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Glomerular-specific protein kinase C-β-induced insulin receptor substrate-1 dysfunction and insulin resistance in rat models of diabetes and obesity

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Insulin resistance has been associated with the progression of chronic kidney disease in both diabetes and obesity. In order to determine the cellular mechanisms contributing to this, we characterized insulin signaling in renal tubules and glomeruli during diabetic and insulin-resistant states using streptozotocin-diabetic and Zucker fatty-insulin-resistant rats. Compared with nondiabetic and Zucker lean rats, the insulin-induced phosphorylation of insulin receptor substrate-1 (IRS1), Akt, endothelial nitric oxide synthase, and glycogen synthase kinase 3α were selectively inhibited in the glomeruli but not in the renal tubules of both respective models. Protein, but not mRNA levels of IRS1, was decreased only in the glomeruli of streptozotocin-diabetic rats likely due to increased ubiquitination. Treatment with the protein kinase C-β inhibitor, ruboxistaurin, enhanced insulin actions and elevated IRS1 expression. In glomerular endothelial cells, high glucose inhibited the phosphorylation of Akt, endothelial nitric oxide synthase, and glycogen synthase kinase 3α; decreased IRS1 protein expression and increased its association with ubiquitin. Overexpression of IRS1 or the addition of ruboxistaurin reversed the inhibitory effects of high glucose. Thus, loss of insulin's effect on endothelial nitric oxide synthase and glycogen synthase kinase 3α activation may contribute to the glomerulopathy observed in diabetes and obesity.

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KEYWORDS: diabetic nephropathy; insulin receptor substrate-1; insulin resistance; obesity; protein kinase C-β

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Diabetic nephropathy is the most common cause of chronic kidney disease and end-stage renal disease.[1–3] Insulin resistance, observed in both diabetes and obesity, has been associated with increased risks of renal dysfunction and chronic kidney disease.[4] However, a comprehensive and comparative characterization of insulin signaling in renal glomeruli and tubules has not been reported in these diseases.

Physiological studies have shown that renal tissues are responsive to insulin, specifically in the renal tubules affecting sodium uptake and glucose metabolism.[5,6] The insulin's effect on renal sodium reabsorption has been reported to be unaffected in diabetes or insulin resistance, manifested by increased fluid retention in diabetic patients after the initiation or intensification of insulin therapy.[7] However, systemic insulin resistance has been associated with the progression of nephropathy in type 1 diabetic patients.[8,9] Thus, insulin may have actions in the glomeruli and the proximal tubules. A potential site of insulin's glomerular action is the endothelial cells, regulating endothelial nitric oxide synthase (eNOS) and altering nitric oxide (NO) production and actions.[10] The role of NO and eNOS in renal function and pathology is significant as eNOS-null mice exhibit glomerular and peritubular capillary endothelium injury with progressive renal disease.[11,12] Insulin can increase NO production by increasing eNOS actions in endothelial cells,[13] which can be impaired in insulin-resistant or diabetic animals.[14,15] NO production has been reported to be decreased in the renal cortex of diabetic[16] and Zucker fatty (ZF) rats[17] and patients with chronic kidney disease.[18]

This study characterized insulin signaling and actions in renal glomeruli and tubules of rat models of diabetes with insulin deficiency and insulin resistance due to obesity. The mechanisms for the selective loss of insulin glomerular actions were further studied in cultured rat glomerular endothelial cells (RGECs).

RESULTS

Physiological characteristics of the experimental groups

Increases in blood glucose by 3.9 ± 0.5-fold, kidney weight by 1.6 ± 0.2-fold, and albuminuria by 24 ± 7-fold were observed in diabetic rats compared with control Sprague-Dawley (SD) rats.

Table 1 | General characteristics of the experimental groups

	Cont.	Cont.+RBX	DM	DM+RBX	ZL	ZL+RBX	ZF	ZF+RBX
Number	6	6	6	5	6	6	6	6
After 1 week								
Body weight (g)	165 ± 4	165 ± 2	168 ± 8	166 ± 1	188 ± 8	164 ± 3	262 ± 23	261 ± 12
Blood glucose (mg/dl)	98 ± 8	94 ± 7	405 ± 183*	412 ± 24*	124 ± 9	100 ± 13	159 ± 34	138 ± 9
After 8 weeks								
Body weight (g)	527 ± 45	541 ± 56	320 ± 78*	321 ± 38*	391 ± 30	402 ± 58	617 ± 23†	621 ± 36†
Blood glucose (mg/dl)	106 ± 9	108 ± 15	415 ± 50*	456 ± 66*	114 ± 24	130 ± 16	157 ± 46	170 ± 49
Kidney weight (g)	2.3 ± 0.1	2.2 ± 0.2	3.7 ± 0.4*	3.5 ± 0.3*	2.0 ± 0.1	1.8 ± 0.3	3.0 ± 0.3†	2.8 ± 0.3
Albuminuria (mg/day)	0.2 ± 0.1	0.2 ± 0.1	4.7 ± 1.6*	2.6 ± 1.2*$^{,\#}$	0.1 ± 0.1	0.2 ± 0.1	0.6 ± 0.3‡	0.4 ± 0.2‡
Insulin (ng/ml)	2.4 ± 0.5	2.5 ± 0.4	0.2 ± 0.1*	0.2 ± 0.1*	2.9 ± 0.5	2.8 ± 0.4	42 ± 3†	43 ± 5†

Abbreviations: Cont., control rat; Cont.+RBX, control rat with ruboxistaurin (RBX) treatment; DM, diabetic rat; DM+RBX, diabetic rat with RBX treatment; ZL, Zucker lean rat; ZL+RBX, Zucker lean rat with RBX treatment; ZF, Zucker fatty rat; ZF+RBX, Zucker fatty rat with RBX treatment.
The data are expressed as the means ± s.d. *$P<0.001$ vs Cont.; $^{\dagger}P<0.001$ vs ZL; $^{\ddagger}P<0.05$ vs ZL; $^{\#}P<0.05$ vs DM.

After 8 weeks of diabetes, body weight in diabetic SD rats were less than the control SD rat group by 39 ± 15% ($P<0.001$, Table 1), although all the final weights of the diabetic rats were higher than their weights at the initiation of the study. Body weights of ZF rats were significantly greater than Zucker lean (ZL) rats by 1.6 ± 0.7-fold ($P<0.001$, Table 1).

Renal histology in experimental groups

Mesangial matrix expansion was prominent in diabetic rats (control SD rats; 3.1 ± 0.6% vs diabetic SD rats; 5.5 ± 2%, $P<0.05$, Figure 1a and b). Area in the glomeruli stained for type IV collagen was also increased in diabetic rats compared with control SD rats (control SD rats; 2.9 ± 0.9% vs diabetic SD rats; 4.9 ± 0.7%, respectively, $P<0.05$, Figure 1a and c).

Insulin's effect on the phosphorylation of Akt and ERK/MAPK (extracellular signal-regulated kinase/mitogen-activated protein kinase)

In the glomeruli, insulin stimulated phosphorylated Akt (p-Akt) by at least 18 ± 3-fold vs control SD or ZL rats. In diabetic SD and ZF rats, insulin-induced p-Akt levels were inhibited by 51 ± 4% ($P<0.001$, Figure 2a) and 69 ± 9% ($P<0.001$, Figure 2b) compared with non-diabetic SD control and ZL rats, respectively. In contrast, insulin increased p-Akt in the tubules by 15 ± 3-fold to 25 ± 4-fold in all groups ($P<0.001$, Figure 2a and b), which were unaffected by diabetes.

Immunohistochemistry indicated that the number of p-Akt-positive cells in the glomeruli of control SD rats treated with insulin was increased significantly by 9.1 ± 1.6-fold when compared with control SD rats without insulin. In diabetic SD rats treated with insulin, the number of p-Akt-positive cells were decreased by 42 ± 10% when compared with control SD rats with insulin ($P<0.05$, Figure 2c and d).

Insulin increased Erk1/2 phosphorylation (p-Erk1/2) levels in both the glomeruli by up to 5.8 ± 0.2-fold ($P<0.001$, Figure 2e and f) and the tubules by up to 7.6 ± 0.4-fold $P<0.001$, Figure 2e and f) when comparing streptozotocin (STZ)-diabetic SD and ZF rats with their respective controls. Moreover, the levels of phosphorylation peaked similarly (88 ± 5–95 ± 3%; p-Erk1/2/Erk1/2, Figure 2e and f). Basal levels of p-Erk1/2 were increased in both glomeruli and tubules of diabetic SD rats (42 ± 3 and 27 ± 1%, respectively; p-Erk1/2/Erk1/2, $P<0.001$, Figure 2e and f) and ZF rats (40 ± 1 and 23 ± 1%, respectively; p-Erk1/2/Erk1/2, $P<0.05$, Figure 2e and f) when compared with non-diabetic and ZL rats.

In addition, we studied insulin's effect on renal tubular cell line (RPTEC). As in the case *in vivo*, insulin-induced p-Erk1/2 and p-Akt were not inhibited when exposed to high glucose condition (Supplementary Figure S1E and F online).

Phosphorylation of eNOS and glycogen synthase kinase 3α (GSK3α)

Insulin increased p-eNOS in the glomeruli of SD nondiabetic and ZL rats by 6.4 ± 2.9-fold and 13 ± 3-fold, respectively. However, insulin's effect to increase p-eNOS was reduced by 15 ± 6% ($P<0.05$, Figure 3a) in STZ-diabetic SD compared with non-diabetic rats and was reduced by 68 ± 1% in ZF compared with ZL rats ($P<0.001$, Figure 3b).

