PKC-δ Promotes Renal Tubular Cell Apoptosis Associated with Proteinuria

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ABSTRACT

Proteinuria may contribute to progressive renal damage by inducing tubulointerstitial inflammation, fibrosis, and tubular cell injury and death, but the mechanisms underlying these pathologic changes remain largely unknown. Here, in a rat kidney proximal tubular cell line (RPTC), albumin induced apoptosis in a time- and dose-dependent manner. Caspase activation accompanied albumin-induced apoptosis, and general caspase inhibitors could suppress this activation. In addition, Bcl-2 transfection inhibited apoptosis and attenuated albumin-induced Bax translocation to mitochondria and cytochrome c release from the organelles, further confirming a role for the intrinsic pathway of apoptosis in albuminuria-associated tubular apoptosis. We observed phosphorylation and activation of PKC-δ early during treatment of RPTC cells with albumin. Rottlerin, a pharmacologic inhibitor of PKC-δ, suppressed albumin-induced Bax translocation, cytochrome c release, and apoptosis. Moreover, a dominant-negative mutant of PKC-δ blocked albumin-induced apoptosis in RPTC cells. In vivo, we observed activated PKC-δ in proteinuric kidneys of streptozotocin-induced diabetic mice and in kidneys after direct albumin overload. Notably, albumin overload induced apoptosis in renal tubules, which was less severe in PKC-δ-knockout mice. Taken together, these results suggest that activation of PKC-δ promotes tubular cell injury and death during albuminuria, broadening our understanding of the pathogenesis of progressive proteinuric kidney diseases.


Proteinuria or albuminuria is a common feature of chronic kidney diseases, including diabetic nephropathy and nephrotic syndromes.1–4 Although proteinuria is a result of renal injury, it can also be a causative or aggravating factor for progressive renal damage. Excessive protein load can induce tubulointerstitial inflammation, fibrosis, and tubular cell injury and death.1 However, the mechanisms behind these pathologic changes remain largely unclear.

Apoptosis of renal tubular cells has been shown in both in vitro and in vivo models of proteinuria or albuminuria. In 2001, Erkan and colleagues5 showed that albumin overload induced apoptosis in LLC-PK1 proximal tubular cells. Interestingly, the apoptosis was associated with up-regulation of Fas signaling and caspase-8 activation, suggesting a

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1115
role for this extrinsic apoptotic pathway in albumin-induced apoptosis in LLC-PK<sub>1</sub> cells. These in vitro observations were later shown to be relevant to renal tubular apoptosis associated with proteinuria in kidney biopsy specimens from children with focal segmental glomerulosclerosis. However, in HKC-8 human proximal tubular cells, albumin-induced apoptosis was shown to be mainly mediated by the intrinsic pathway of apoptosis, characterized by Bax translocation to mitochondria and cytochrome c release from the organelles. In addition, Ohse et al.<sup>8</sup> demonstrated endoplasmic reticulum (ER) stress in albumin-treated rat kidney proximal tubular cells and proteinuric kidneys of puromycin aminonucleoside-treated rats, suggesting the involvement of ER stress in tubular cell apoptosis. Recent work by Lindenmeyer et al.<sup>9</sup> further demonstrated ER stress in proteinuric kidney biopsy of established diabetic nephropathy. Together, these studies suggest that multiple pathways of apoptosis can be activated in renal tubular cells during proteinuric kidney diseases.

However, despite the recognition of multiple apoptotic pathways, very little is known about the upstream signaling that is activated by protein overload, leading to tubular cell apoptosis. In this regard, Erkan et al.<sup>7</sup> showed that albumin-induced apoptosis in HKC-8 cells could be suppressed by bisindolylmaleimide-I, a general pharmacologic inhibitor of protein kinase C (PKC). Nonetheless, it remains unclear which PKC member(s) is activated during albumin treatment and plays a key role in the induction or regulation of tubular cell apoptosis. PKC is a family of serine/threonine protein kinases that has been implicated in the regulation of a variety of cellular activities, from cell growth, migration, and survival to cell death.<sup>10</sup> Depending on their activation mechanisms, PKC members are divided into classical, novel, and atypical subfamilies. In this study, we show that PKC-δ, a novel PKC, is activated during albumin treatment of tubular cells in vitro and in proteinuric renal tissues in vivo. Importantly, inhibition of PKC-δ pharmacologically or genetically can attenuate tubular cell apoptosis under these pathologic conditions. The results have, therefore, revealed a novel role for PKC-δ in the regulation of proteinuria-associated tubular injury, apoptosis, and atrophy.

