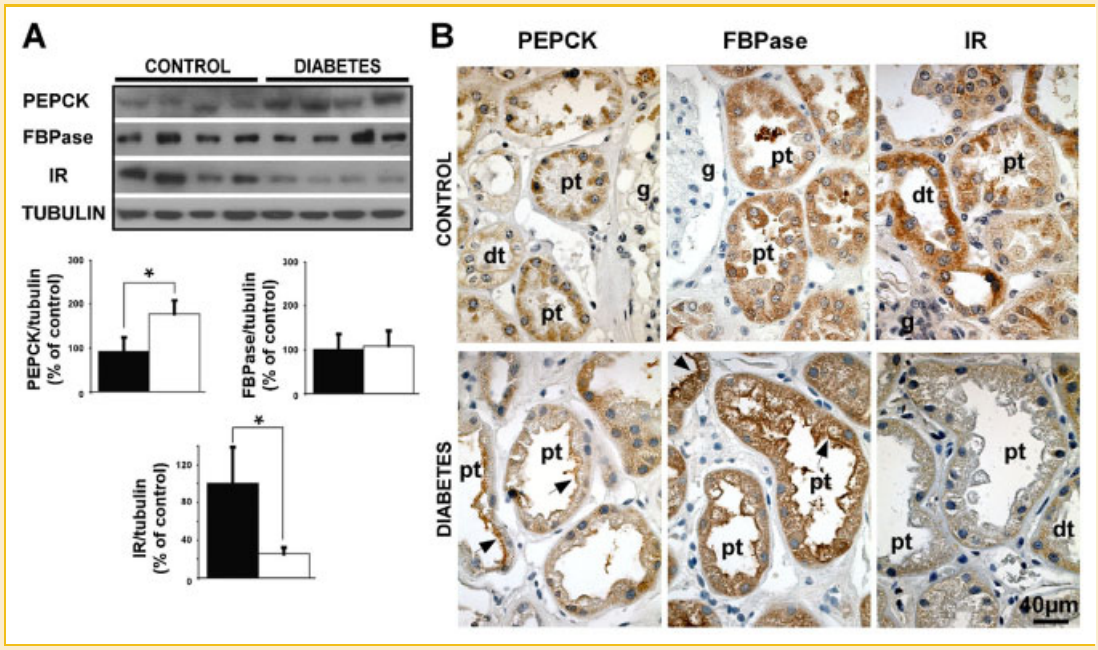


Fig. 3. Expression of gluconeogenic enzymes and IR in human kidney. A: Western blot analysis of FBPase, PEPCK, IR and tubulin in renal cortex biopsies from control and type 2 diabetic human patients. B: Immunohistochemistry of FBPase, PEPCK and IR in renal cortex biopsies from control and type 2 diabetic patients. Black arrows: apical distribution. $^{*}P < 0.05$; n = 4 for each group.

lation of IR and down-regulation of renal PEPCK during feeding of control rats was observed, whereas refeeding did not stimulate down-regulation of PEPCK protein levels in the diabetic kidney (Fig. 2D).

EXPRESSION OF GLUCONEOGENIC ENZYMES AND IR IN TYPE 2 DIABETIC HUMAN KIDNEY

Laboratory animal models are widely used in clinical research, but data cannot be always extrapolated to humans. Notably, repeating the same analysis as in rat kidney, we also detected increased protein levels of PEPCK but not FBPase in renal biopsies from diabetic patients, in comparison to the non-diabetic controls (Fig. 3A). Moreover, IR protein showed a significant reduction in renal biopsies from diabetic patients (Fig. 3A). As it was detected in diabetic rat kidney, FBPase and PEPCK redistribution to the apical membrane compartment was also observed in PT from diabetic patient biopsies (Fig. 3B). A critical reduction of IR immunoreactivity was also detected in human diabetic kidney (Fig. 3C) but, in contrast to the results obtained in the rat model, a pronounced down regulation of IR level was observed preferentially in PT, with less effect in distal tubules.