To confirm that activation of phosphatidylinositol 3-kinase (PI3K)/Akt is selectively inhibited in the glomeruli, we investigated insulin-stimulated phosphorylation of GSK3α (p-GSK3α), another target of insulin signaling induced by the activation of the insulin receptor substrate (IRS)/PI3K pathway.[19] Insulin increased p-GSK3α in the glomeruli of all rat groups by at least 7.6 ± 1.2-fold. Similar to eNOS activation, GSK3α phosphorylation was reduced by 23 ± 3% in STZ-diabetic rats and 62 ± 3% in ZF rats when compared with control SD and ZL rats, respectively ($P<0.001$, Figure 3c and d). In contrast, insulin-induced increases of p-GSK3α in the tubules were comparable in control and diabetic rats by 7.9 ± 0.5-fold to 10 ± 1fold ($P<0.001$, Figure 3c and d). Lastly, GSK3β phosphorylation induced by insulin was reduced by 57 ± 3% in STZ-diabetic SD rats and 53 ± 1% in ZF rats when compared with control SD and ZL rats, respectively ($P<0.001$, Figure 3e and f).

Characterization of mRNA and protein levels of IRS1/2

To identify possible mechanisms of insulin resistance on the activation of Akt/eNOS in the renal glomeruli, the protein

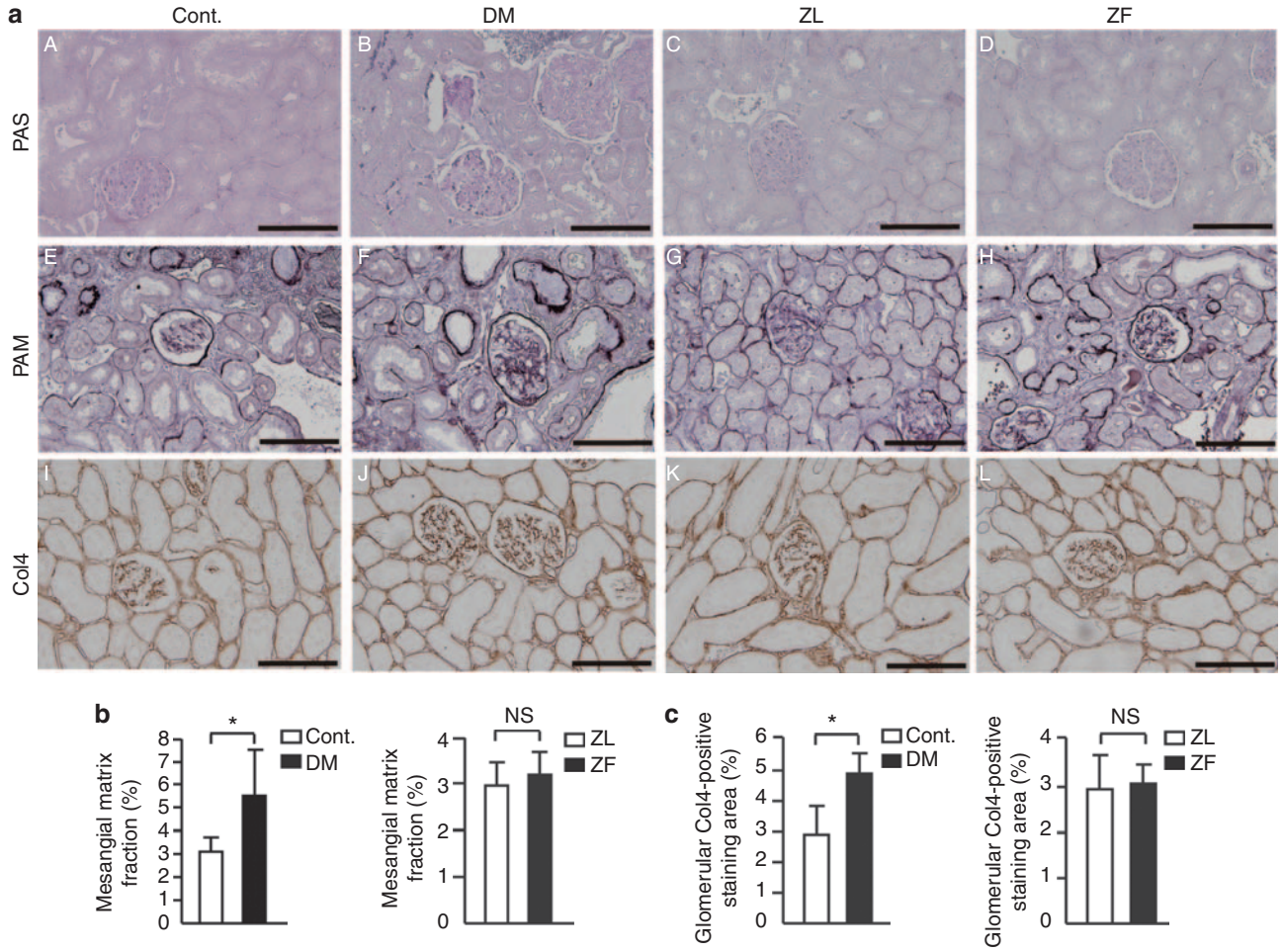


Figure 1 | Renal morphology and immunohistochemical staining for type IV collagen in the experimental groups. (**a**) Representative light microscopic appearance of glomeruli (periodic acid-Schiff (PAS) and periodic acid–methenamine-silver (PAM) staining) and immunohistochemistry of Col4 for control rats (A, E, I), STZ-induced diabetic SD rats (B, F, J), Zucker lean rats (C, G, K), and Zucker fatty rats (D, H L). Bar = 100 μm. (**b**) Morphometric analysis of PAM-positive staining area. The glomerular PAM-positive area was measured as described in the Materials and Methods; $n = 6$ in each group, $^*P < 0.05$. (**c**) Morphometric analysis of the glomerular expression of Col4. The glomerular staining area of Col4 was measured as described in the Materials and Methods; $n = 6$ in each group, $^*P < 0.05$. The data are expressed as means ± s.d. Cont., control rats; DM, STZ-induced diabetic Sprague-Dawley (SD) rats; NS, not significant; ZL, Zucker lean rats; ZF, Zucker fatty rats.

and mRNA levels of IRS1/2 were assessed. Protein levels of IRS1, measured by immunoblot analysis, in the glomeruli of STZ-diabetic SD rats were reduced by 54 ± 9% when compared with controls ($P < 0.05$, Figure 4a). In contrast, IRS1 protein levels in the glomeruli from ZF rats were not changed vs ZL rats. No significant differences in the expression of mRNA levels of IRS1/2 and IRS2 protein levels in the glomeruli and tubules were observed in all four groups of rats (Figure 4b and c).

Studies using immunohistochemistry showed that the number of IRS1-positive cells was significantly decreased in STZ-diabetic SD rats by 36 ± 6% when compared with control SD rats ($P < 0.05$, Figure 4d and e).

Evaluation of insulin receptors and IRS1/2 activation

Insulin-induced tyrosine phosphorylation of IR in both glomeruli and tubules were increased by 8.5 ± 0.1-fold to 16 ± 1-fold and 7.8 ± 0.4-fold to 13 ± 3-fold, respectively ($P < 0.001$, Supplementary Figure S1A and B online), and did not differ significantly when compared with their respective controls. In contrast, tyrosine phosphorylation of IRS1 was significantly reduced in the glomeruli of diabetic and ZF rats by 21 ± 2% and 64 ± 1% compared with control SD and ZL rats, respectively ($P < 0.001$, Figure 4f and g). Insulin increased IRS1 tyrosine phosphorylation in the tubules by 5.5 ± 0.5-fold to 23 ± 2-fold, and no differences were observed between STZ-diabetic SD and ZF rats and their controls ($P < 0.001$, Figure 4f and g).

Association of ubiquitin with IRS1/2 in the glomeruli

The results suggest that the decreases of IRS1 in the diabetic SD rats are because of changes in the degradation of IRS1. The association of IRS1/2 with ubiquitin was evaluated by immunoprecipitation studies.[20] Figure 4h showed that there was a significant increase by 2.3 ± 0.7-fold in the association of ubiquitin with IRS1 in the glomeruli of diabetic SD rats compared with non-diabetic control ($P < 0.001$). No increases

