Targeting the protein kinase C family in the diabetic kidney: lessons from analysis of mutant mice

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Abstract The protein kinase C (PKC) superfamily comprises proteins that are activated in response to various pathogenic stimuli in the diabetic state. Hyperglycaemia is the predominant stimulus that induces the activation of distinct PKC isoforms within a cell, each mediating specific functions, probably through differential subcellular localisation. The contribution of individual PKC isoforms can be directly addressed in vivo using innovative PKC-isoform-specific knockout (KO) mouse models, which are providing key insights into the physiological function of PKC isoform diversity in the development of diabetic nephropathy. Such studies can be a valuable complementary approach to more commonly used pharmacological analyses using agents such as ruboxistaurin mesylate (Arxxant, LY333531), which is claimed to specifically inhibit the PKC- β -isoform. As expected given the multiple and specific properties of the isoforms in vitro, deletion of different PKC isoform signalling pathways leads to distinct phenotypes in mice. Notably, KOs of the individual PKCs assigned specific non-redundant biological functions to each isoform, which were not compensated for by the others. Thus, PKC isoform specificity and cellular diversity seem to be responsible for the divergent outcomes leading to albuminuria and/or renal fibrosis according to studies on the streptozotocin-induced mouse model of diabetes. This review discusses the role of individual PKC isoforms in diabetic nephropathy and their potential therapeutic implications. Defining and targeting mediators of increased intracellular activation in the diabetic microvasculature will

have important clinical and therapeutic benefits and help in the design of novel effective therapies in the near future.

Keywords Diabetic · Kidney · Mutant · Mice · Protein kinase C

Abbreviations

ACR	Albumin:creatinine ratio
aPKC	Atypical protein kinase C
cPKC	Classical protein kinase C
ECM	Extracellular matrix
KO	Knockout
MAPK	Mitogen-activated kinase
NEFA	Non-esterified fatty acid
nPKC	Novel protein kinase C
PKC-DMES	PKC- β Inhibitor Diabetic Macular Edema Study
PKC-DRS	PKC- β Inhibitor Diabetic Retinopathy Study
PKC	Protein kinase C
ROS	Reactive oxygen species
VEGF	Vascular endothelial growth factor
WT-1	Wilms tumour-1

Introduction

Hyperglycaemia plays a key role in the pathogenesis of long-term complications in diabetes mellitus, although this process is influenced by individual susceptibility (i.e. genetic determinants) and by accelerating factors such as hypertension and dyslipidaemia. Vascular complications primarily affect those parts of the body comprising cells that do not require insulin for glucose uptake, such as the blood vessels, kidneys and nervous system. These cells are

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subjected to high intracellular glucose concentrations during hyperglycaemia, which leads to the activation of various intracellular metabolic pathways. As described by Brownlee in his 2004 Banting Medal Award Lecture [1], several attractive hypotheses have been postulated to explain the role of hyperglycaemia in the pathogenesis of long-term complications. Such hypotheses include (1) increased polyol pathway flux, (2) increased formation of AGEs, (3) increased hexosamine pathway flux, and (4) activation of protein kinase C (PKC). The PKC signalling pathway is the postulate that has received the most attention lately. Although hyperglycaemia is the main stimulus that leads to PKC activation, other triggers related to the diabetic state, such as non-esterified fatty acids (NEFAs), and various growth factors, including angiotensin II, have been identified [2–4]. PKC activation is involved in the regulation of vascular permeability and contractility, endothelial cell activation and vasoconstriction, extracellular matrix (ECM) synthesis and turnover, abnormal angiogenesis, excessive apoptosis, leucocyte adhesion, abnormal growth factor signalling and cytokine action, as well as abnormal cell growth and angiogenesis, all of which are involved in the pathophysiology of diabetic vascular complications (Fig. 1) [2, 5, 6]. Although there are other examples of PKC acting in the pathogenesis of renal diseases, e.g. in renal cell carcinoma or certain immune disorders such as IgA nephropathy [7–9], the present review aims to provide an overview of the general role of PKC and its activation in the kidney, and to report on the role and modulation of distinct PKC isoforms in the development of diabetic nephropathy. The following summary is not intended to be comprehensive but instead focuses on some of the more

recent research developments in this field, with particular focus on knockout (KO) mouse studies. For further information, we would like to refer the reader to other recent reviews [6, 7, 10–12].