INSULIN SIGNALING AND GSK3β PHOSPHORYLATION IN THE DIABETIC KIDNEY

To compare the activation of the insulin-signaling cascade between the control and the diabetic kidney, we centered our attention on GSK3β, a key kinase involved in deregulated glucose metabolism and insulin resistance. Since GSK3β is expressed in the entire kidney, isolated proximal tubules from control and diabetic human and rat kidney were used in order to detect specific changes in this particular renal structure, in relation with the expression of PEPCK. Expression of total GSK3β in isolated renal PT from diabetic rats showed a significant reduction of the expected protein of 46 kDa, with an increase of degradation products (Fig. 4A). The analysis also demonstrated a proportional reduction of phosphorylation on serine 9 (pGSK3β) of the 46 kDa GSK3β band. Ser9 phosphorylation was not detected on the lower MW bands (Fig. 4A). In PT from diabetic patients, total GSK3β expression was not significantly altered, but again a reduction of the corresponding phosphorylated form was observed (Fig. 4B).

DISCUSSION

DN is the commonest cause of end-stage renal disease and is an increasing worldwide health care problem to study. Indeed, kidneys are major sites of deregulated glucose production in diabetic patients. Taking into account that renal gluconeogenesis is negatively modulated by insulin, it is expected that this inhibitory effect is absent during diabetes. Nevertheless, the exact mechanism of how insulin deficiency or resistance produces the specific pattern of deregulated cellular functions is not yet fully understood. Hence, the aim of this study was to compare the effect of diabetes over IR expression in relation with PEPCK, in human and rat kidney. Here, we demonstrated that in the kidney of both human type 2 diabetic patients and in a type 1 diabetic rat model, a significant reduction in the protein levels of IR and a consequent increase of PEPCK is produced. These data support the similarities of the renal alterations between type 1 and type 2 diabetic patients, and also show that despite the dissimilar etiology of both forms of diabetes, even between different species such as human and rat, deregulation of the

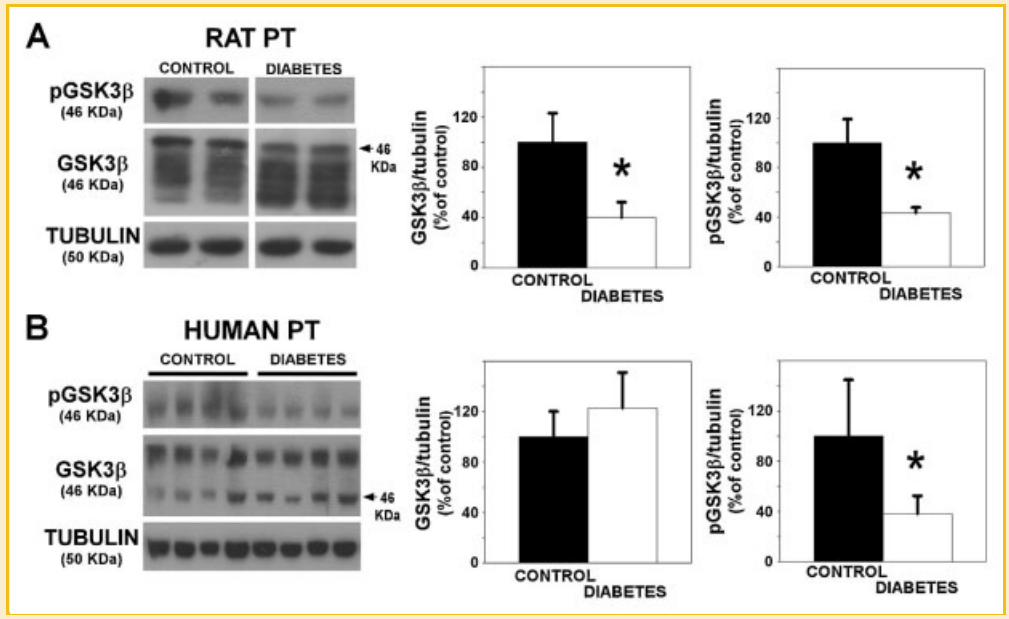


Fig. 4. GSK3β activation in human and rat diabetic kidney. Western blot analysis of total and Ser9 phosphorylated GSK3b in isolated PT from A: control and diabetic rat kidney; and B: non-diabetic and type 2 diabetic patients. $^{*}P < 0.05$; n = 5 for each rat group and n = 4 for each human group.