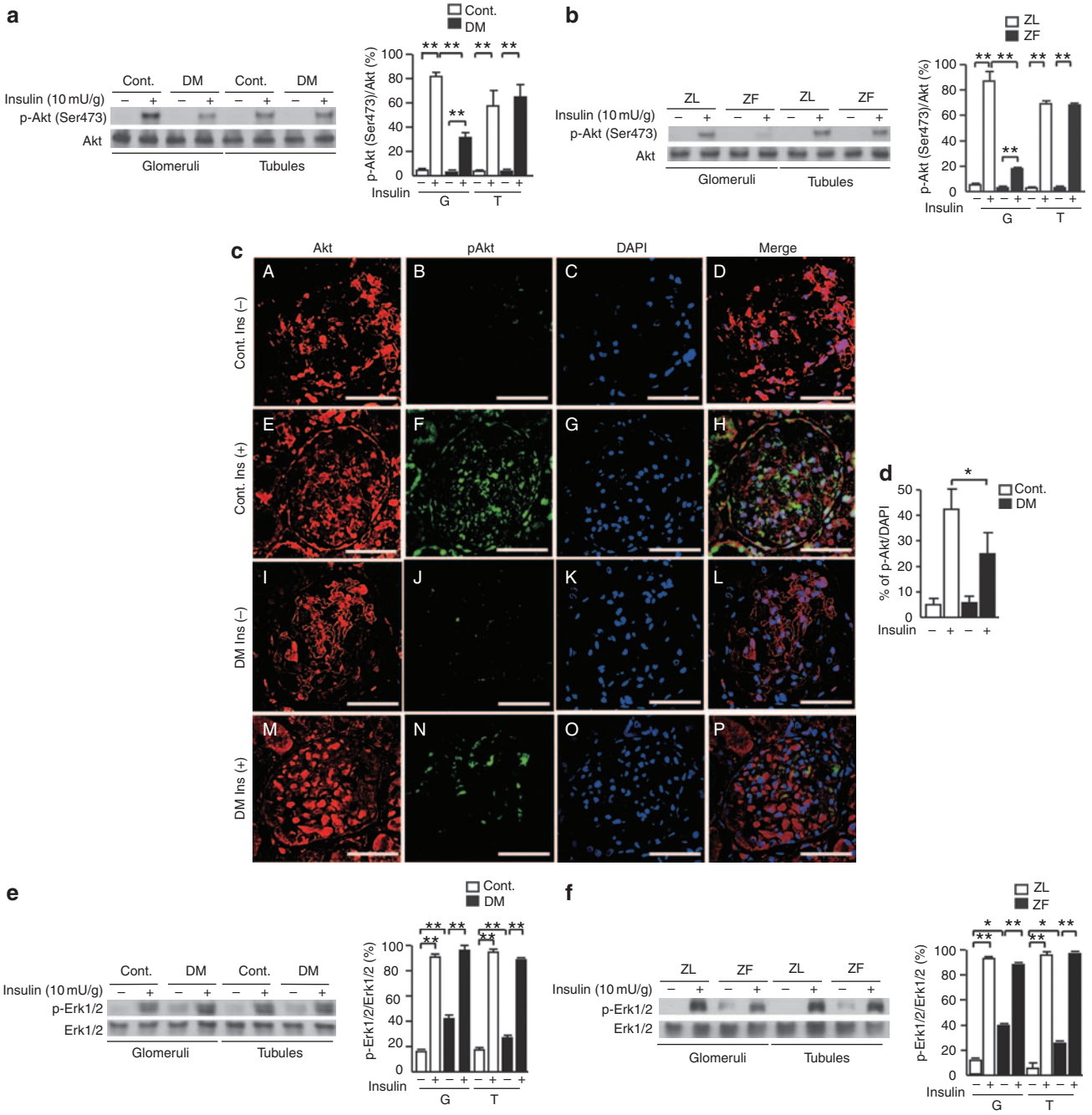


Figure 2 | Insulin's effect on p-Akt and p-Erk1/2 in the glomeruli and tubules of SD and Zucker rats. (**a**, **b**) Representative immunoblots of p-Akt from glomerular and tubular fractions. Data from three experiments were quantitated by densitometry. (**a**) Cont. vs DM, (**b**) ZL vs ZF, $n = 6$ in each group, $^{**}P < 0.001$. (**c**) Immunostaining for Akt (A, E, I, M), p-Akt (B, F, J, N), DAPI (C, G, K, O), and merge images (D, H, L, P) in the glomeruli of control rats without insulin, control rats with insulin, STZ-induced diabetic SD rats without insulin, and STZ-induced diabetic SD rats with insulin. (**d**) Percentage of p-Akt-positive cells per DAPI; $n = 6$ in each group, $^{*}P < 0.05$. (**e**, **f**) Representative immunoblots of p-Erk1/2 from glomerular and tubular fractions. Data from three experiments were quantitated by densitometry. (**e**) Cont. vs DM, (**f**) ZL vs ZF; $n = 6$ in each group, $^{*}P < 0.05$, $^{**}P < 0.001$. These data are expressed as means ± s.d. Cont., control rats; DM, STZ-induced diabetic Sprague-Dawley (SD) rats; G, glomeruli; p-Erk, phosphorylated extracellular signal-regulated kinase; T, tubules; ZF, Zucker fatty rats; ZL, Zucker lean rats.

in association between ubiquitin and IRS2 were observed in the glomeruli of diabetic vs control SD rats. In addition, no changes in the association of IRS1/2 with ubiquitin were observed in the glomeruli of ZL vs ZF rats (Figure 4i).

Nuclear factor-κB (NF-κB) activation in kidney

Previous reports have indicated that GSK3α/β phosphorylation is decreased in the renal cortex and associated with increases in NF-κB activity.[21,22] Thus, we evaluated the

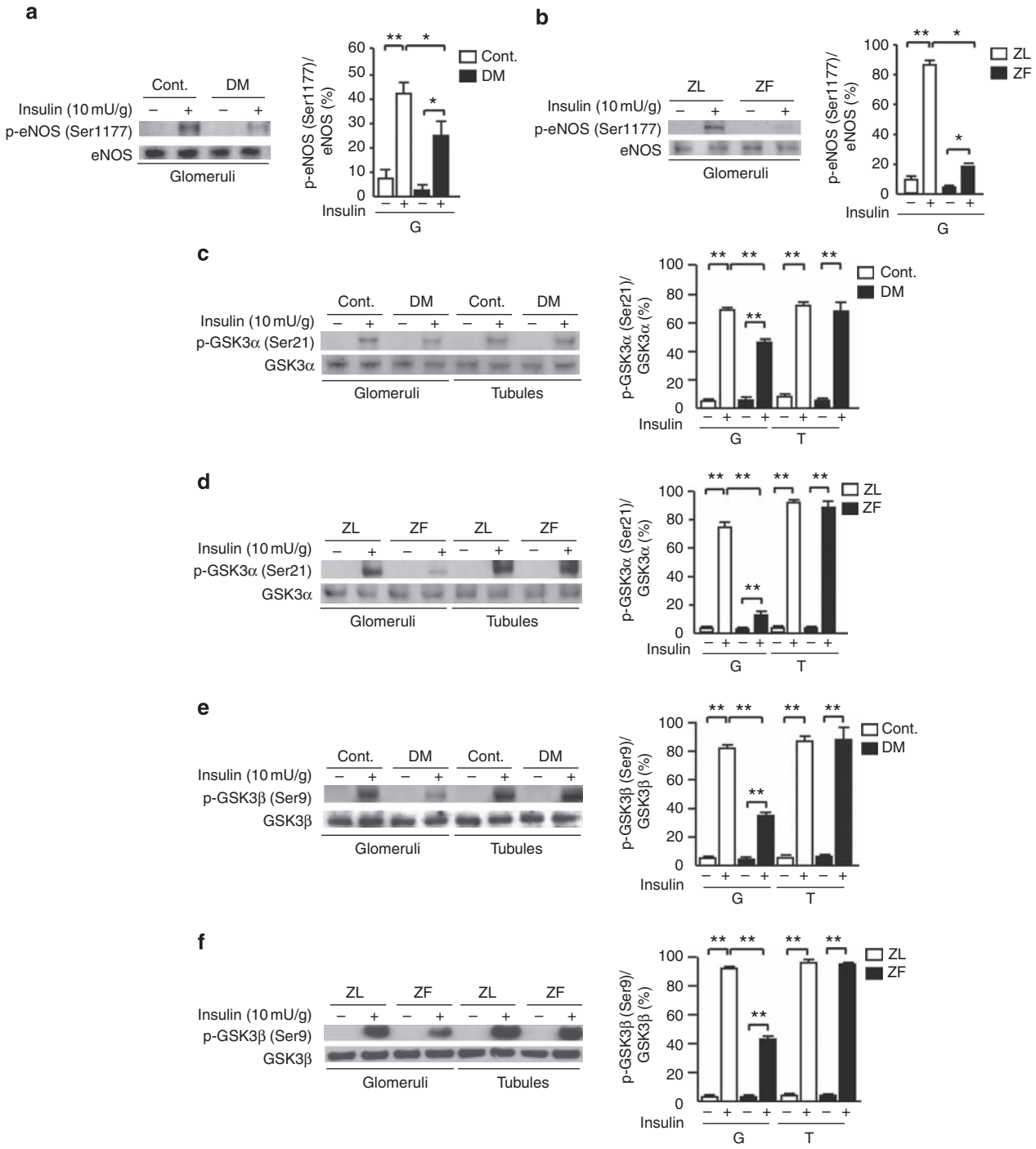


Figure 3 | Insulin-induced p-eNOS and p-GSKα in the glomeruli and tubules of diabetic and control SD rats and ZL and ZF rats. (**a**, **b**). Representative immunoblots of p-eNOS from glomerular proteins. Data from three experiments were quantitated by densitometry. (**a**) Cont. vs DM, (**b**) ZL vs ZF; $n=6$ in each group, $^{*}P<0.05$, $^{**}P<0.001$. (**c**, **d**). Representative immunoblots of p-GSKα from glomerular and tubular fractions. Data from three experiments were quantitated by densitometry. (**c**) Cont. vs DM, (**d**) ZL vs ZF; $n=6$ in each group, $^{**}P<0.001$. (**e**, **f**). Representative immunoblots of p-GSKβ from glomerular and tubular fractions. Data from three experiments were quantitated by densitometry. (**e**) Cont. vs DM, (**f**) ZL vs ZF; $n=6$ in each group, $^{**}P<0.001$. These data are expressed as means ± s.d. Cont., control rats; DM, STZ-induced diabetic Sprague-Dawley (SD) rats; G, glomeruli; p-eNOS, phosphorylated endothelial nitric oxide synthase; p-GSKα, phosphorylated glycogen synthase kinase 3α; T, tubules; ZF, Zucker fatty rats; ZL, Zucker lean rats.

activation of NF-κB in the kidney. In the glomeruli of diabetic SD rats and ZF rats, NF-κB activation was increased by 6.4 ± 0.2-fold in the glomeruli of diabetic SD rats when compared with control SD rats and by 7.8 ± 0.9-fold in the glomeruli of ZF rats when compared with ZL rats ($P<0.001$, Figure 5a). However, no increases were observed in the tubules of diabetic SD rats and ZF rats. Similar to immunoblot study, NF-κB binding assay only exhibited