PKC at a glance

The PKC superfamily comprises homologous serine/threonine kinases that are involved in many signalling events [11, 13–15]. In mammals, a gene family of nine independent loci are distributed over the whole genome [12]. The PKC family is divided into three subfamilies according to biochemical properties and sequence homologies: conventional or classical PKCs (cPKCs; PKC- α , the two splice variants PKC- β_1 and PKC- β_2 , and PKC- γ), novel PKCs (nPKCs; PKC- δ , PKC- ϵ , PKC- η and PKC- θ) and atypical PKCs (aPKCs; PKC- ζ and PKC- λ/ι) (Fig. 2) [15]. The cPKC isoforms are sensitive to diacylglycerol and are responsive to Ca^{2+} through an archetypal C_2 domain. Diacylglycerol represents the main physiological activator of PKC; phosphatidylserine, phorbol ester, NEFAs and various growth factors, such as angiotensin II or vascular endothelial growth factor (VEGF), are also known stimulants [12, 15]. The nPKC isoforms are diacylglycerol-sensitive but Ca^{2+} -insensitive; their C_2 -related domains do not retain Ca^{2+} -coordinating residues [15]. The aPKC isoforms have altered C_1 domains and are not diacylglycerol-sensitive; regulation occurs in part through the N-terminal Phox and Bem1p (PB1) domain. The structures of the C_1 and C_2 domains have been determined, and kinase domain models have been built (Fig. 2) [15]. Unfortunately, the constitutive,

Fig. 1 Under hyperglycaemic conditions, glucose enters vascular and renal cells via the GLUT1 transporter independently of insulin and is metabolised by glycolysis. Accumulation of glycolysis intermediates such as glyceraldehyde 3-phosphate leads to de novo synthesis of diacylglycerol (DAG), which in turn activates one or more PKC isoforms. PKC isoform-mediated phosphorylation of transcription factors, cytoskeletal proteins, enzymes and transporters affects many of the pathophysiological features of diabetic nephropathy. PGE_2 , prostaglandin E_2 ; PGI_2 , prostacyclin

Fig. 2 The PKC kinase family members have distinct kinase domains but some related regulatory properties. The three PKC subgroups are structurally and functionally distinct. The cPKC isoforms (PKC- α , - β_1 and - β_2 , - γ) are diacylglycerol (DAG)-sensitive and Ca^{2+} -responsive through an archetypal C_2 domain. The nPKC isoforms (PKC- δ , - ϵ , - η , - θ) are DAG-sensitive but Ca^{2+} -insensitive; their C_2 -related domains do not retain Ca^{2+} -coordinating residues. The aPKC isoforms (PKC- ζ and - τ/λ) have altered C_1 domains and are not DAG-sensitive; regulation occurs in part through the N-terminal Phox and Bem1p (PB_1) domain

lipid-dependent protein kinase activity of purified PKC delayed the realisation that PKC phosphorylation plays a fundamental role in the catalytic activities of these proteins [12, 15, 16]. PKC phosphorylation *in vivo* is well documented and plays an important role in the maturation of the enzyme into a fully functional form localised correctly in the cell (Fig. 3) [12, 15]. As more information becomes available regarding the activation of PKC isoforms, it is likely that other mechanisms of phosphorylation and activation of specific isoforms will be elucidated. The activation and degradation of the PKC isoforms is controlled spatially and temporally, since all cells and/or tissues produce more than one PKC isoform that can act in a functionally redundant manner while targeting the same or overlapping sets of substrates [11, 12, 15]. There is now ample evidence that the wide spectrum of PKC-mediated signalling is organised by isotype specificity that, despite the broad overlapping substrate specificities, is defined via unique expression patterns, intracellular localisation and adaptor proteins [13, 17].