RESULTS

**Albumin-Induced Apoptosis in RPTC**

Our initial experiments characterized albumin-induced apoptosis in RPTC, a rat proximal tubular cell line. RPTC was incubated for 24 hours in serum-free medium in the presence of 0 to 40 mg/ml bovine serum albumin or transferrin. The cells were then stained with Hoechst 33342 to examine cellular and nuclear morphology or fixed for TdT-mediated dUTP nick-end labeling (TUNEL) assay. As shown in Figure 1A, in the albumin-treated groups there were many cells that developed the typical morphology of apoptosis, showing shrunken configuration, noticeable apoptotic bodies, and condensed and fragmented nuclei. In contrast, very few or no apoptotic cells were detected in the control cells or the cells incubated with transferrin (Figure 1A). Consistently, significantly more TUNEL-positive cells were shown after albumin incubation (Figure 1A). Quantification by cell counting showed that treatment with 10, 20, and 40 mg/ml albumin induced 16%, 43%, and 62% of apoptosis in 24 hours, respectively, whereas less than 3% of apoptosis was shown in the control and transferrin-treated cells (Figure 1B). Albumin-induced apoptosis in RPTC was also treatment time dependent. As a result, 20 mg/ml albumin induced 4%, 13%, 36%, and 43% apoptosis in 12, 16, 20 and 24 hours, respectively (Figure 1C). The morphologic observations of apoptosis were confirmed by measurement of caspase activation (Figure 1D and Supplemental Figure 1, A and B). As shown in Figure 1D, significant caspase activation was induced by 20 mg/ml albumin within 16 hours. As expected, caspase activation was blocked by Z-VAD, a broad-spectrum peptide inhibitor of caspases. Z-VAD also suppressed RPTC apoptosis during albumin treatment (Figure 1E). For example, 20 mg/ml albumin induced over 40% apoptosis in 24 hours, which was suppressed to 17% by Z-VAD. Albumin treatment for 24 hours did not induce significant necrosis as shown by lack of propidium iodide staining (data not shown). Together, these results indicate that albumin mainly induced caspase-dependent apoptosis in RPTC cells.

**Effects of Bcl-2 Expression on Albumin-Induced Cytochrome c Release and Apoptosis**

Recent work by Erkan and colleagues<sup>7</sup> has suggested the activation of the intrinsic mitochondrial pathway of apoptosis during albumin treatment of HKC-8 human proximal tubular cells. In line with those findings, we detected the release of mitochondrial cytochrome c into cytosol during albumin treatment of RPTC (Figure 2A, lane 2). Notably, albumin-induced cytochrome c release was blocked in RPTC stably transfected with Bcl-2 (Figure 2A, lane 5). Albumin-induced apoptosis was also attenuated in these Bcl-2 cells. As shown in Figure 2B, 20 mg/ml albumin induced 47% apoptosis in RPTC in 24 hours, but only 3% in Bcl-2 cells. Consistently, albumin-induced caspase activation was completely blocked in these cells. The results using the stable Bcl-2 cell line were confirmed by transient transfection experiments. As shown in Supplemental Figure 2, transient transfection of Bcl-2 into RPTC suppressed albumin-induced cytochrome c release and apoptosis, whereas vector transfection was ineffective. Together, the results support the recent work by Erkan et al.<sup>7</sup> that the mitochondrial pathway of apoptosis plays an important role in albumin-induced apoptosis in renal tubular cells.