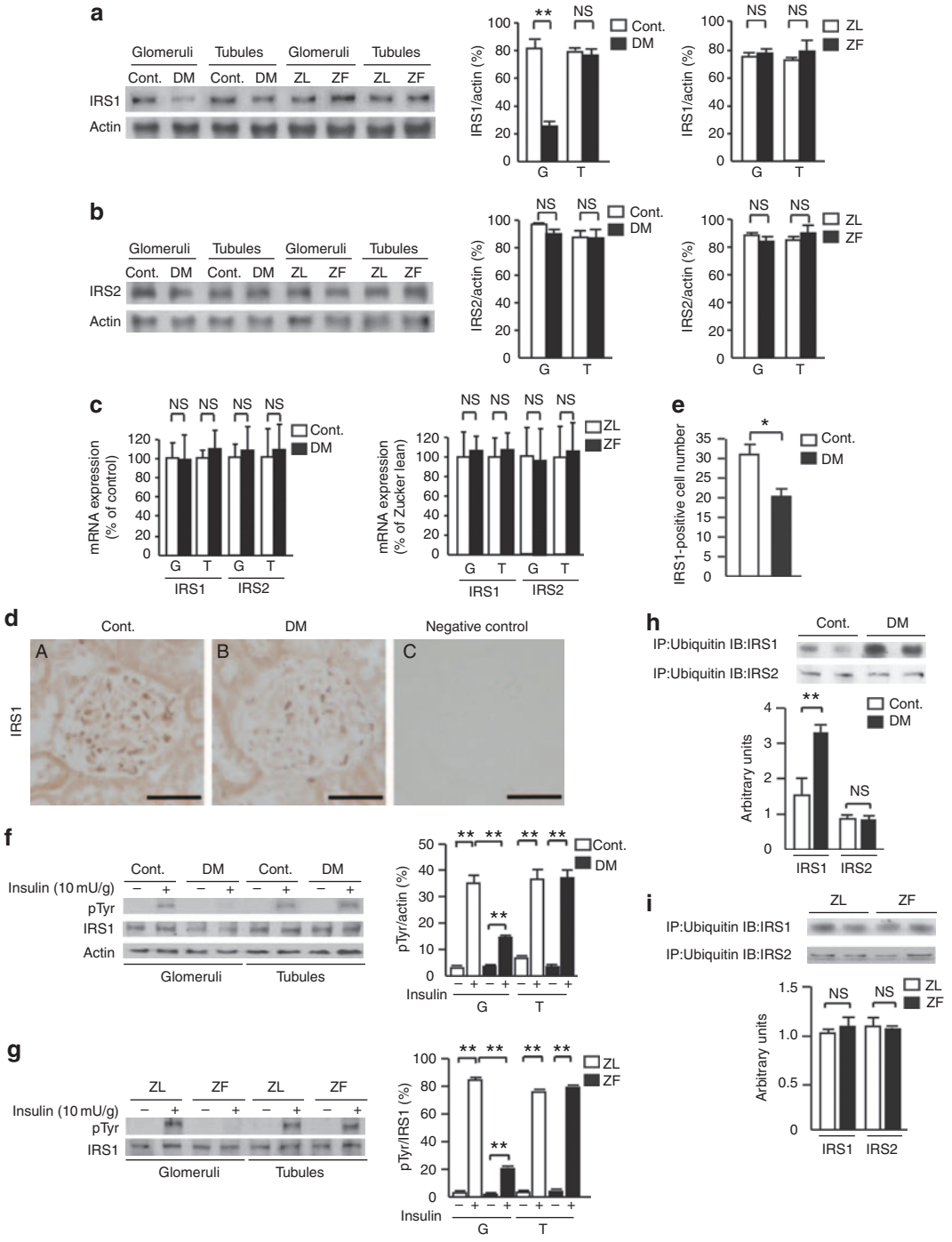


Figure 4 | Expression of IRS1/2 proteins and mRNA levels and insulin's effect on the tyrosine phosphorylation of IRS1 in the glomeruli and tubules. (**a**, **b**) Representative immunoblots of IRS1 and IRS2 from glomerular and tubular fractions. Data from three experiments were quantitated by densitometry. (**a**) IRS1, (**b**) IRS2; $n = 6$ in each group, $^{**}P < 0.001$. (**c**) Glomerular and tubular fractions in diabetic and Zucker rats. mRNA expressions for IRS1/2 were measured by real-time reverse transcriptase-PCR (RT-PCR); $n = 6$ in each group. (**d**) Immunostaining for IRS1 and representative pictures in (A) control rats, (B) STZ-induced diabetic SD rats, and (C) negative control. Bar = 50 μm. (**e**) Number of IRS1-positive cells per glomerulus in control rats and STZ-induced diabetic SD rats; $n = 6$ in each group, $^{*}P < 0.05$. (**f**, **g**) Representative immunoblots of tyrosine phosphorylation of IRS1 from glomerular and tubular fractions. Solubilized glomeruli and tubular fractions were isolated and subjected to immunoprecipitation followed by immunoblotting. Data from three experiments were quantitated by densitometry. (**f**) Cont. vs DM, (**g**) ZL vs ZF; $n = 6$ in each group, $^{**}P < 0.001$. (**h**, **i**) Solubilized glomeruli fractions were immunoprecipitated with antibodies against ubiquitin and subsequently immunoblotted with anti-IRS1 or anti-IRS2 antibodies. Data from three experiments were quantitated by densitometry; $n = 6$ in each group, $^{**}P < 0.001$. These data are expressed as means ± s.d. Cont., control rats; DM, STZ-induced diabetic Sprague-Dawley (SD) rats; G, glomeruli; IRS, insulin receptor substrate; NS, not significant; T, tubules; ZF, Zucker fatty rats; ZL, Zucker lean rats.

increases in the glomeruli of diabetic SD rats and ZF rats when compared with control SD rats and ZL rats (5.7 ± 0.8-fold and 7.5 ± 0.9-fold, respectively, $P<0.001$, Figure 5b).

Effects of ruboxistaurin (RBX) on insulin-induced Akt, eNOS, and GSKα phosphorylation

We have previously reported that activation of protein kinase C (PKC), especially PKCβ, inhibited insulin-stimulated p-Akt and p-eNOS.[15] Therefore, we evaluated whether inhibition by RBX can decrease insulin resistance in the glomeruli of diabetic SD and ZF rats. RBX treatment did not affect insulin-induced phosphorylation of Akt or its actions in the glomeruli of control SD rats and ZL rats. In contrast, RBX treatment partially normalized Akt phosphorylation by 67 ± 14% and by 43 ± 12%, respectively ($P<0.001$, $P<0.05$, Figure 5c and d) in the glomeruli of diabetic SD rats and ZF rats. Treatment with RBX also normalized eNOS phosphorylation by 122 ± 20% and by 144 ± 48%, respectively ($P<0.001$, Figure 5e and f) and GSK3α phosphorylation by 68 ± 4% and by 136 ± 10%, respectively ($P<0.001$, Figure 5g and h) in diabetic SD and ZF rats. In addition, RBX treatment partially normalized insulin-induced levels of p-Erk1/2 and basal p-Erk1/2 (Supplementary Figure S1C online).

Effect of RBX on IRS1 function and NO synthesis

In the glomeruli of diabetic SD rats and ZF rats, RBX partially normalized insulin-induced tyrosine phosphorylation of IRS1 by 165 ± 21 and by 164 ± 11%, respectively ($P<0.001$, Figure 6a and b). Moreover, RBX decreased the degradation of IRS1 by 26 ± 11% ($P<0.001$, Figure 6a) and its association with ubiquitin by 35 ± 7% ($P<0.001$, Figure 6c) in the glomeruli of diabetic SD rats compared with diabetic SD rats. NO release induced by insulin in the isolated glomeruli of diabetic SD rats and ZF rats were reduced by 40 ± 6 and by 41 ± 5%, respectively ($P<0.001$, Figure 6d and e) compared with control and ZL rats. In the isolated glomeruli from diabetic SD rats and ZF rats, RBX treatment improved insulin-induced NO release by 30 ± 10 and by 31 ± 11%, respectively ($P<0.05$, Figure 6d and e).

Effect of glucose levels on IRS1 expression and ubiquitination

To investigate whether hyperglycemia is responsible for the increase in IRS1 degradation, we studied the effect of high glucose on IRS mRNA and protein levels in RGECs, cultured for 72 h, in the presence of low (5.5 mmol/l) and high (25 mmol/l) glucose levels. Levels of IRS1 and IRS2 mRNA and the protein levels of IRS2 were not changed during the experiments (Figure 7a).

The protein levels of IRS1 decreased in high glucose condition after 48 and 72 h of incubation by 21 ± 2 and 30 ± 1% compared with basal, respectively ($P<0.05$, $P<0.001$, Figure 7b). Similar to the *in vivo* condition, polyubiquitination for IRS1 in RGECs was increased by 1.7 ± 0.2-fold when cells were incubated with high glucose for 72 h compared with low glucose condition ($P<0.001$, Figure 7c). No difference for IRS2 immunoreactive band associated with ubiquitin between control and diabetic rats was detected (Figure 7c).

We also checked the differences of insulin receptor and IRS expression among the glomerular cell types. In podocytes, both insulin receptor and IRS1 expression were higher than other cells (insulin receptor/actin: 85 ± 13% in podocytes, 74 ± 10% in mesangial cells, and 80 ± 15% in RGECs, respectively, IRS/actin: 87 ± 11% in podocytes, 70 ± 11% in mesangial cells, and 82 ± 14% in RGECs, $P<0.05$, Supplementary Figure S1G online).