Entry of glucose into vascular and renal cells through GLUT1 raises the intracellular concentration of glycolysis metabolites, directly translating the extracellular hyperglycaemic state into cellular activation [18]. Accumulation of intermediates in glycolysis, such as glyceraldehyde 3-phosphate during hyperglycaemia, leads to *de novo* synthesis of diacylglycerol, which directly or indirectly activates PKC isoforms through the direct effects of high glucose described above or the autocrine and paracrine action of vasoactive peptides such as angiotensin II, VEGF and endothelin 1 [19]. In the diabetic state, all of these pathways probably contribute to the activation of the diacylglycerol–PKC signalling cascade and, upon activation,

the development of vascular complications [5]. Interestingly, cultured vascular cells usually require exposure to high glucose levels for hours to days before increased diacylglycerol levels are observed, suggesting that induction of protein synthesis may be required [5, 6, 10, 20]. Thus, diabetes mellitus may also lead to increased transcription of distinct PKC isoforms, as recently demonstrated by Langham et al. in human diabetic kidney biopsies [21].

As summarised above, the mechanism of activation is clearly different for each of the three PKC subgroups. However, for a long time it was debated whether each isoform of a subgroup has a specific function or activation mechanism [13]. This is because *in vitro* most PKCs behave very similarly and targeted experimental therapeutics for the inhibition of individual PKC isoforms had been largely unavailable [12]. Whiteside and Dlugosz have demonstrated that, in cultured mesangial cells, excess glucose metabolism leads to *de novo* synthesis of both diacylglycerol and phosphatidic acid, which may account for the increased PKC- α , - β , - δ , - ϵ , and - ζ activation/translocation observed in these cells within 48 h of exposure to high glucose levels [20]. Most previous experimental studies used pharmacological analyses that included ruboxistaurin mesylate (LY333531, Arxxant; Eli Lilly, Indianapolis, IN, USA), a rather selective inhibitor of the PKC- β isoform [5, 22]. However, novel molecular technologies such as transfection of cultured cells with small interfering RNA targeting individual PKC isoforms have lately been introduced to study PKC isoform specificity [23]. Furthermore, the three-dimensional structures of the distinct PKC isoforms have now been elucidated, providing valuable information for selective drug design [24]. Nevertheless, a clear picture of PKC isoform specificity

Fig. 3 The activation of cPKC and nPKC isoforms typically involves recruitment to membranes and interaction with, or allosteric activation by, DAG whereas aPKC isoforms remain DAG-insensitive owing to an incomplete C₁ domain. Agonist-induced production of DAG is effected by multiple mechanisms such as receptor tyrosine kinases and receptors linked to non-receptor tyrosine kinases [15]. AKAP79, A-kinase anchoring protein 79; EGFR, epidermal growth factor receptor; ER, endoplasmic reticulum; GAP43, growth-associated protein 43; IP₃, inositol 1,4,5-trisphosphate; LIP, λ -interacting protein; MARCKS,

myristoylated alanine-rich C kinase substrate; MEK5, mitogen-activated protein kinase kinase; NLS/NES, nuclear localisation signal/nuclear export signal; PAR, proteinase-activated receptor; PDK, pyruvate dehydrogenase kinase; PI3K, phosphatidylinositol 3-kinase; PICK-1, protein interacting with C kinase; PKD, protein kinase D; PLC, phospholipase C; RACK-1, receptor for activated C kinase 1; SHP-1, Src homology 2 domain-containing protein tyrosine phosphatase 1; ZIP, zeta inhibitory peptide

in vivo as regards the abovementioned processes that lead to diabetic vascular complications is still lacking.