**PKC-δ Activation during Albumin Treatment of RPTC**

PKC-δ is a member of novel PKCs, which can be activated by diacylglycerol independent of Ca<sup>2+</sup>. Recent studies have further demonstrated several other activation mechanisms of PKC-δ, including proteolysis, dimerization, and phosphorylation. Especially, tyrosine phosphorylation has been recognized...
Figure 1. Albumin induces apoptosis and caspase activation in RPTC cells. (A) Representative images of cellular and nuclear morphology. RPTC cells were untreated (Control), treated with 20 mg/ml low-endotoxin BSA (Albumin), or treated with 20 mg/ml transferrin in serum-free medium for 24 hours. The cells were then stained with Hoechst 33342 to record cellular and nuclear morphology or were subjected to TUNEL staining. Magnification, ×400. (B) Dose dependence of albumin-induced apoptosis in RPTC cells. RPTC cells were incubated for 24 hours with 0 to 40 mg/ml albumin or 40 mg/ml transferrin in serum-free medium. The cells were then examined by microscopy to count cells with typical apoptotic morphology to determine the percentage of apoptosis. (C) Time course of albumin-induced apoptosis in RPTC cells. RPTC cells were treated with 20 mg/ml albumin for 0 to 24 hours. Apoptosis was quantified by morphologic methods. (n = 5). (D) Caspase activation during albumin incubation of RPTC cells. RPTC cells were incubated with 20 mg/ml albumin for indicated durations in the absence or presence of 50 μM Z-VAD to collect lysate for measurement of caspase activity. (E) Inhibition of albumin-induced apoptosis by Z-VAD. RPTC cells were incubated with 20 mg/ml albumin for indicated durations in the absence or presence of 50 μM Z-VAD. Apoptosis was evaluated by counting the cells with typical apoptotic morphology. Data in B to E are expressed as mean ± SD, n = 5. In B and C, *P < 0.01 versus control (0 mg/ml albumin). In D and E, bP < 0.01 between two compared groups.
as a distinct activation mechanism for PKC-δ that is not shared by other PKC members.13 Our immunoblot analysis detected an albumin treatment time and dose-dependent PKC-δ phosphorylation at Tyr-311 (Figure 3). As shown in Figure 3A, PKC-δ Tyr-311 phosphorylation started after 4 hours of 20 mg/ml albumin treatment, reached high levels at 10 to 12 hours, and then decreased toward basal levels (Figure 3A). Total PKC-δ was relatively constant during albumin treatment (Figure 3A). The time course results suggest that PKC-δ was activated by albumin before apoptosis, which became noticeable at 16 hours (Figure 1C). A correlation of PKC-δ activation and apoptosis was also suggested by the dose responses. Obvious PKC-δ Tyr-311 phosphorylation was induced by 10 to 40 mg/ml albumin (Figure 3B). Densitometry of immunoblots from separate experiments showed that 5 mg/ml albumin induced a marginal PKC-δ activation, which was increased dramatically to 4- to 6-fold of control by 10 to 40 mg/ml albumin (Figure 3C). In sharp contrast, 40 mg/ml transferrin did not induce PKC-δ phosphorylation, suggesting that the observed PKC-δ activation was a specific cellular response to albumin and not a result of nonspecific protein overload. To further confirm albumin-induced PKC-δ activation in RPTC, we conducted in vitro immunocomplex protein kinase activity assay. As shown in Supplemental Figure 3, immunoprecipitation pulled down similar amounts of PKC-δ from lysates of control and albumin-treated cells; however, the PKC-δ precipitated from albumin-treated cells induced significantly higher histone phosphorylation during the in vitro kinase assay (lane 4 versus 3).

Inhibition of Albumin-Induced Apoptosis and PKC-δ Activation in RPTC by Rottlerin

To determine whether PKC-δ is involved in tubular cell injury by albumin overload, we initially tested the effects of Rottlerin, a pharmacologic inhibitor of PKC-δ.14,15 We first titrated the concentrations of Rottlerin and found the Rottlerin was toxic to RPTC at over 5 μM (data not shown). We then examined the effects of 0.5 to 4 μM Rottlerin on RPTC apoptosis during 20 mg/ml albumin treatment. As shown in Figure 4A, Rottlerin was marginally inhibitory to albumin-induced apoptosis at 0.5 μM but diminished apoptosis to approximately 50% at 1, 2, and 4 μM. The results of morphologic observation were confirmed by analyzing caspase activity (Figure 4B). Clearly, Rottlerin suppressed albumin-induced caspase activation in a analyzed by densitometry. The results were normalized with the value of the control (0 mg/ml albumin), which was arbitrarily set as 100. Data are expressed as mean ± SD, n = 3. aP < 0.05 compared with control; bP < 0.01 compared with control.
To this end, RPTC cells were incubated with 20 mg/ml albumin for 24 hours with 0 to 4 μM Rottlerin. Apoptosis was evaluated by morphologic methods. (B) Cell lysates were collected to determine caspase activity by an enzymatic assay. (C) RPTCs were incubated for 12 hours without (−) or with (+) 20 mg/ml albumin in the absence (−) or presence (+) of 1 μM Rottlerin. Whole-cell lysates were collected for immunoblot analysis of Tyr-311-phosphorylated PKC-δ, total PKC-δ, and β-actin. (D) RPTCs were incubated with 20 mg/ml albumin for 24 hours. Rottlerin (1 μM) was added at 0, 8, 12, or 16 hours of albumin treatment. Another group of cells was treated with albumin only without Rottlerin. Apoptosis was evaluated by morphologic methods. Data in A to C are expressed as mean ± SD, n = 3. *P < 0.05 and 9P < 0.01 versus albumin-only group.

dose-dependent manner. To minimize the potential toxicity of Rottlerin, we chose 1 μM Rottlerin for further study. We showed that 1 μM Rottlerin could effectively block PKC-δ Tyr-311 phosphorylation during albumin treatment (Figure 4C, lane 3 versus 2). On the basis of these results, we further determined the effect of Rottlerin added at different time points after albumin treatment. As shown in Figure 4D, Rottlerin added at 0, 8, and 12 hours of albumin treatment could protect against albumin-induced apoptosis, whereas Rottlerin became ineffective when added at 16 hours of albumin treatment, a time point after PKC-δ activation. These results suggested the first evidence for a role of PKC-δ in albumin-induced apoptosis in renal tubular cells.

