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Nephrin loss in experimental diabetic nephropathy is prevented by deletion of protein kinase C alpha signaling *in-vivo*

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Albuminuria in diabetic nephropathy is due to endothelial dysfunction, a loss of negative charges in the basement membrane, and changes a of the slit-membrane diaphragm composition. We have recently shown that protein kinase C alpha (PKCα)-deficient mice are protected against the development of albuminuria under diabetic conditions. We here tested the hypothesis that PKCα mediates the hyperglycemia-induced downregulation of the slit-diaphragm protein nephrin. After 8 weeks of streptozotocin (STZ)-induced hyperglycemia the expression of glomerular nephrin was significantly reduced. In contrast, other slit-diaphragm proteins such as podocin and CD2AP were unaltered in diabetic state. In PKCα$^{-/-}$ mice, hyperglycemia-induced downregulation of nephrin was prevented. Podocin and CD2AP remained unchanged. In addition, the nephrin messenger RNA expression was also reduced in hyperglycemic wild-type mice but remained unaltered in PKCα$^{-/-}$ mice. We postulate that the underlying mechanism of the hyperglycemia-induced regulation of various proteins of the glomerular filtration barrier is a PKCα-dependent regulation of the Wilms' Tumor Suppressor (WT1) which previously has been shown to act as a direct transcription factor on the nephrin promoter. Our data suggest that PKCα activation may be an important intracellular signaling pathway in the regulation of nephrin expression and glomerular albumin permeability in the diabetic state.

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The incidence of diabetes mellitus is steadily rising and diabetic nephropathy is the leading cause of end-stage renal disease. An early marker for diabetic nephropathy is the occurrence of microalbuminuria. The glomerular vasculature consists of three structures that act in concert to prevent the development of albuminuria and proteinuria. These structures are the fenestrated endothelium, the glomerular basement membrane and the epithelial slit diaphragm. Several authors have shown that hyperglycemia leads to a downregulation of negatively charged proteoglycans in the basement membrane of the glomerulus.[1] Recently, a role of the slit membrane and especially of nephrin in the pathogenesis of diabetic albuminuria has been suggested.[2] An open question is which signaling pathways are involved in mediating the effects of hyperglycemia on the basement membrane and the slit-membrane proteins.[3]

We have recently demonstrated that protein kinase C alpha (PKCα)$^{-/-}$ animals are protected against the development of albuminuria.[4] When we analyzed the basement membrane in the PKCα-deficient diabetic mice we found that the loss of the negatively charged heparan sulfate proteoglycans was almost completely prevented and the glomerular basement membrane only modestly affected by hyperglycemia. Furthermore we were able to demonstrate, that PKCα influence the vascular endothelial growth factor system, which could contribute to an increased endothelial permeability. These findings suggest that PKCα is a major intracellular mediator of hyperglycemia – induced changes in the basement membrane. However, the second molecular system regulating glomerular permeability, that is, slit-diaphragm proteins has not been analyzed so far.

We therefore tested the hypothesis that PKCα mediates the hyperglycemia-induced downregulation of the slit-diaphragm protein nephrin because several groups previously demonstrated that nephrin expression is reduced in diabetic animals with albuminuria.[5–7] Furthermore, similar data were obtained in human when kidney biopsies from patients with Type I as well as Type II diabetes mellitus were studied.[8–12]

RESULTS

Hyperglycemia was induced in 7-week-old mice by intraperitoneal injection of streptozotocin (STZ) on day 1 and 4. After 8 weeks of hyperglycemia the nonfasting blood glucose levels were 25.9 ± 3.4 mmol/l in the diabetic wild-type (WT) ($n = 6$) and 32.2 ± 1.6 mmol/l in the PKC$\alpha^{-/-}$ mice ($n = 7$). In the sham-injected mice the levels were 12.2 ± 0.9 mmol/l in WT ($n = 7$) and 10.9 ± 0.6 mmol/l in the PKC$\alpha^{-/-}$ mice ($n = 7$).

After 8 weeks of hyperglycemia a significant higher urinary albumin excretion was detected in WT diabetic mice (24.78 ± 8.98 g/mol creatinine) compared with WT control mice (7.37 ± 0.47 g/mol creatinine) ($P < 0.05$). Notably, no significant increase of the albumin/creatinine ratio was observed in the diabetic PKC$\alpha^{-/-}$ (9.88 ± 1.67 g/mol creatinine) in comparison to control PKC$\alpha^{-/-}$ mice (6.94 ± 1.81 g/mol creatinine).

To further reveal the mechanism responsible for the prevention in albuminuria in the diabetic PKC$\alpha^{-/-}$ animals, we studied the expression of nephrin, a key protein of the glomerular slit membrane. We first assessed the expression of nephrin by immunochemistry. As shown in Figure 1 there was a strong podocytary expression of nephrin in the glomerular of nondiabetic WT (A) and PKC$\alpha^{-/-}$ mice (C). Notably, the glomerular expression of nephrin was significantly reduced in the diabetic WT animals and was barely detectable (Figure 1b). Importantly, this diabetes-induced loss of glomerular nephrin expression was completely prevented in the diabetic PKC$\alpha^{-/-}$ mice (Figure 1d).