Role of PKC in hyperglycaemia-induced renal injury

Diabetic nephropathy is the leading cause of end-stage renal disease worldwide and an independent risk factor for all-cause and cardiovascular mortality in diabetic patients [25]. It is characterised by initial glomerular hyperfiltration, progressive amassing of ECM with thickening of glomerular and tubular basement membranes and increased amounts of mesangial matrix, which ultimately progress to glomerulosclerosis and tubulointerstitial fibrosis [26]. Albuminuria represents the clinical hallmark of diabetic nephropathy, the development of which may involve PKC signalling events in distinct parts of the glomerular filtration barrier, namely, the glomerular endothelial cell layer, the basement membrane and the podocyte cell layer [27, 28]. If this were proved to be the case, it would mean that all the

cellular elements of the kidney, i.e. glomerular endothelial cells, mesangial cells, podocytes and tubular epithelial cells, are affected by the pivotal hyperglycaemic injury, and that PKC signalling events are contributing to this process.

In addition, there is evidence that oxidants and AGEs also increase diacylglycerol levels and activate PKC [21, 29–31]. Reactive oxygen species (ROS) have been demonstrated to play a central role in the synthesis and degradation of ECM in the glomeruli and tubulointerstitium, which leads to renal fibrosis [2, 32]. High glucose levels produce cellular ROS through PKC-dependent activation of NADPH oxidase and altered mitochondrial metabolism [2, 33]. ROS are further generated from injured mitochondria and activate a signal transduction cascade involving the mitogen-activated protein kinases (MAPKs) and the Janus kinase/signal transducers and activators of transcription [30, 34]. Subsequently, the profibrotic cytokine TGF- β 1 is upregulated, along with other profibrotic factors that further promote formation of AGEs and enhanced ECM synthesis (Fig. 1) [32].

Craven and DeRubertis [35] and Lee et al. [36] were the first to provide data indicating that activation of the PKC system by hyperglycaemia may represent an important pathway by which glucotoxicity is transduced in susceptible cells in diabetic nephropathy. These initial results have been confirmed in various cell culture models, such as endothelial and vascular smooth muscle cells and distinct renal cells (mainly mesangial cells, proximal tubular cells, podocytes), implicating different PKC isoforms in the development of diabetic kidney disease [37]. The importance of individual PKC isoform activation in the diabetic milieu initiating early diabetic nephropathy was first described by Haneda et al. [37]. Exposure of cells to high concentrations (27.8 mmol/l) of glucose for 5 days resulted in a significant elevation of PKC activities in the membrane fraction. MAPK was also activated in these cells. Of the PKC isoforms, the levels of PKC- α and PKC- ζ were significantly increased, indicating a potential role for these isoforms in the development of diabetic nephropathy. These results were later confirmed in a study that demonstrated high-glucose-induced activation of the PKC- α and the PKC- ε isoforms in whole kidney lysate from a streptozotocin-induced rat model of diabetes [38]. Furthermore, as mentioned above, de novo synthesis of both diacylglycerol and phosphatidic acid occurs in response to excess glucose metabolism, which may account for the increased PKC- α , - β , - δ , - ε and - ζ activation/translocation observed in mesangial cells after 48 h exposure to high glucose [20]. Notably, King and coworkers postulated that among the various PKC isoforms, PKC- β and PKC- δ are activated preferentially in the vasculatures of diabetic animals, although other PKC isoforms are also increased in the renal glomeruli and retina [5]. This group of investigators also discovered that the pharmacological compound ruboxistaurin, which is claimed to specifically inhibit both PKC- β splice variants (PKC- β_1 and - β_2), when administered orally to streptozotocin-induced diabetic rats, not only prevented an elevated glomerular filtration rate, increased albumin excretion rate, and increased retinal circulation time but also overproduction of mRNA for glomerular TGF- β_1 and ECM proteins [39, 40]. Ruboxistaurin also prevented progressive mesangial expansion in the *db/db* mouse model of type 2 diabetes [41]. Although the PKC- β isoform has been shown to contribute to early progressive diabetic nephropathy, it should be noted that the other abovementioned results suggest that this isoform is not exclusively involved [28].

One major obstacle to progress in current clinical research in human diabetic microangiopathies is the lack of a suitable assay system for measurement of intracellular PKC activation in humans. However, it has previously been shown that PKC activity is acutely regulated by plasma glucose concentrations in human monocytes [42] and

human platelets [43] *in vivo*. These studies suggest that intracellular PKC activation is at least involved in the development of human diabetic vascular complications, mainly accelerated atherosclerosis in type 2 diabetic patients.