Effects of Rottlerin on Albumin-Induced Bax Translocation and Cytochrome c Release in RPTC
Erkan and colleagues have recently demonstrated a role for the mitochondrial/intrinsic pathway of apoptosis in albumin-induced apoptosis in HKC-8 renal tubular cells. Consistently, we showed cytochrome c release from mitochondria during albumin incubation of RPTC, which was suppressed by Bcl-2 expression (Figure 2). To understand the mechanism of the mitochondrial events of apoptosis; i.e., Bax accumulation in mitochondria and cytochrome c release from the organelles. To this end, RPTC cells were incubated with 20 mg/ml albumin in the absence or presence of 1 μM Rottlerin. The cells were then fractionated into cytosolic and mitochondrial fractions for immunoblot analysis. As shown in Figure 5A, albumin treatment led to marked increases of Bax in the mitochondrial fraction at 16 and 24 hours (Figure 5A, lanes 3 and 4). Notably, the accumulation was suppressed by Rottlerin (Figure 5A, lanes 6 and 7). Rottlerin also inhibited the release of cytochrome c from mitochondria during albumin treatment (Figure 5B, lanes 3 and 4 versus 6 and 7). Together, the results suggest that by inhibiting PKC-δ, Rottlerin can suppress the mitochondrial pathway of apoptosis during albumin treatment to protect renal tubular cells.

Effects of Dominant-Negative PKC-δ on Albumin-Induced Apoptosis in RPTC
To confirm the pharmacologic observations with Rottlerin, we determined the effects of expression of a dominant-negative mutant of PKC-δ (dn-PKC-δ), which had a point mutation in the active site and lost its kinase activity. RPTC cells were transiently cotransfected with GFP and dn-PKC-δ, dn-PKC-α, or empty vector. The cells were then incubated with 20 mg/ml albumin. Apoptosis was analyzed in the transfected (GFP-labeled) cells. Representative cell images are shown in Figure 6A. Albumin induced apoptosis in vector/GFP-transfected cells, showing cellular condensation and nuclear fragmentation, whereas obviously fewer cells were apoptotic in dn-PKC-δ/
GFP-transfected group (Figure 6A). Quantification by cell counting showed about 40% apoptosis in both empty vector and dn-PKC-α-transfected cells but only 19% in dn-PKC-δ-transfected cells. The results provided further support for a role for PKC-δ in tubular cell apoptosis under pathologic conditions of proteinuria or albuminuria.

**Albumin Overload-Induced PKC-δ Activation and Tubular Cell Apoptosis in Mice: Suppressed by PKC-δ Deficiency**

To investigate the involvement of PKC-δ in proteinuria or albuminuria-associated apoptosis *in vivo*, we used a PKC-δ knockout mouse model. Albuminuria was induced in mice by albumin overload as described in previous studies. Briefly, albumin was injected for 6 weeks in PKC-δ knockout mice and wild-type littermates. Control groups were injected with saline. As shown in Figure 7A, albumin overload led to a marked increase in PKC-δ expression and Tyr-311 phosphorylation in wild-type mice (lanes 3 and 4 versus lanes 5 and 6). As expected, regardless of the treatment, PKC-δ was not detected in tissues of PKC-δ knockout mice (Figure 7A, lanes 2, 5, and 6). We also compared tissue lysates collected after 3 and 6 weeks of albumin overload treatment. PKC-δ phosphorylation was detected at 3 weeks and continued to increase at 6 weeks (Supplemental Figure 4). Importantly, albumin overload induced apoptosis in renal tissues in wild-type mice, which was significantly ameliorated in PKC-δ knockout littermates (Figure 7, B and C). As shown by TUNEL assay, about 37 apoptotic cells were detected in 20 fields of wild-type renal tissues under 200× magnification microscopy, but only 15 were detected in renal tissues of PKC-δ knockout mice after albumin treatment (Figure 7B). As expected, very few or no apoptotic cells were observed in saline controls (Figure 7B). Representative images of TUNEL staining are shown in Figure 7C. Apoptotic cells were mainly shown in renal tubules, with occasional occurrence in the interstitium. Of note, albumin injection induced comparable increases in urinary protein excretion in PKC-δ knockout and wild-type mice (Supplemental Figure 5). Thus,

**Figure 6.** Dominant negative PKC suppresses albumin-induced RPTC apoptosis. RPTCs were cotransfected with pEGFP-C3 and dominant-negative PKC-δ (dn-PKC-δ), dominant-negative PKC-α (dn-PKC-α), or empty pcDNA3.1 vector. The cells were then incubated for 24 hours with 20 mg/ml albumin and stained with Hoechst 33342 for morphologic examination of apoptosis. (A) Representative cell morphology. Magnification, ×400. Arrows show transfected cells that showed typical apoptotic morphology. (B) Percentage of apoptosis in GFP-labeled transfected cells. Data are mean ± SD, n = 3. hP < 0.01 versus vector control.