Effect of glucose and the overexpression of IRS1 on insulin signaling in RGECs

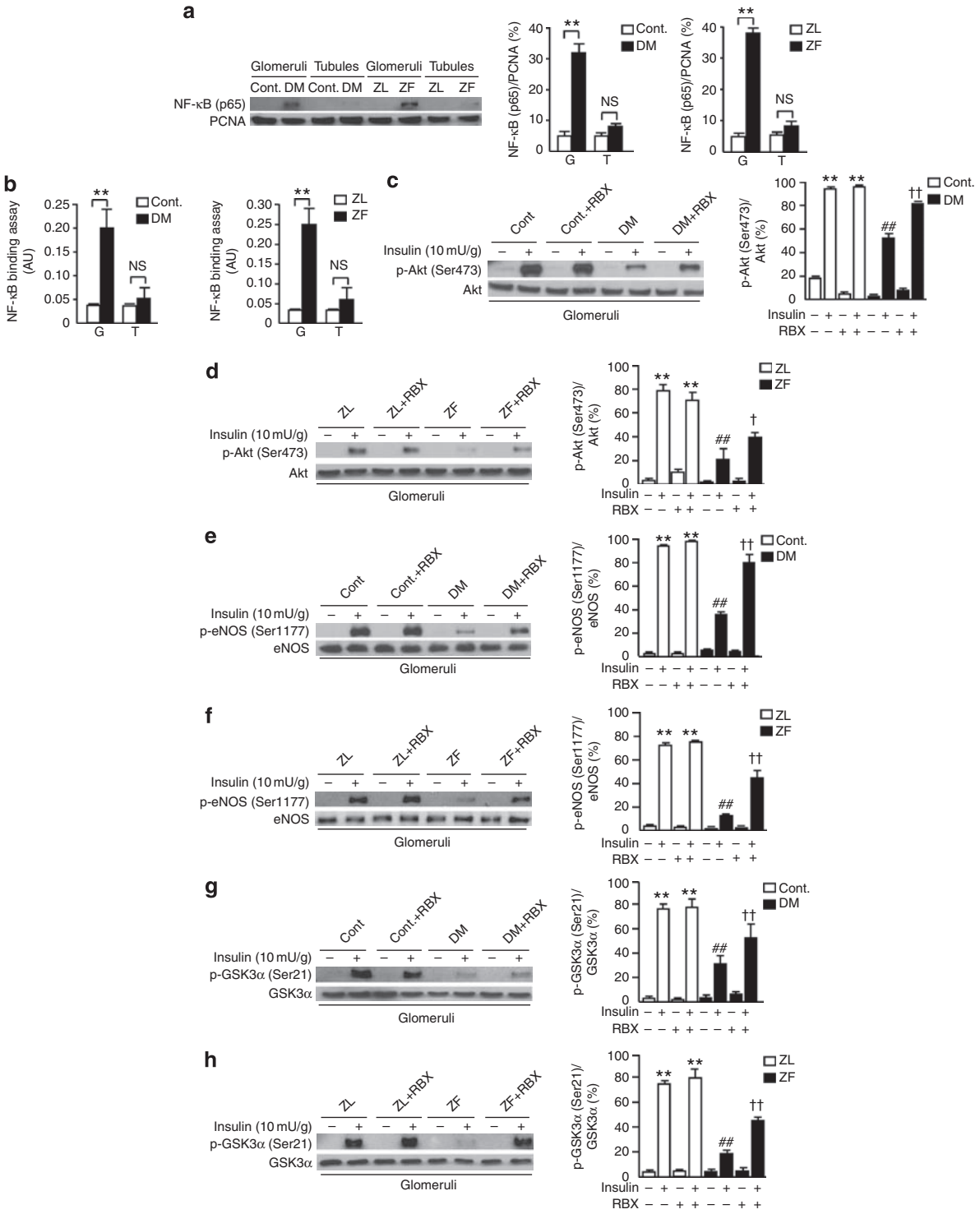
As eNOS is selectively expressed in the endothelial cells and inhibited by diabetes, we characterized the direct effect of glucose levels on insulin signaling and activation of eNOS in RGECs. As shown in Figure 6d and f, insulin at 5.5 mmol/l glucose significantly increased p-Akt (Ser473), p-eNOS (Ser1177), and p-Erk1/2 by 3- to 4-fold ($P<0.001$) with maximum effects observed at 30, 30, and 15 min after the addition of insulin, respectively.

Infection with Ad-IRS1 increased IRS1 protein expression similarly in low and high glucose levels by 9.1 ± 1.9-fold and 9.4 ± 0.3-fold, respectively ($P<0.001$, Supplementary Figure S1C). Insulin increased p-Akt (Ser473)/Akt to 91 ± 2% of total Akt in Ad-green fluorescent protein (GFP)-infected cells (Figure 7g), which were not different from noninfected RGECs (95 ± 2% of total Akt protein, Figure 7d). Infection of Ad-IRS1 increased basal p-Akt/Akt to 74 ± 1% of total Akt. Insulin still significantly increased p-Akt in Ad-IRS1-infected cells, although the maximum did not change between Ad-GFP vs Ad-IRS1-infected cells. In RGECs cultured in high glucose, the maximal stimulation of p-Akt in control or Ad-GFP-infected cells showed a 15 ± 3% inhibition compared with low glucose condition ($P<0.05$). The infection of Ad-IRS1 in RGECs reversed the loss of insulin's activation of p-Akt in RGECs incubated in high glucose conditions (Figure 7g) without infection with Ad-IRS1. RGECs, incubated without insulin but with Ad-IRS1 infection, had elevated basal p-eNOS levels (Figure 7h). Lastly, insulin's effect on p-GSK3α was inhibited by 17 ± 2% in RGECs incubated with high glucose when compared with low glucose conditions ($P<0.001$). Overexpression of Ad-IRS1 in RGECs totally normalized the maximum responses per p-GSK3α induced by insulin in high glucose conditions (Figure 7i).

Effect of antioxidant, PKCβ inhibitor, and proteosome inhibitor on RGECs

To characterize the possible role of PKC activation in RGECs, we examined the effects of bisindolylmaleimide I (GF109203X, GFX), a general PKC inhibitor, or RBX in RGECs. In RGECs cultured with high level of glucose, insulin's activation of p-Akt was inhibited, compared with low glucose condition ($P<0.001$). Addition of GFX and RBX reversed the inhibitory effect of high glucose on p-Akt activation by 32 ± 2 and by 17 ± 2%, respectively ($P<0.05$).

The addition of *N*-acetyl-L-cystein (NAC), an antioxidant, also partially normalized this inhibition by 30 ± 4% ($P < 0.05$, Figure 8a). Similarly, inhibitions of p-eNOS and p-GSK were also partially normalized by NAC, GFX, or RBX ($P < 0.05$, Figure 8b and c). Next, we tested the effect of NAC, GFX, RBX, and proteasome inhibitor, MG132, on proteasomal IRS1 degradation in RGECs. When the cells were incubated with high glucose for 72 h, IRS1 protein levels in RGECs were decreased by 30 ± 2%. NAC, GFX, RBX, and MG132 significantly increased IRS1 proteins by 51 ± 7, 39 ± 3, 12 ± 5, and 54 ± 4%, respectively (Figure 8d). For NO production in RGECs, insulin induced its production by 4.9 ± 0.7-fold. When incubated with high glucose, NO release was inhibited by 45 ± 11% compared with low glucose condition ($P < 0.001$, Figure 8e). NAC, GFX, and RBX increased NO production in RGECs exposed to high glucose



level by 41 ± 8, 40 ± 7, and 23 ± 7%, respectively ($P<0.05$, Figure 8e).

DISCUSSION

This is the first comparative analysis of insulin signaling and cellular actions between renal glomeruli and tubules in control, insulin-resistant, and diabetic states. The results demonstrated that the renal tubules are protected from the loss of insulin action as a consequence of metabolic abnormalities induced by insulin resistance or diabetes. In contrast, insulin signaling and actions in the renal glomeruli are significantly inhibited in a selective manner, similar to the endothelium of all the other vascular tissues exposed to insulin resistance and diabetes.[23,24] Our findings of the selective loss of insulin action in the glomeruli but not in the tubules in both diabetes and insulin resistance has suggested a biochemical explanation for the glomerular pathologies shared by both of these pathological conditions.[25,26]

Resistance to insulin signaling and actions in the renal glomeruli is also selective for the activation of the IRS1/PI3K/Akt cascade, whereas the activation of the Erk/MAPK pathway by insulin remained fully active. This pattern of selective loss of insulin signaling in insulin-resistant and diabetic states has been reported in many vascular beds, such as in the microvessels from adipose tissues and the aorta.[13,24] The diminution of eNOS activation induced by insulin suggests the presence of glomeruli endothelial dysfunction and is consistent with previous reports regarding decreasing NO production in the renal cortex of ZF rats and diabetic rodents.[16,17] The loss of insulin-induced eNOS activation and endothelial dysfunction in the glomeruli can contribute to changes in glomerular blood flow and loss of antioxidative and inflammatory actions of NO.[12] Our results have also shown for the first time that there is also a parallel selective loss of insulin's inhibitory actions on GSK3α, limited to the glomeruli. Our data have demonstrated that this decrease in GSK3α phosphorylation is limited to the glomeruli and is partially related to the loss of insulin action, which is known to inhibit GSK3α activities by increasing its phosphorylation.[19] The increases in GSK3α activity in the glomeruli can be equally important as the diminution of eNOS activation, as GSK3α can regulate multiple critical actions in renal cells,[27,28] such as increases in oxidative stress via the activation of NF-κB and regulation of endothelial cell[29] and podocyte apoptosis via Wnt signaling.[26,30]

For Erk1/2 phosphorylation, the basal levels are increased in both diabetes and insulin resistance, which is consistent with previous reports.[24,31,32] The increase of basal p-Erk in these pathological states is probably because of the activation of PKC,[33] which is known to increase MAPK.[15] Insulin-induced increased ratio of p-Erk in diabetic SD rats and ZF rats are decreased because the basal p-Erk level is increased. However, their maximal effects induced by insulin are similar in control and diabetic mice.[24]

In diabetes, our results clearly suggest that hyperglycemia can induce a decrease in the protein level of IRS1, selectively, but not in IRS2, in parallel with the loss of insulin action. The suggestion of enhanced degradation of IRS1 induced by hyperglycemia is supported by the increased association of polyubiquitination with IRS1, which was observed in both the glomeruli of diabetic rats and RGECs exposed to high concentrations of glucose. These findings indicate that hyperglycemia by an unknown mechanism increases IRS1 being targeted for proteasomal degradation.