Development and characterisation of PKC isoform-selective inhibitors

As summarised above, there is growing evidence that individual PKC isoforms serve unique (and in some cases, opposing) functions in cells, at least in part as a result of isoform-specific patterns of subcellular compartmentalisation, protein–protein interactions and post-translational modifications that influence catalytic function. This has prompted several pharmaceutical companies to try to develop selective PKC inhibitors for these enzymes, which may play a role in the manifestation of the early and/or late stages of nephropathy [12, 44]. Generating PKC isoform-selective inhibitors that target the active enzyme has proven to be a challenge [12, 45]. Pharmacological probes for the action of members of the PKC superfamily include membrane-permanent allosteric activators (e.g. phorbol esters) and catalytic inhibitors (Fig. 3) [45]. Early PKC inhibitors developed for experimental use, such as staurosporine and 1-(5-isoquinoliny)sulfonyl-2-methylpiperazine (*H7*) were only capable of blocking the activity of all PKCs rather than individual PKC isoforms [22, 46]. They also have the ability to modulate other C₁-domain-containing proteins or protein kinases [22, 46].

Staurosporine-like bisindolylmaleimide compounds, such as CGP41251, Go-6850, Ro-31–8220 and UCN-01, were subsequently developed, which act at the ATP-binding site of the enzyme [44]. Various ATP-competitive inhibitors have been developed in the meantime, but these are associated with problems of specificity [12]. Partial responses in the prevention of the progress of malignancies were found in early phases of clinical trials of UCN-01 and CGP41251, which are partially isoform-specific inhibitors [45]. The macrocyclic bisindolylmaleimide compound ruboxistaurin was synthesised and shown to be a competitive, reversible inhibitor of both PKC- β_1 and - β_2 , with a half-maximal inhibitory concentration (IC₅₀) of approximately 5 nmol/l [47, 48]. This value is 50 times lower than the IC₅₀ of ruboxistaurin for other PKC isoforms and 1,000 times lower than that for proteins other than PKCs [47]. The orally active pharmacological compound ruboxistaurin has potential as a causal therapy for diabetic microangiopathies [5, 48].

More recent approaches used to modulate PKC isoforms include oligonucleotide antisense technology and peptide fragments to either inhibit or promote translocation of PKC

isoforms to specific anchoring proteins [11]. One possible new approach to developing novel, specific therapeutics aimed at PKC would be to target the signalling termination pathways of the enzyme [12]. The elucidation of the three-dimensional structures of distinct PKC isoforms will certainly provide valuable information for more selective drug design [12, 24].

From bench to bedside: human trials of PKC- β inhibition

Owing to the large amount of preclinical data demonstrating that ruboxistaurin can prevent retinal and renal pathologies with very little evidence of toxicity [5, 39–41, 49, 50], this compound has been used in several clinical trials to determine its efficacy for the treatment of diabetic microvascular complications [51–62]. Pharmacokinetic measurements were performed on a subset of healthy volunteer patients as well as patients with type 2 diabetes mellitus to study efficacy, safety and tolerability of ruboxistaurin [51]. Dyspepsia and increased blood creatine phosphokinase level were common adverse drug reactions observed in the ruboxistaurin-treated patients [62]. Large international, randomised, controlled trials were subsequently initiated to evaluate the safety and efficacy of ruboxistaurin at different doses over a longer administration period in larger groups of diabetic patients with diverse diabetic microangiopathies [52–57].