To evaluate the role of other slit-diaphragm proteins, we secondly performed tissue analysis of other slit-diaphragm proteins, podocin and CD2AP. Podocin showed a podocytary expression comparable to nephrin in nondiabetic WT mice (Figure 2a). However, in contrast to nephrin, hyperglycemia did not induce any change in the expression level of podocin in the diabetic WT (Figure 2b). We also did not observe any changes in the nondiabetic (Figure 2c) and diabetic PKC$\alpha^{-/-}$ mice (Figure 2d). Immunohistochemistry of the third slit-diaphragm protein, CD2AP, displayed comparable results to podocin as shown in (Figure 3a–d). Hyperglycemia did not have any effect on the expression of CD2AP in WT and PKC$\alpha^{-/-}$ mice.

As we observed a prevention of the hyperglycemia-induced nephrin loss in our diabetic PKC$\alpha^{-/-}$ mice, we next measured the nephrin expression by western blot analysis as well as the podocin and CD2AP expression to confirm and quantitatively analyze the results obtained from the immunohistological tissue evaluation. The results are shown in Figure 4a–c. Nephrin was expressed as a single band with a molecular weight (MW) of 136 kDa. Hyperglycemia of 8 weeks reduced the expression of nephrin significantly as observed with immunohistochemical analysis. The high-glucose-induced nephrin loss is again prevented in the PKC$\alpha^{-/-}$ mice (Figure 4a). In contrast, Western blot analysis

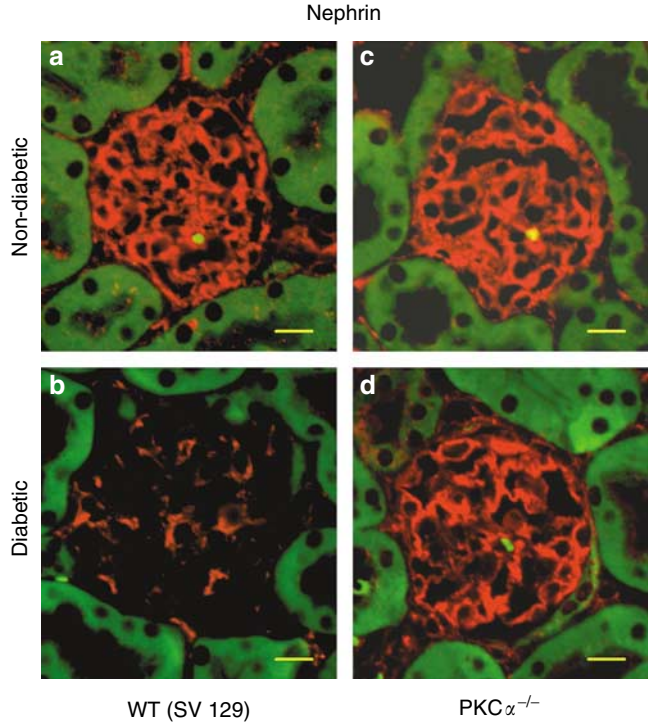

Figure 1 | Immunohistochemistry of nephrin expression in WT and PKCα$^{-/-}$ mice. Immunohistochemistry of nephrin expression in (**a**, **b**) WT and (**c**, **d**) PKC$\alpha^{-/-}$ mice shows markedly reduced expression of nephrin in (**b**) diabetic WT mice compared to the other groups. Paraffin sections; bar = 50 μm. (**b**) hyperglycemia-induced nephrin loss is prevented in the diabetic PKC$\alpha^{-/-}$ mice.

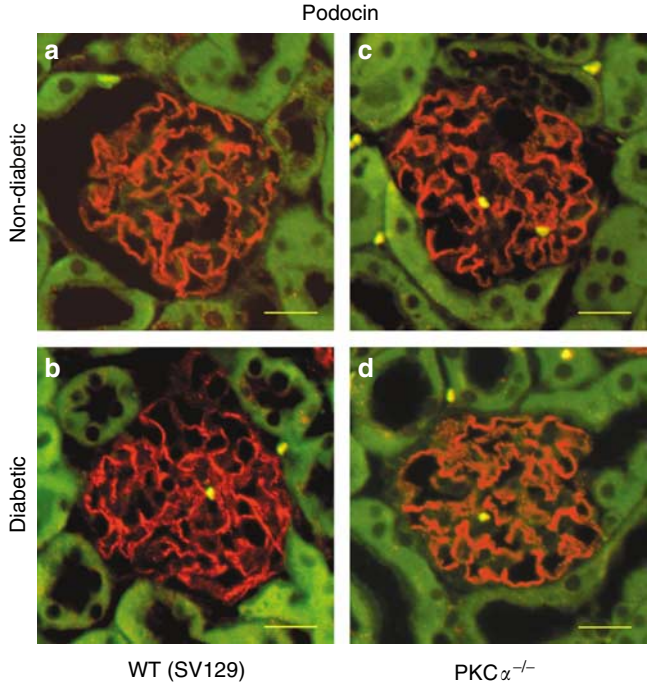

Figure 2 | Immunohistochemistry of podocin expression in WT and PKCα$^{-/-}$ mice. Immunohistochemistry of podocin expression in (**a**, **b**) WT and (**c**, **d**) PKC$\alpha^{-/-}$ mice displays no hyperglycemia-induced change in the expression of podocin in the diabetic WT animals compared to healthy controls. Furthermore, WT and PKC$\alpha^{-/-}$ mice shows comparable expression levels. Paraffin sections; bar = 50 μm.