Several mechanisms, such as the activation of PKC, have been identified to induce the selective inhibition of the IRS/PI3K/Akt pathway of insulin in the endothelial cells.[15] The selective loss of IRS1 but not IRS2 is interesting, but has also been reported in macrophages and adipocytes in association with diabetes.[34,35] The potential mechanism for the selective loss of insulin's activation of IRS/PI3K/Akt/eNOS pathways appears to be the activation of PKC, possibly by the β-isoform. The results indicated that hyperglycemia activated several PKC isoforms, including β to selectively inhibit the IRS/PI3K

Figure 5 | NF-κB activation and effect of RBX, PKCβ inhibitor on p-Akt, p-eNOS, and p-GSK3α in the glomeruli of diabetic SD rats and ZF rats. (**a**) Representative immunoblots of NF-κB (p65) from nuclear proteins of glomerular and tubular fractions. Data from three experiments were quantitated by densitometry; $n=6$ in each group, $^{**}P<0.001$. (**b**) Transcriptional binding activity assay of NF-κB in glomerular and tubular fractions; $n=6$ in each group, $^{**}P<0.001$. (**c**, **d**) Representative immunoblots of p-Akt from glomerular fractions. Data from three experiments were quantitated by densitometry. (**c**) Cont. vs Cont. + RBX vs DM vs DM + RBX; $n=6$ in Cont., Cont. + RBX, and DM; $n=5$ in DM + RBX, $^{**}P<0.001$ vs Cont./insulin(−)/RBX(−). $^{\#\#}P<0.001$ vs Cont./insulin(+)/RBX(−). $^{\dagger\dagger}P<0.001$ vs DM/insulin(+)/RBX(−). (**d**) ZL vs ZL + RBX vs ZF vs ZF + RBX ; $n=6$ in each group, $^{**}P<0.001$ vs ZL/insulin(−)/RBX(−). $^{\#\#}P<0.001$ vs ZL/ insulin(+)/RBX(−). $^{\dagger}P<0.05$ vs ZF/insulin(+)/RBX(−). (**e**, **f**) Representative immunoblots of p-eNOS from glomerular fractions. Data from three experiments were quantitated by densitometry. (**e**) Cont. vs Cont. + RBX vs DM vs DM + RBX; $n=6$ in Cont., Cont. + RBX, and DM; $n=5$ in DM + RBX, $^{**}P<0.001$ vs Cont./insulin(−)/RBX(−). $^{\#\#}P<0.001$ vs Cont./insulin(+)/RBX(−). $^{\dagger\dagger}P<0.001$ vs DM/insulin(+)/RBX(−). (**f**) ZL vs ZL + RBX vs ZF vs ZF + RBX $n=6$ in each group, $^{**}P<0.001$ vs ZL/insulin(−)/RBX(−). $^{\#\#}P<0.001$ vs ZL/ insulin(+)/RBX(−). $^{\dagger\dagger}P<0.001$ vs ZF/insulin(+)/RBX(−). (**g**, **h**) Representative immunoblots of p-GSK3α from glomerular fractions. Data from three experiments were quantitated by densitometry. (**g**) Cont. vs Cont. + RBX vs DM vs DM + RBX; $n=6$ in Cont., Cont. + RBX, and DM; $n=5$ in DM + RBX, $^{**}P<0.001$ vs Cont./insulin(−)/RBX(−). $^{\#\#}P<0.001$ vs Cont./insulin(+)/RBX(−). $^{\dagger\dagger}P<0.001$ vs DM/insulin(+)/RBX(−). (**h**) ZL vs ZL + RBX vs ZF vs ZF + RBX; $n=6$ in each group, $^{**}P<0.001$ vs ZL/insulin(−)/RBX(−). $^{\#\#}P<0.001$ vs ZL/ insulin(+)/RBX(−). $^{\dagger\dagger}P<0.001$ vs ZF/insulin(+)/RBX(−). These data are expressed as means ± s.d. Cont., control rats; Cont. + RBX, control rats treated with ruboxistaurin; DM, STZ-induced diabetic rats; DM + RBX, STZ-induced diabetic rats treated with ruboxistaurin; G, glomeruli; NF-κB, nuclear factor-κB; NS, not significant; PCNA, proliferating cell nuclear antigen; p-eNOS, phosphorylated endothelial nitric oxide synthase; p-GSKα, phosphorylated glycogen synthase kinase 3α; PKCβ, protein kinase C-β; T, tubules; ZF, Zucker fatty rats; ZF + RBX, Zucker fatty rats treated with ruboxistaurin; ZL, Zucker lean rats; ZL + RBX, Zucker lean rats treated with ruboxistaurin.

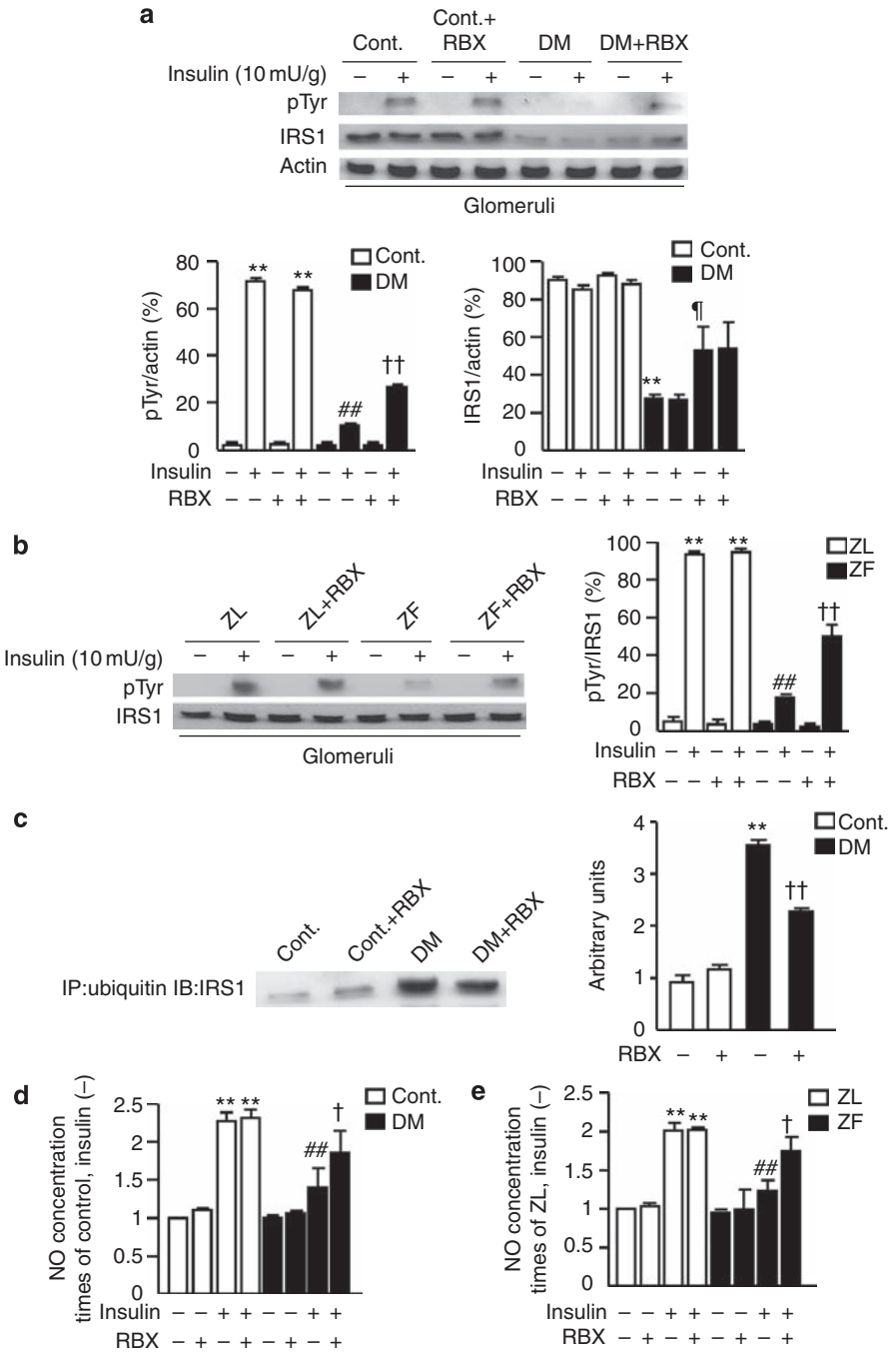


Figure 6 | Effect of RBX on IRS1 and NO synthesis in the glomeruli of diabetic Sprague-Dawley (SD) rats and ZF rats. (**a**, **b**) Representative immunoblots of tyrosine phosphorylation of IRS1 from glomerular and tubular fractions. Solubilized glomeruli and tubular fractions were isolated and subjected to immunoprecipitation followed by immunoblotting. Data from three experiments were quantitated by densitometry. (**a**) Cont. vs Cont. + RBX vs DM vs DM + RBX; $n = 6$ in Cont., Cont. + RBX, and DM; $n = 5$ in DM + RBX, $^{**}P < 0.001$ vs Cont./insulin(−)/RBX(−). $^{\#\#}P < 0.001$ vs Cont./insulin(+)/RBX(−). $^{\dagger\dagger}P < 0.001$ vs DM/insulin(+)/RBX(−). $^{\P}P < 0.001$ vs DM/insulin(−)/RBX(−). (**b**) ZL vs ZL + RBX vs ZF vs ZF + RBX ; $n = 6$ in each group, $^{**}P < 0.001$ vs ZL/insulin(−)/RBX(−). $^{\#\#}P < 0.001$ vs ZL/ insulin(+)/RBX(−). $^{\dagger\dagger}P < 0.001$ vs ZF/ insulin(+)/RBX(−). (**c**) Solubilized glomeruli fractions were immunoprecipitated with antibodies against ubiquitin and subsequently immunoblotted with anti-IRS1 antibodies. Data from three experiments were quantitated by densitometry; $n = 6$ in Cont., Cont. + RBX, and DM; $n = 5$ in DM + RBX, $^{**}P < 0.001$ vs Cont./RBX(−), $^{\dagger\dagger}P < 0.001$ vs DM/RBX(−). (**d**, **e**) The effect of RBX on NO synthesis in the glomeruli of diabetic SD rats and ZF rats. Isolated glomeruli from each group were incubated with insulin (100 nM for 30 min). After being homogenized and centrifuged, supernatants were collected. NO levels in the supernatant were measured with the Nitric Oxide Colorimeric Assay Kit. The results were derived from three separate experiments. (**d**) $^{**}P < 0.001$ vs Cont./insulin(−)/RBX(−). $^{\#\#}P < 0.001$ vs Cont./insulin(+)/RBX(−). $^{\dagger}P < 0.05$ vs DM/insulin(+)/RBX(−). (**e**) $^{**}P < 0.001$ vs ZL/insulin(−)/RBX(−). $^{\#\#}P < 0.001$ vs ZL/insulin(+)/RBX(−). $^{\dagger}P < 0.05$ vs ZF/insulin(+)/RBX(−). These data are expressed as means ± s.d. Cont., control rats; Cont. + RBX, control rats treated with ruboxistaurin; DM, STZ-induced diabetic rats; DM + RBX, STZ-induced diabetic rats treated with ruboxistaurin; IRS1, insulin receptor substrate-1; NO, nitric oxide; ZF, Zucker fatty rats; ZF + RBX, Zucker fatty rats treated with ruboxistaurin; ZL, Zucker lean rats; ZL + RBX, Zucker lean rats treated with ruboxistaurin.