**Figure 7.** Apoptosis induced by albumin overload in renal tissues requires PKC-δ. Male PKC-δ knockout mice and their wild-type littermates were injected with albumin at 10 mg/g body weight for 5 consecutive days per week for 6 weeks. Control animals received comparable volumes of saline. (A) At the end of albumin injections, kidney tissues were collected for immunoblot analysis of Tyr-311-phosphorylated PKC-δ and total PKC-δ. The blot was reprobed for cyclophilin B to monitor protein loading and transferring. (B) Kidneys were fixed in 4% paraformaldehyde and paraffin embedded for TUNEL assay. Apoptosis in renal tissues was quantified by counting the total of TUNEL-positive cells in 20 random fields at the magnification of ×200. Data are mean ± SD, n = 3. kP < 0.01, WT versus KO. (C) Representative images of TUNEL assay. Hoechst 33342 costaining was performed at the end of TUNEL staining to identify the nuclei. Magnification: ×400 in images; ×850 in inserts. Arrows show nuclei with positive TUNEL staining. Insets show TUNEL-positive nuclei at high magnifications.
the apoptosis difference between these two groups of animals was not due to differences in their protein exposure; rather, it was due to the difference in tubular sensitivity to apoptosis. Together, these results provided direct *in vivo* evidence for a role of PKC-δ in tubular cell apoptosis during proteinuria or albuminuria.

**DISCUSSION**

Proteinuria or albuminuria may contribute to the development and progression of chronic kidney diseases by inducing tubulointerstitial inflammation, fibrosis, and tubular cell injury and death. Tubular cell apoptosis has been demonstrated during albumin treatment of cultured renal tubular cells, in proteinuric animal models and also in human patients with nephrotic syndromes. Although both extrinsic and intrinsic pathways of apoptosis have been shown to contribute to tubular apoptosis during proteinuria, recent studies have further suggested the involvement of ER stress. Despite these findings, very little is known about the upstream signaling that leads to the activation of the apoptotic pathways. In this study, we have confirmed a role for the intrinsic pathway mediated by mitochondrial injury in albumin-induced apoptosis in renal tubular cells. Importantly, we have demonstrated compelling evidence for a role of PKC-δ in tubular apoptosis during albumin treatment *in vitro* and proteinuria *in vivo*.

Previous studies have suggested the involvement of PKC in tubular response to protein overload. In HK-2 cells, Morigi et al. showed that albumin induced a rapid oxidative stress and NF-κB activation and consequent expression of inflammatory genes, all of which were blocked by the PKC inhibitor calphostin C. Consistent results were shown by other studies. More relevant to our current findings, Erkan et al. showed that bisindolylmaleimide-1, a general inhibitor of PKC, protected HKC-8 cells from albumin overload-induced apoptosis. These pharmacologic results suggested that PKC may have an important role in the regulation of tubular cell response to proteinuria *in vivo*, but the identity of the involved PKC member(s) was not known. Our study has now demonstrated a critical role for PKC-δ in tubular cell apoptosis under the pathologic condition. In cultured rat proximal tubular RPTC cells, we demonstrated an early PKC-δ phosphorylation at the Tyr-311 site and further confirmed albumin-induced PKC-δ activation by immunocomplex kinase activity assay (Supplemental Figure 4). Inhibition of PKC-δ with Rottlerin suppressed albumin-induced apoptosis. Of note, the protective effects of Rottlerin in our study were verified by experiments using a dominant-negative mutant of PKC-δ. Interestingly, in HKC-8 cells Erkan et al. did not show significant effects of Rottlerin on albumin-induced apoptosis (not shown results in Erkan et al.). The lack of effects of Rottlerin could be related to the experimental conditions, such as cell type and concentrations of Rottlerin. We noticed in our study that the optimal protective concentrations of Rottletin were between 1 and 4 μM; Rottlerin was toxic to RPTC cells and induced apoptosis by itself at higher concentrations.