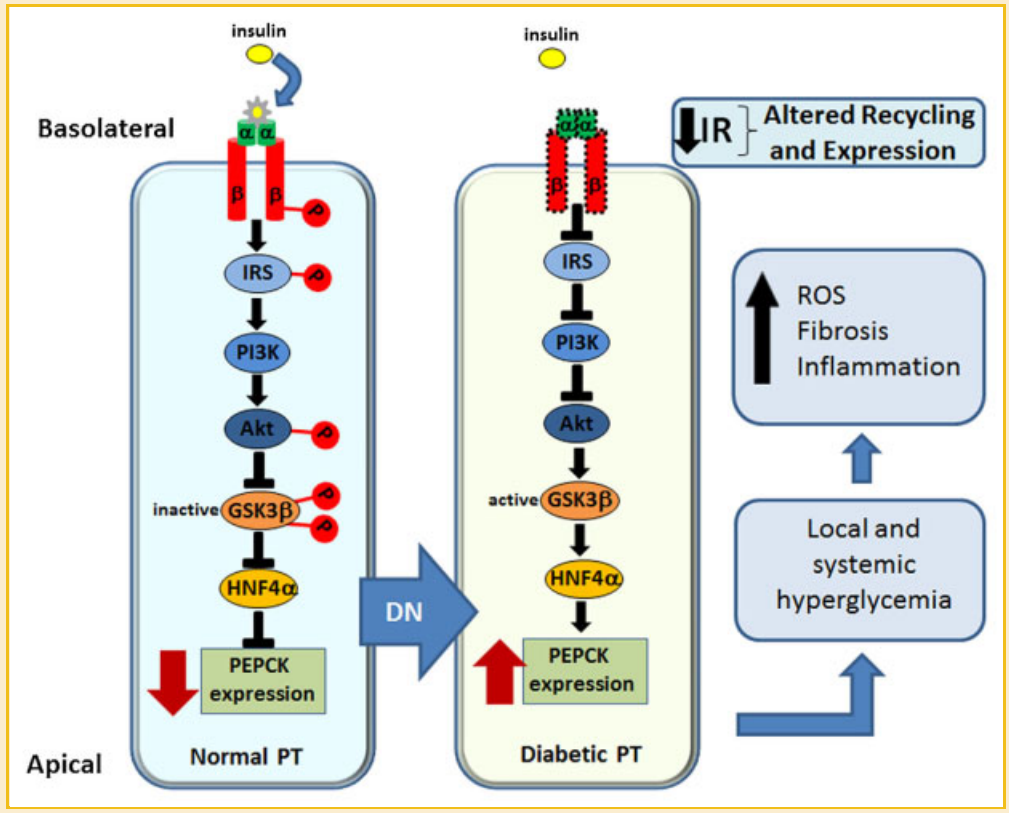
renal insulin pathway could be attributed to its main regulator, the IR. On the contrary, diabetes induces an increase on the mRNA and protein levels of IR in the liver, indicating that alterations of the insulin pathway are different between organs. Moreover, by analyzing isolated PT we demonstrated that this specialized epithelium, which is the exclusive responsible for renal gluconeogenesis, contains a large amount of IR protein. Hence, altered expression of IR in human diabetic kidney could be one of the main triggers for enhanced gluconeogenesis and the consequent dysfunction of PT in DN, regardless the etiology of the diabetic condition.