Retinopathy The overall result for two studies of diabetic retinopathy (PKC- β Inhibitor Diabetic Retinopathy Study [PKC-DRS], PKC- β Inhibitor Diabetic Macular Edema Study [PKC-DMES]) was largely equivocal; however, subanalysis demonstrated that, compared with placebo, 32 mg/day of ruboxistaurin was associated with a reduced risk of visual loss [46, 54]. The treatment effect on moderate visual loss was statistically assessed using a Cox proportional hazards model that adjusted for several important baseline covariates identified among potential effect modifiers, such as ACE inhibitor/angiotensin receptor blocker (ARB) use [46, 54]. However, it did not prevent progression of diabetic retinopathy, or the combined outcome of progression of diabetic retinopathy or the application of laser photocoagulation in patients with moderately severe to very severe non-proliferative diabetic retinopathy [46, 54]. Failure to reach the primary outcomes in this study was probably due to the design of the trial as a combined Phase 2/3 trial which studied 252 patients in the diabetic retinopathy arm (PKC-DRS) and 426 patients in the diabetic macular oedema arm (PKC-DMES) [46, 54]. One likely possibility is that the study was underpowered (i.e. power calculation was based on a previously docu-

mented retinopathy progression rate when glycaemic and blood pressure control were likely to be less intensive) [46]. Therefore, two large, high-powered, single-dose studies (PKC-DRS2 and PKC-DMES2), evaluating the impact of ruboxistaurin 32 mg as add-on therapy to ‘usual care’ have subsequently been carried out [55, 56]. Oral ruboxistaurin treatment reduced vision loss, need for laser treatment and macular oedema progression, while increasing occurrence of visual improvement in patients with non-proliferative diabetic retinopathy [56]. Ruboxistaurin also increased the chance of visual acuity improvement in patients with moderately severe to very severe non-proliferative diabetic retinopathy [57].

Neuropathy In a multinational Phase 2 randomised controlled trial to assess sensory symptoms and nerve function in patients with diabetic neuropathy, ruboxistaurin improved sensory symptoms and nerve fibre functions in a subgroup of patients with less severe diabetic neuropathy but did not prevent the progression of diabetic neuropathy as measured by nerve conduction velocity [58].

Nephropathy Currently available therapeutic approaches for diabetic nephropathy are focused on intensive blood glucose control and blockade of the renin–angiotensin system; a causal therapy, however, is still lacking [59]. The therapeutic potential of ruboxistaurin has also been observed in human diabetic nephropathy [60, 61]. First, a multicentre, double-blind, placebo-controlled study suggested an additive effect of ruboxistaurin compared with placebo in 123 type 2 diabetic patients with proteinuria (mean albumin:creatinine ratio [ACR] 764 mg/g) or near-normal serum creatinine who were already treated optimally with an ACE inhibitor or angiotensin receptor blocker and intensive glycaemic control [60]. The investigators reported that 32 mg of ruboxistaurin daily for up to 1 year resulted in a reduction in urinary ACR in the ruboxistaurin group compared with the placebo group. Renal function, examined by estimated GFR, was maintained with ruboxistaurin treatment. In contrast, participants in the placebo group had a significant decline in estimated GFR that was within the range reported in recent clinical trials of ARBs and ACE inhibitors in persons with type 2 diabetes and nephropathy [60]. It is important to note, however, that the reduction in ACR compared with the baseline level was not significant compared with the decrease achieved by placebo, according to conventional intergroup comparison [46]. Another recent meta-analysis of the three major diabetic retinopathy trials (PKC-DRS, PKC-DMES, PKC-DRS2) demonstrated that kidney outcome rates after 3 years did not differ by treatment assignment (serum creatinine doubling was 6.0%; progression to higher stages of nephropathy, 3.5%; decline in GFR, $\sim 3\text{--}4\text{ ml min}^{-1}\text{ year}^{-1}$)

[61]. There are, however, certain limitations to this study: the GFR changes were not the primary endpoint; a very early stage in nephropathy progression was used (stage 2 nephropathy; $86.8 \pm 27.6 \text{ ml min}^{-1} 1.73 \text{ m}^{-2}$); there was a relatively high dropout rate (25%); and ACR values were missing for a significant proportion of the study population (33%) [61, 62]. It is possible that this intervention may not offer a significant benefit beyond maximal inhibition of the renin–angiotensin–aldosterone system and blood pressure and glucose control [62]. Thus, the expected benefits from preclinical trials have so far not translated into meaningful outcome improvements in clinical trials. In summary, the current data highlight that prospective Phase 3 clinical trials in diabetic nephropathy are needed to assess the therapeutic effects of ruboxistaurin on diabetic nephropathy.