*In vivo* in kidney tissues, PKC-δ was activated by albumin overload in mice. Interestingly, the expression of total PKC-δ was also increased, whereas the levels of total PKC-δ did not change significantly during albumin treatment of RPTC cells. The cause of PKC-δ expression by direct albumin overload in mouse kidneys is currently unclear. To determine the role of PKC-δ in proteinuric tubular apoptosis *in vivo*, we used the PKC-δ-null mouse model. It was shown that albumin overload induced apoptosis in renal tissues of wild-type mice, which was markedly suppressed in PKC-δ-null littermates. Together with the *in vitro* cell culture results, these data support a critical role for PKC-δ in the regulation of tubular cell injury and death during proteinuria. Of note, in the present study, albumin overload-induced apoptosis in kidney tissues was relatively low. Consistently, renal tissue pathology was also minimal in these animals (Figure 7). The low apoptosis and renal injury rate was likely caused by the genetic background of the mouse line used in our study. Ishola and colleagues reported that the renal response to albumin overload is mouse strain dependent. Although 129/Sv mice are generally sensitive to albumin-induced renal injury, C57BL/6 mice are much more resistant. The PKC-δ-null mice and their wild-type littermates used in our study had a mixed genetic background of C57BL/6 to 129/Sv and thus are expected to be somewhat resistant to albumin treatment. Despite the apoptosis rate, our experiments showed significantly lower apoptosis in PKC-δ-null mice, supporting the involvement of PKC-δ in albuminuric or proteinuric apoptosis in renal tissues. In addition, we detected PKC-δ activation in STZ-induced diabetic mice that were proteinuric (data not shown), although the involvement of PKC-δ in tubular cell apoptosis in this model remains to be determined by further studies.

It is unclear how PKC-δ is activated by albumin in RPTC cells and proteinuria *in vivo* in renal tissues. An interesting possibility is that protein overload may induce oxidative stress in the cells, which may then lead to PKC-δ activation. However, Morigi et al. showed that in HK2 cells, inhibition of PKC abrogated albumin-induced H$_2$O$_2$ production, suggesting that oxidant stress in HK-2 cells occurs downstream of PKC activation. Consistently, Erkan et al. showed that albumin-induced apoptosis in HKC-8 cells was independent of oxidant stress. Because albumin induced PKC-δ phosphorylation at Tyr-311, we speculate that a protein tyrosine kinase is involved in PKC-δ activation in renal tubular cells during proteinuria. Further investigation should gain insights into the responsible protein tyrosine kinase.

The mechanism underlying PKC-δ regulation of tubular cell apoptosis during proteinuria is currently unclear. One possibility is that PKC-δ may be involved in the regulation of albumin uptake. Hryciw et al. showed that general PKC inhibitors were effective in blocking albumin uptake by opossum kidney proximal tubule cells. They further demonstrated the inhibitory effects of specific inhibitors and dominant-negative mutants of PKC-α. These observations support a specific role.
for PKC-α in albumin uptake by renal tubular cells, although it remains unclear whether other PKC isoforms, such as PKC-δ, are also involved. On the other hand, PKC-δ has been suggested to be directly involved in the regulation of apoptosis. A proapoptotic role for PKC-δ has been demonstrated in a variety of experimental models. Mechanistically, PKC-δ can promote apoptosis at multiple levels, including the activation of apoptotic gene expression, phosphorylation of caspases, interaction with apoptotic regulators, remodeling of cell membranes, and regulation of mitochondria. A recent study further suggested a role for PKC-δ in ER-mitochondria crosstalk during ER-stress-induced apoptosis. It remains unknown how PKC-δ regulates Bax during apoptosis. Our results do not show clear evidence for a direct interaction and phosphorylation of Bax by PKC-δ (unpublished observations). We thus speculate that PKC-δ may be involved in the signaling pathway that leads to Bax activation under conditions of albumin overload. Unfortunately, despite extensive effort, the mechanism(s) underlying Bax activation in apoptosis remains poorly understood, making it difficult to determine how PKC-δ is involved in Bax regulation. Nevertheless, it would be important to investigate in the future to gain in-depth understanding of the regulation of tubular cell apoptosis by PKC-δ during proteinuric stress.

In conclusion, this study has demonstrated PKC-δ activation during albumin treatment of renal tubular cells in vitro and proteinuria in vivo. Under the experimental conditions, blockade of PKC-δ pharmacologically or genetically can protect the cells against apoptosis. The results suggest that it is possible to target PKC-δ for renoprotection in diabetic nephropathy and nephrotic syndromes.