It is well established that the excessive release of glucose into the circulation is a major factor responsible for fasting hyperglycemia in diabetes, and an increase of renal glucose delivery has been demonstrated [Gerich et al., 2001]. The diabetic human kidney contributes to hyperglycemia through two different mechanisms, both occurring in the PT: enhanced glucose reabsorption and gluconeogenesis [Yáñez et al., 2005; Mitrakou, 2011]. Whereas the former is insulin-independent, the latter is negatively regulated by the hormone [DeFronzo et al., 2012], explaining why in a gram–gram basis the kidney is able to release more glucose than the liver during diabetes [Meyer et al., 1998]. Over-expression of rate-limiting gluconeogenic enzymes has been observed in diabetes [Krones-Herzig et al., 2006]. However, these enzymes are not all regulated by mass. For instance, a distinct regulation of PEPCK and FBPase has been reported in the liver from control and STZ-induced diabetic rats [Bertinat et al., 2012]. Here, in both human and rat kidney we observed the same modulation of FBPase and PEPCK expression than previously reported in rat liver, suggesting that different regulation of these enzymes is maintained between organs and species, maybe providing metabolic plasticity for regulation of the gluconeogenic flux. Indeed, we observed over-expression and hyperactivity of PEPCK in the diabetic kidney, whereas FBPase did not change. This result supports the fact that FBPase in the kidney might be modulated at the post-translational level as well [Bertinat et al., 2012]. Of interest is the apical redistribution of both enzymes in diabetic human and rat PT, suggesting that enhanced gluconeogenesis could mainly takes place from increased luminal substrate uptake, and this activation can be related to insulin signaling regulation. PT hypertrophy and extracellular matrix accumulation are early features of DN, and hyperglycemia-induced oxidative stress is implicated in the etiology of these alterations [Brezniceanu et al., 2008]. Peritubular capillaries are essential for normal structure and function of renal tubules; but tubulo-interstitial injury and fibrosis induce loss of these capillaries during diabetic progression [Singh et al., 2008]. In this context, we can speculate that basolateral transport is being altered due to the interstitial fibrogenic process and the activated gluconeogenic machinery moves to the apical compartment, where the enriched glomerular ultrafiltrate is available.

Once activated, insulin signaling cascade also turns on negative mechanisms to stop insulin action itself, avoiding undesired hypoglycemia [Knutson, 1991; Ueki et al., 2004; Fagerholm et al., 2009]. STZ-induced diabetic rats display an increase in liver IR mRNA and protein, which is reversed by insulin treatment [Amessou et al., 1999]. Hepatic IR is also elevated in diabetic patients, with little increase in binding affinity [Arner et al., 1986]. On the contrary, in diabetic rat kidney, the increase of IR mRNA is not corresponded with enhanced IR protein [Tiwari et al., 2007] and present data]. At this regard, IR is detectable in a polarized fashion in renal epithelial cells: basolateral IR may senses blood insulin and apical IR may be involved in insulin reabsorption. The kidney plays a major role in insulin clearance and megalin has been described as the main receptor-mediated endocytic machinery of filtered insulin