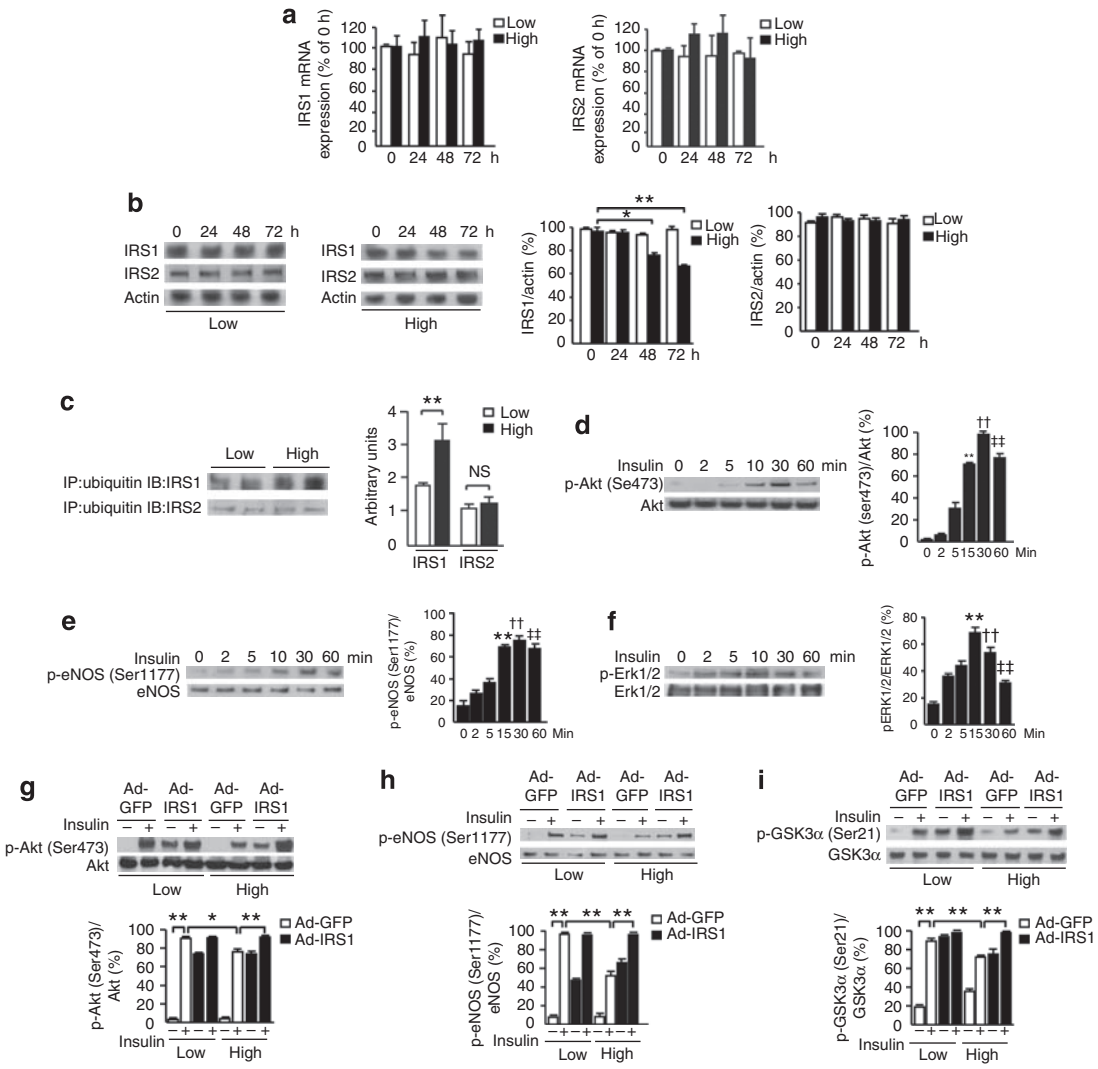


Figure 7 | Effect of glucose levels on the association of insulin receptor substrate (IRS)1/2 with ubiquitin and glucose levels on the activation of Akt, endothelial nitric oxide synthase (eNOS), extracellular signal-regulated kinase (Erk)1/2, and overexpression of IRS1 in rat glomerular endothelial cells (RGECs). (**a**) Time course for the effect of high glucose levels on mRNA gene expression for IRS1 and IRS2 as measured by real-time reverse transcriptase-PCR (RT-PCR). RGECs were incubated with low glucose (5.5 mmol/l) or high glucose (20 mmol/l) as indicated. One of three independent experiments is shown. (**b**) Time course for the effect of high glucose levels on the protein expression of IRS1 and IRS2. RGECs were incubated with low glucose (5.5 mmol/l) or high glucose (20 mmol/l) as indicated. Data from three experiments were quantitated by densitometry. $^{*}P<0.05$, $^{**}P<0.001$. (**c**) Immunoprecipitation with antibodies against ubiquitin and subsequent immunoblotting analyses of the precipitate with anti-IRS1 or anti-IRS2 antibodies showed increased amounts of polyubiquinated IRS1 in high glucose condition. Data from three experiments were quantitated by densitometry. $^{**}P<0.001$. (**d**–**f**) Time course of phosphorylation of (**d**) Akt, (**e**) eNOS, and (**f**) Erk1/2 by insulin. RGECs were incubated with 100 nmol/l insulin for the indicated time. One of three independent experiments is shown. $^{**}P<0.001$, $^{\dagger\dagger}P<0.001$, $^{\ddagger\ddagger}P<0.001$ vs 0 min. (**g**–**i**) Effect of IRS1 overexpression on RGECs on insulin-stimulated (**g**) p-Akt, (**h**) phosphorylated eNOS (p-eNOS), and (**i**) phosphorylated glycogen synthase kinase 3α (p-GSK3α). After RGECs were infected with Ad-green fluorescent protein (GFP) or with Ad-IRS1, cells were stimulated with insulin (100 nmol/l, 30 min) as indicated in low glucose (5.5 mmol/l) or high glucose (20 mmol/l). One of three independent experiments is shown. $^{*}P<0.05$, $^{**}P<0.001$. These data are expressed as means ± s.d.

pathway, resulting in the loss of eNOS and GSK3α actions. The target of PKC activation could be IRS1, which has been reported to be phosphorylated by PMA in nonvascular cells.[36] The finding that the inhibition of PKCβ can improve glomerular endothelial function and insulin actions is consistent with previous reports of RBX being able to improve endothelial dysfunction in diabetes and insulin-resistant states.[13,15]

Like diabetes, insulin resistance can also induce the selective loss of insulin action through the IRS/PI3K/Akt pathway.[37] However, the mechanism of this selective loss of insulin action in the glomeruli by insulin resistance appears to be different from diabetes, as no decreases in IRS1 protein or mRNA were found. This lack of change of IRS1 protein in the glomeruli and endothelial cells is consistent with other vascular beds that exhibit endothelial dysfunction.[15,36,38] In obesity, free fatty acid is known to be elevated and can activate PKC.[39] Our results indicate that abnormal metabolic factors, such as hyperglycemia and free fatty acids, can induce