CONCISE METHODS

Antibodies and Special Reagents
Antibodies were purchased from the following sources: polyclonal anti-PKC-δ from Calbiochem (San Diego, CA) and Cell Signaling Technology, polyclonal anti-phospho-PKC-δ Tyr-311 and polyclonal anti-Bax (N-20) from Santa Cruz Biotechnology; and monoclonal anti-cytochrome c from BD Biosciences (San Diego, CA). All secondary antibodies for Western blot analysis were from Jackson ImmunoResearch (West Grove, PA), and secondary antibodies for immunofluorescence were from Chemicon (Temecula, CA). Plasmids of dominant-negative mutant PKC-δ (PKC-δ-KD) and PKC-α (PKC-α-KD) were generously given by Drs. Jae-Won Soh (Inha University, Inchun, Korea) and Fushin Yu (Wayne State University, Detroit, MI). Bcl-2 plasmids were kindly provided by Dr. David W. Andrews (McMaster University, Hamilton, ON, Canada). In situ Cell Death Detection kit was obtained from Roche Applied Science (Indianapolis, IN). The caspase inhibitor (Z-VAD) and fluorogenic substrate DEVD-7-amino-4-trifluoromethylcoumarin (AFC) were purchased from Enzyme Systems Products (Dublin, CA). [γ-32P] ATP was from MP Biochemicals (Solon, OH). Protein A/G plus-agarose was from Santa Cruz Biotechnology (Santa Cruz, CA). Enhanced chemiluminescence kit was purchased from Pierce Biotechnology (Rockford, IL). Rottlerin was obtained from Calbiochem. Other reagents, including fatty-acid-free, low-endotoxin bovine serum albumin were obtained from Sigma (St. Louis, MO).

Albumin Treatment of RPTC
The immortalized RPTC line was originally obtained from Dr. U. Hopfer (Case Western Reserve University, Cleveland, OH). The RPTC cells stably transfected with Bcl-2 were generated and used in our previous study. The cells were maintained for experiments as described previously. For albumin treatment, the cells were washed twice with phosphate-buffered saline and once with serum-free medium, and then they were incubated with albumin in serum-free Dulbecco modified Eagle medium/F-12 medium. To test the effects of specific inhibitors, the inhibitors were given during the incubation period. At the end of incubation, cells were evaluated for apoptotic morphology or lysed to collect cell lysates for biochemical analysis.

Albumin Overload in Mice
The PKC-δ knockout mouse model was established by the laboratory of Dr. Robert O. Messing as described previously. The mouse line had a mixed genetic background of C57Bl/6 to 129/Sv. Heterozygous breeders were used to produce PKC-δ knockout mice and wild-type littermates for this study. Albumin overload was conducted by a method modified from previous studies. Briefly, male PKC-δ knockout mice and their wild-type littermates of 6 to 8 weeks were injected intraperitoneally with low-endotoxin BSA (10 mg/g body weight, dissolved in saline) for 5 consecutive days per week for indicated time. Control animals were injected with a comparable volume of saline. To monitor urinary protein, urine samples were collected before and after the first albumin injection, as well as after 3 and 6 weeks of injection. Urinary proteins were measured by the bicinchoninic acid (BCA) method. After the injections, some mice were sacrificed to collect kidney tissues for biochemical analysis. For other mice, their kidneys were perfused with ice-cold heparin saline followed by ice-cold saline for fixation in 4% paraformaldehyde for histologic examination and TUNEL assay.

Measurement of Urinary Proteins
Urine samples were collected in 24 hours using metabolic cages while the mice were allowed to intake water and food freely. Urinary proteins were measured using the BCA Protein Assay kit (Pierce).

Transient Transfection of RPTC
Transient transfection was conducted using Lipofectamine 2000 (Invitrogen) as described previously. Briefly, cells were plated on collagen-coated glass coverslips at 0.4–0.5 × 10⁶ per 35-mm dish. The cells were then transfected with 1.0 µg of PKC plasmids, Bcl-2 plasmids, or the empty vector pcDNA3.1 (Invitrogen). pEGFP-C3 (0.2 µg per dish; Clontech) was cotransfected to label the transfected cells with green fluorescence protein (GFP). The transfected cells were used in the next day for experimental treatment.

Morphologic Examination of Apoptosis
Apoptosis was evaluated by morphologic methods as described previously. Briefly, cells were fixed with 4% paraformaldehyde and stained with Hoechst 33342. Cellular and nuclear morphologies were...
examined by phase contrast and fluorescence microscopy. Typical apoptosis was indicated by cellular shrinkage, nuclear condensation and fragmentation, and the formation of apoptotic bodies. At least four fields with approximately 200 cells per field were checked in each dish to estimate the percentage of apoptosis. For GFP/PKC cotransfected cells, the morphologic examination was focused on the GFP-labeled cells.

**Determination of Caspase Activity**
Caspase activity was measured by an enzymatic assay as described previously.32–35 Briefly, cell lysate collected with 1% Triton X-100 was added to an enzymatic reaction containing 50 μM DEVD-AFC, a fluorogenic substrate for caspases. Fluorescence generated during 60 minutes of reaction was monitored at excitation 360 nm/emission 530 nm. A standard curve was constructed using free AFC in each measurement. Caspase activity was calculated on the basis of the fluorescence measurement and expressed as the nanomolar amount of liberated AFC by each milligram of protein of cell lysate.