in PT [Orlando et al., 1998]. However, megalin is primarily expressed in PT segment 1 (S1), with lesser amounts in S2 and S3 [Orlando et al., 1998]. An opposite IR expression (present data) suggests a complementary non-redundant system with a first low affinity (megalin) and a second high affinity (IR) reaction for insulin removal from the filtrate. This tandem system for insulin reabsorption may produce a great impact on the bioavailability of insulin at the level of PT and downstream nephron segments. Expression of several hepatic genes are controlled by metabolites rather than hormones [Dentin et al., 2012], and long-term culture with high glucose has shown to increase IR and its kinase activity [Hauguel-De-Mouzon et al., 1995]. Nevertheless, renal proximal epithelial cells do not metabolize glucose efficiently [Bolon et al., 1997], which may explain why IR protein expression is reduced despite chronic hyperglycemia. Besides, studies by Tiwari et al. [2007] have shown a decreased expression of IR in distal and connecting tubules in the inner medulla of obese Zucker- and high-fat diet-insulin resistant rats. These models of diabetes are both characterized by elevated circulating levels of free fatty acids (FFA), which are recognized as inhibitors of glucose transport and glycolysis [Boden, 2002]. In the kidney, the enzymatic machinery for glycolysis is compartmentalized to non-PT tubules [Yáñez et al., 2005; Mitrakou, 2011]. If IR expression is regulated by glycolytic intermediates, inhibition of glucose metabolism by excess FFA in distal and collecting tubules could account for greater down-regulation of IR in these specific structures. Moreover, one of the first changes in the diabetic kidney is glycogen accumulation in the epithelial cells of the distal tubular system [Nannipieri et al., 2001], which are not observed in the animal models used by Tiwari et al. [2007]. Instead, we have detected glycogen accumulation as soon as 2 weeks after diabetes-induction (unplublished data), suggesting a distinct regulation of glucose metabolism in the distal tubular system of different animal models of diabetes. In contrast, we found that the major reduction of IR occurs in the renal cortex and outer medulla of STZ-induced diabetic rats and human renal biopsies, suggesting that this is the most sensitive region of insulin pathway in the models studied in this work. Here, we reported for the first time that IR protein levels are also reduced in the kidney from diabetic patients. Notably, IR down-regulation was almost equal in proximal and distal tubules from diabetic rats, whereas it was much more prominent in proximal than in distal tubules from diabetic patients. These differences might be explained by the different models of diabetes: STZ-induced diabetic rats display type 1 diabetes whilst examined biopsies were from type 2 diabetic patients. Moreover, specie-specific differences could also be involved. However, both models highlight the relevance of insulin regulation and the role of IR in insulin signaling activation in PT and the appearance of insulin resistance in these specific tubules during progression of DN. In addition, IR expression deficiency cannot be attributed to processing problems from the precursor form, since we detected a drastic down-regulation of pro-IR as well. On this regard, the uncoupling mechanism, which induces IR mRNA accumulation in the diabetic kidney, is an open question. One possible explanation comes from the cytoplasmic polyadenylation element binding (CPEB) protein which functions as a translational repressor of specific genes [Villalba et al., 2011]. Certainly, the new described function of CPEB family in adult tissues together with the fact that mRNAs usually contain multiple regulatory elements, provides an enormous combinatorial potential for translational control in diverse pathologies, however no studies have examined the potential role of CPEB during DN progression yet. Together with the low protein levels, we also observed an altered subcellular localization of IR in diabetes (i.e., cytoplasmic accumulation), but attribution to increased internalization or impaired translocation to the plasma membrane, or both, remains to be established. In this sense, caveolae have been recognized as key membrane structures for proper IR signaling, and caveolin-1-deficient mice display hyperinsulinemia after feeding with a high-fat diet [Cohen et al., 2003]. Interestingly, a 90% reduction of IR protein levels, without changes in IR mRNA, where observed in fat tissue but not in the liver from this caveolin-1 null model [Cohen et al., 2003]. Unfortunately, the kidney was not evaluated in that study, but down-regulation and mislocation of caveolin-1 protein has been reported in diabetic rat kidney [Komers et al., 2006].

Contrary to the vast majority of kinases involved in insulin signaling, GSK3β is normally active under unstimulated conditions and is negatively regulated in response to insulin, which is considered an essential step for a normal insulin catabolic response [Patel et al., 2008]. Initially, activation of GSK3β has been implicated in altered glucose metabolism, insulin resistance and diabetes through phosphorylation and inactivation of glycogen synthase [Patel et al., 2008]. Several studies have also correlated GSK3β over-expression and activation with other pathological processes, including renal interstitial fibrosis [Powell et al., 2009; Yan et al., 2012]. It has been recently reported that GSK3β positively regulates PEPCK expression in the liver [Sakamaki et al., 2012]. Since one of the normoglycemic effects of the GSK3 inhibitor L803-mts is down-regulation of PEPCK mRNA levels in liver and muscle from diabetic mice [Kaidanovich-Beilin and Eldar-Finkelman, 2006], we suggest that a GSK3β-mediated mechanism is triggering PEPCK over-expression in the diabetic PT. At this regard, the renal PT cell resembles a periportal hepatocyte in many metabolic aspects, such as its ability to produce endogenous glucose but a low efficiency to metabolize it [Katz, 1992; Bolon et al., 1997]. In fact, the hepatic nuclear factor 4α (HNF4α) has been implicated in GSK3β-mediated PEPCK induction in the liver [Sakamaki et al., 2012]. Kanazawa et al. [2010] have demonstrated that HNF4α is exclusively expressed in PT from adult kidney, and we have observed a significant alteration of HNF4α mRNA levels in response to different metabolic conditions: HNF4α mRNA was down-regulated in control refed versus fasted rat kidney cortex, whereas in the diabetic kidney HNF4α mRNA expression was proportionally induced with the progression of the alterations (Supplementary Fig. 1). We propose that a similar positive regulation of PEPCK expression through GSK3β and HNF4α is operating in the PT during diabetes, leading to enhanced glucose release (Scheme I). Local and systemic hyperglycemia caused by insulin resistance/deficiency induces toxicity by activation of sorbitol and hexosamine pathways, and accumulation of advanced glycation end-products and reactive oxygen species [Brownlee, 2005]. These cytotoxic agents progressively alter protein function and lead to perturbation of cellular behavior, triggering release of pro-inflammatory mediators and inflammatory infiltration. Indeed, PT cells exposed