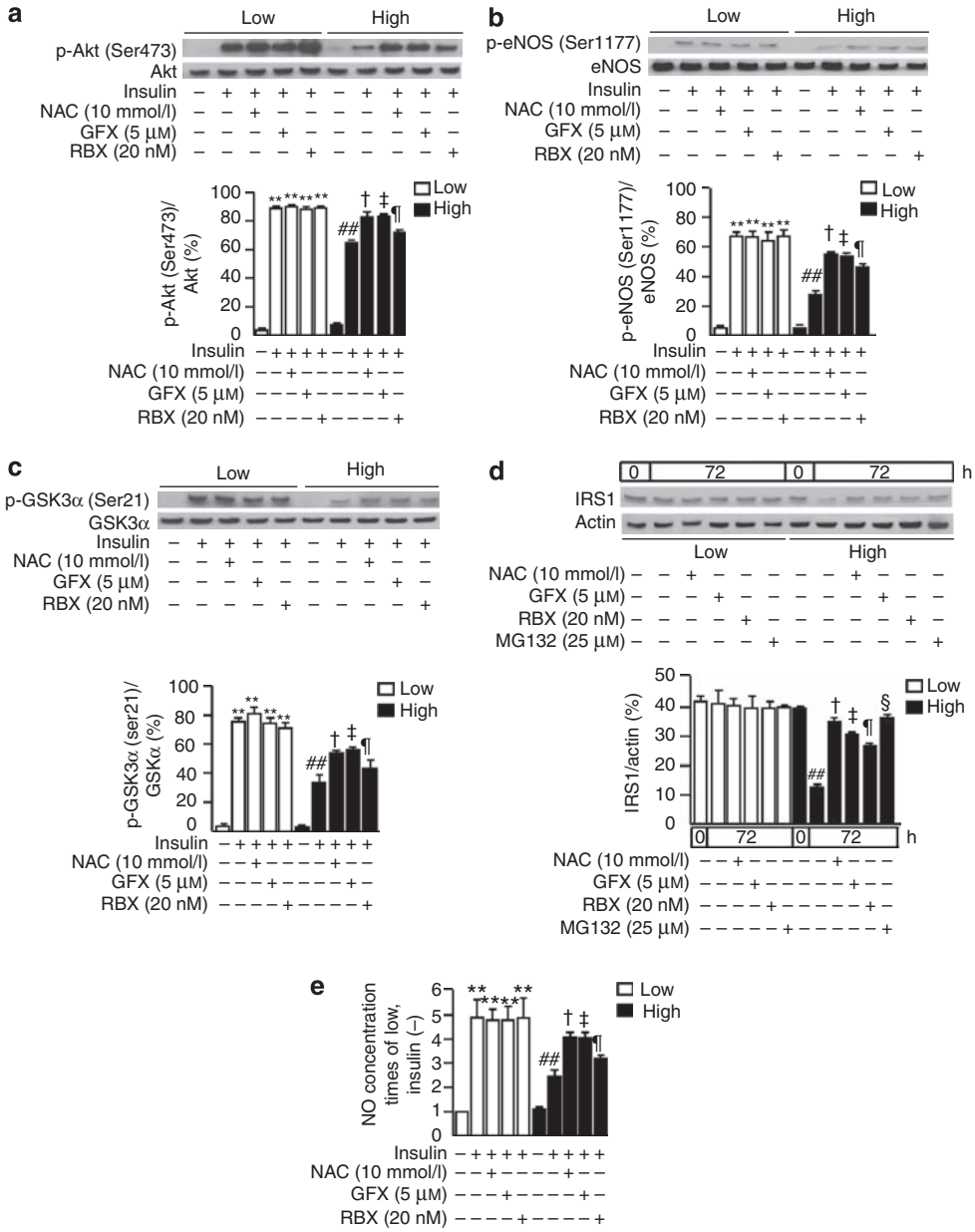


Figure 8 | Effect of NAC, GFX, RBX, and proteasome inhibitor on insulin signaling and degradation of insulin receptor substrate-1 (IRS1) in rat glomerular endothelial cells (RGECs). (**a**–**c**) After 48 h of exposure to low glucose (5.5 mmol/l) or high glucose (20 mmol/l), RGECs were stimulated with insulin (100 nmol/l, 30 min) with or without an antioxidant, *N*-acetyl-L-cystein (NAC, 10 mmol/l), or a protein kinase C (PKC)-specific inhibitor, GF109203X (GFX, 5 μM), or PKCβ-specific inhibitor, LY333531 (RBX, 20 nM). One of three independent experiments is shown. $^{**}P<0.001$ vs low/insulin(−)/NAC(−)/GFX(−)/RBX(−). $^{\#\#}P<0.001$ vs Low/insulin(+)/NAC(−)/GFX(−)/RBX(−). $^{\dagger}P<0.05$ vs High/insulin(+)/NAC(−)/GFX(−)/RBX(−). $^{\ddagger}P<0.05$ vs High/insulin(+)/NAC(−)/GFX(−)/RBX(−). $^{\P}P<0.05$ vs High/insulin(+)/NAC(−)/GFX(−)/RBX(−). (**a**) P-Akt, (**b**) phosphorylated endothelial nitric oxide synthase (p-eNOS), and (**c**) phosphorylated glycogen synthase kinase 3α (p-GSK3α). (**d**) RGECs were cultured in low glucose (5.5 mmol/l) or high glucose (20 mmol/l) media for 72 h with or without NAC (10 mmol/l), GFX (5 μM), RBX (20 nM), or proteasome inhibitor, MG132 (25 μM). One of three independent experiments is shown. $^{\#\#}P<0.001$ vs 0 h/High/NAC(−)/GFX(−)/RBX(−)/MG132(−). $^{\dagger}P<0.001$ vs 72 h/High/NAC(−)/GFX(−)/RBX(−)/MG132(−). $^{\ddagger}P<0.001$ vs 72 h/High/NAC(−)/GFX(−)/RBX(−)/MG132(−). $^{\P}P<0.001$ vs 72 h/High/NAC(−)/GFX(−)/RBX(−)/MG132(−). $^{\S}P<0.001$ vs 72 h/High/NAC(−)/GFX(−)/RBX(−)/MG132(−). (**e**) After 48 h of exposure to low glucose (5.5 mmol/l) or high glucose (20 mmol/l), RGECs were stimulated with insulin (100 nmol/l, 30 min) with or without NAC (10 mmol/l), GFX (5 μM), or RBX (20 nM). Nitric oxide (NO) levels in the culture media were measured with the Nitric Oxide Colorimeric Assay Kit. $^{**}P<0.001$ vs Low/insulin(−)/NAC(−)/GFX(−)/RBX(−). $^{\#\#}P<0.001$ vs Low/insulin(+)/NAC(−)/GFX(−)/RBX(−). $^{\dagger}P<0.05$ vs High/insulin(+)/NAC(−)/GFX(−)/RBX(−). $^{\ddagger}P<0.05$ vs High/insulin(+)/NAC(−)/GFX(−)/RBX(−). $^{\P}P<0.05$ vs High/insulin(+)/NAC(−)/GFX(−)/RBX(−). These data are expressed as means ± s.d.

selective insulin resistance in the renal glomeruli, probably because of different mechanisms between diabetes and obesity. The pathophysiological significance of the findings suggests that glomerular endothelial dysfunction alone will not cause glomerulopathy as observed in diabetes. This is reflected by the lack of significant pathologies in the renal glomeruli in the ZF insulin-resistant rats and the reduced level of nephropathy in obese and insulin-resistant population without diabetes. However, the contribution of glomerular endothelial dysfunction may contribute significantly to the initiation and progression of glomerular lesions in diabetes when it is combined with abnormalities in the mesangial cells and podocytes.

In summary, these observations have identified glomeruli as the site of insulin resistance in diabetic, obese, and other insulin-resistant states. Furthermore, these findings suggest that increasing IRS1 levels or inhibiting PKCβ action as a possible therapeutic target could prevent or improve renal function in diabetic and insulin-resistant states.

MATERIALS AND METHODS

Animal studies

All protocols for animal use were approved by the animal care committee of the Joslin Diabetes Center and were in accordance with the National Institutes of Health guidelines. We used age-matched male SD (Harlan, Indianapolis, IN) and ZF rats and their lean matched controls, ZL rats. Diabetes was induced in 6-week-old SD rats by a single intravenous injection of STZ (55 mg/kg body weight; Sigma, St Louis, MO) in 0.05 mol/l citrate buffer (pH 4.5) or citrate buffer for controls. Blood glucose levels, determined 2 days after the injections by glucose analyzer (Yellow Spring Instruments, Yellow Springs, OH) and levels >16.7 mmol/l, were defined as having diabetes. The rats were randomly divided into eight groups: Control, Control with the PKCβ-selective inhibitor RBX (LY333531) (Lilly, Indianapolis, IN) treatment, STZ-induced diabetic (DM), DM with RBX treatment, ZL, ZL with RBX treatment, ZF, and ZF with RBX treatment. RBX was given orally using mixed chow (5 mg/kg body weight per day) from the age of 7–14 weeks. Rats were anesthetized with 100 mg/kg of sodium pentobarbital injected intraperitoneally 8 weeks after diabetes or at 14 weeks of age for ZF and ZL. Regular human insulin (10 mU/g; Lilly) or diluents were injected into the inferior vena cava for studying insulin signaling and action. After 10 min of injection, kidneys were harvested and all the procedures were performed within 30 min.

Cell culture

Glomeruli were isolated from the kidneys of SD rats at 6 weeks of age under sterile conditions. The digested glomeruli were filtered through a 100 mm cell strainer (BD Biosciences, San Jose, CA) twice. After centrifugation, the cells were mixed with sheep anti-rat IgG beads (Invitrogen, Carlsbad, CA) coated with anti-ICAM2 antibody or with streptavidin-coupled beads (Invitrogen) with biotin anti-CD31 (BD Biosciences) at the antibody concentration of 3 μg for 1×10^7 beads in 1 ml Dulbecco's modified Eagle's medium containing 0.1% bovine serum albumin. After 1 h, RGECs were isolated using a MPC-50 magnet (Dynal, Hamburg, Germany).[40] The cells were cultured in 10 cm dishes precoated with rat collagen I (5 mg/cm^2; BD Biosciences) at 37 °C in a humidified 5% CO_2 atmosphere. On days 5–7 after seeding, outgrowths of individual glomeruli were detached by trypsin-EDTA (Invitrogen) and were washed with Dulbecco's modified Eagle's medium and subsequently treated with 0.1% collagenase type I (Worthington, Lakewood, NJ) in Dulbecco's modified Eagle's medium containing 0.1% bovine serum albumin at 37 °C for 1 h. Endothelial cell purity $>90\%$ was assessed by immunofluorescence staining with CD31.

Table 2 | Sequences of primers

Gene	Sequence (5′–3′)
18s rRNA	CGCGGTTCTATTTTGTTAGT; AGTCGGCATCGTTTATGGTC
IRS1	GCCAATCTTCATCCAGTTGC; CATCGTGAAGAAGGCATAGG
IRS2	CTACCCACTGAGCCCAAGAG; CCAGGGATGAAGCAGGACTA

Abbreviation: *IRS*, insulin receptor substrate.

Real-time PCR analysis

IRS1/2 mRNA were assayed by real-time PCR and normalized to 18S rRNA as described previously[41] (Table 2).

Data analysis

The data are expressed as mean ± s.d. Comparison among more than two groups was performed by one-way analysis of variance followed by the *post hoc* analysis with paired or unpaired *t*-test to evaluate statistical significance between the two groups. Statistical significance was defined as $P<0.05$.

Additional methodology

Reagents; measurement of urinary albumin; isolation of glomeruli and tubules; mesangial cell, podocyte, and RPTEC culture; adenoviral vector infection; immunoblot analysis; quantification of NO; NF-κB activation; and immunohistochemistry are described in the Supplementary Methods online.

DISCLOSURE

All the authors declared no competing interests.

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SUPPLEMENTARY MATERIAL

Figure S1: Characterization of insulin signalling in renal tissues and cells in culture.

Supplementary material is linked to the online version of the paper at http://www.nature.com/ki

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