**TUNEL Assay of Apoptosis**
TUNEL assay was conducted to examine apoptosis in RPTCs and kidney tissues as described previously.32,36,37 Briefly, the cells and tissues were fixed in 4% paraformaldehyde. After permeabilized in 0.1% Triton X-100, the samples were incubated with a TUNEL reaction mixture containing terminal deoxynucleotidyl transferase and nucleotides, including Cy3-labeled dUTP. Positive staining with DNA breakage in cell nuclei was identified by fluorescence microscopy. For kidney tissue samples, the slides were incubated with 1 μg/ml Hoechst 33342 in PBS for 5 minutes after TUNEL staining to identify nuclei and kidney structures. Apoptosis in kidney tissues was quantified by counting TUNEL-positive cells in 20 nonoverlapping fields in each slide at the magnification of ×200. Representative images were taken at ×400 magnification.

**Immunofluorescence of Cytochrome c in RPTCs**
Immunofluorescence was conducted as described in our previous work.33,36 Bcl-2 or empty vector-transfected RPTCs were grown on collagen-coated glass coverslips. After 24 hours of albumin treatment, the cells were fixed with a modified Zamboni fixative containing picric acid and 4% paraformaldehyde. After blocking in 2% normal goat serum, the cells were incubated with a mouse monoclonal anti-cytochrome c, followed by exposure to Cy3-labeled goat anti-mouse secondary antibody. Signals were examined by fluorescence microscopy.

**Cell Fractionation and Analysis of Bax and Cytochrome c Translocation**
To analyze the release of cytochrome c from mitochondria to cytosol and the translocation of Bax from cytosol to mitochondria, cells were fractionated into cytosolic and membrane-bound organelar fractions by using a digitonin buffer as described previously.32–34 Briefly, by the end of the treatment, cells were exposed to 0.05% digitonin in the isotonic buffer (250 mM sucrose, 10 mM Hepes, 10 mM KCl, 1.5 mM MgCl₂, 1 mM EDTA, and 1 mM EGTA; pH 7.1) for 2 minutes at room temperature. The soluble fraction was collected as cytosolic extract. The insoluble part was washed with isotonic buffer and then dissolved in 2% SDS buffer to collect the membrane-bound organellar fraction. The digitonin soluble and insoluble fractions were analyzed for cytochrome c and Bax by immunoblot analysis.

**Immunoblot Analysis**
Protein concentration was determined using the BCA reagent (Pierce Chemical Co., Rockford, IL). The NuPAGE Gel System was used for electrophoresis and protein transferring/blotting. Equal amounts of protein were loaded in each well for electrophoresis. The resolved proteins were then electroblotted onto polyvinylidene fluoride membranes. The membranes were incubated in 5% fat-free milk for blocking and then exposed to the primary antibodies overnight at 4°C. After intensive wash, the blots were incubated with the horseradish peroxidase-conjugated secondary antibody, and the antigens on the blots were revealed using the enhanced chemiluminescence (ECL) kit from Pierce.

**In Vitro Immunocomplex Kinase Assay of PKC-δ Activity**
Cell lysates were collected freshly using the immunoprecipitation lysis buffer in the presence of protease and phosphatase inhibitors as described previously.38 Cell lystate of 500 μg of protein was preclenched with protein A/G plus-agarose and then incubated with rabbit polyclonal anti-PKC-δ antibody (Cell Signaling Technology) overnight at 4°C. PKC-δ immunocomplexes were then pulled down by 3 hours of incubation with 30 μl of protein A/G plus-agarose. The immunocomplexes were resuspended in 2X kinase reaction buffer [300 mM NaCl, 8 mM MnCl₂, 12 mM MgCl₂, 20% (vol/vol) glycerol, 20 μM ATP, 2 mM dithiothreitol, 200 mM NaVO₄, and 100 mM Hepes (pH 7.5)]. The protein kinase reaction was initiated by adding 5 μCi of [γ-32P] ATP and 100 ng of histone into 20 μl of reaction buffer containing immunoprecipitated PKC-δ. After 20 minutes of incubation at 30°C, the reaction was terminated by adding 2% SDS gel loading buffer. The samples were then subjected to gel electrophoresis, and phosphorylated histone was revealed by autoradiography to indicate PKC-δ kinase activity.

**Statistical Analysis**
Quantitative data were expressed as means ± SD (n ≥ 4). Statistical differences between two groups were determined by t test. P < 0.05 was considered significantly different. Qualitative results, including immunoblots, were representatives of at least three separate experiments.

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**DISCLOSURES**
None.
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