Scheme. I. In normal renal proximal tubule (PT) cells, insulin binding to the insulin receptor (IR) activates a cascade of intracellular events which, among others, phosphorylates and inactivates glycogen synthase kinase 3β (GSK3β). Inactivated GSK3β impairs hepatic nuclear factor 4α (HNF4α) transactivation activity over PEPCK promoter. On the contrary, in diabetic PT cells, altered expression of IR (dotted lines) negatively impacts on insulin signaling, relieving GSK3β phosphorylation and activating it. Now HNF4α induces PEPCK gene expression, which in turn activates gluconeogenesis. Local and systemic hyperglycemia leads to increased production of cytotoxic agents (sorbitol, advanced glycation-end products, reactive oxygen species), stimulating inflammatory infiltration and fibrosis in the renal tissue.

to high-glucose produce abnormal high-levels of the fibrogenic transforming growth factor (TGF)-β, which in an autocrine fashion stimulates collagen synthesis and secretion [Han et al., 1999]. Despite alterations produced by a deficient insulin signaling are indirectly attributed to hyperglycemia, a specific IR knockout in podocytes has recently revealed an increase production of matrix proteins under normoglycemia [Welsh et al., 2010], which suggests that IR signaling impairment itself is involved in renal fibrosis. Since cross-talk between IR and integrin signaling pathways is disturbed during diabetes [Gupta and Dey, 2009], altered integration and transduction of extracellular environment signals could account for exaggerated extracellular protein production and fibrosis in the proximal tubules of diabetic kidney. In addition to HNF4α, other transcription factors are also orchestrated in the regulation of PEPCK promoter activity, and the dominant inhibitory action of insulin is exerted by the rapid disruption of the active transcription complex [Hall et al., 2007]. Given the central role of GSK3β in insulin signaling, it is expected that its activation might be involved in enhanced PEPCK activity during diabetes. Since the cell signaling networks of GSK3 resides at the nexus of multiple pathways, comprising insulin signaling, canonical wingless (Wnt), NF-κB, and NF-AT pathways, all of them altered during insulin resistance [Ge et al., 2011], a therapy using GSK3β inhibitors must be extensively studied.

At the present, treatment of DN commonly includes control of hyperglycemia and blood pressure, blockage of the renin-angiotensin system, and restriction of salt and protein intake. However, it is difficult to reach ideal blood pressure and glycemic control. Moreover, metformin, one of the first line drugs for diabetic treatment, is not recommended when renal failure is present. On the other side, the absence of an adaptive increase in IR number in diabetic kidney explains why insulin supplementation has no effect over renal IR levels [Tiwari et al., 2007] and, since most anti-diabetic treatments stimulate insulin release by the β cell, low renal IR expression might be a problem for the proper control of insulin signaling and the desired metabolic effects. It is expected that new anti-diabetic drugs can fulfill both liver and renal insulin responses, correcting and even reverting renal damage.

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