The PKC isoform KO approach: new avenues from animal models

Comparative genomics using the robust genetic methods available in the laboratory, including KOs and transgenic mice, provide an innovative complementary approach to defining the functions of genes that may predispose or contribute to diabetic nephropathy in mice and humans [63]. However, standardised benchmarks of hyperglycaemia, albuminuria and measurements of renal failure remain to be developed for different inbred strains of mice. The most glaring deficiency has been the lack of a mouse model of diabetes that develops progressively worsening renal insufficiency [63], although recent reports have given positive indications that this experimental problem will be overcome [64, 65]. Despite the preceding caveats regarding the difficulties in phenotyping renal function in mice, the use of transgenic mouse models has provided a better understanding of mechanisms that exacerbate diabetic nephropathy [63, 66].

The specific effects of the PKC isoforms on renal and other vascular tissues to induce diabetic complications are just beginning to be determined using genetically altered mice as previous attempts using pharmacological approaches were inconclusive [5, 39–41, 50, 51]. Genetic studies in the mouse have indicated that particular PKC isoforms are essential in a number of specific contexts [67–75]. As would be expected given their multiple and specific properties in vitro, the deletion of individual PKC isoform pathways leads to distinct phenotypes in KO mice [28, 76–79]. Surprisingly, in a given cascade, KOs of the various isoforms assign specific non-redundant biological functions to each isoform, which were not compensated for by the others [28, 76–79]. These results emphasise the notion that, although initiated by the same external stimuli, these

intracellular cascades activate kinase isoforms that each have their own specific role.

The aim of our research team was to identify PKC function in vivo by analysing individual PKC KO mice, which had been generated by Michael Leitges in our group over the past decade [68, 72, 75, 80]. We used the streptozotocin mouse model of diabetes. Using this stress model in various PKC KO mice, we identified a specific role for some of the individual PKC isoforms in the development of diabetic nephropathy [28]. More specifically, we have shown that hyperglycaemia-induced down-regulation of the negatively charged basement membrane heparan sulphate proteoglycan perlecan and increases in VEGF and VEGF receptor II production were prevented in mice lacking PKC- α (*Prkca*^{−/−} mice), which also showed a significant reduction in albuminuria [76]. Interestingly, deletion of PKC- α in vivo also abolished nephrin loss in streptozotocin-induced murine diabetic nephropathy [77]. We have demonstrated that a reduction in Wilms tumour-1 (WT-1) level is detectable in diabetic wild-type mice that is prevented in *Prkca*^{−/−} mice [77]. We are currently analysing whether this event is due to diminished podocyte loss or reduced activity of the nephrin transcription factor WT-1 in diabetic kidneys [77]. The PKC- α isoform therefore seems to be an important mediator of the entire glomerular filtration barrier, including the endothelial cell layer, the glomerular basement membrane and the glomerular visceral epithelial cell (podocyte) layer.

Notably, we observed that increased renal and glomerular hypertrophy and augmented TGF- β 1 levels in the diabetic state were unaltered in diabetic *Prkca*^{−/−} mice [76]. The postulate that albuminuria as well as renal and glomerular hypertrophy may be regulated by distinct PKC signalling pathways is supported by findings that short- and long-term inhibition of TGF- β by neutralising antibodies or treatment with antisense oligonucleotides prevents the development of renal hypertrophy but not albuminuria [81, 82]. Further studies revealed that blockade of VEGF by systemic antibody administration inversely decreased albuminuria in streptozotocin-induced diabetic rats or *db/db* mice with only moderate influence on mesangial expansion and renal hypertrophy [83, 84].

It is likely that PKC activation has an important role in the thickening of basement membranes, as ruboxistaurin was able to prevent both mesangial expansion and basement membrane thickening in diabetic *db/db* mice and hypertensive diabetic rats [5, 41, 50]. Furthermore, a recent clinical trial in patients with diabetic nephropathy who were treated with 32 mg/day of ruboxistaurin speculated that the urinary TGF- β :creatinine ratio reflects a reduction in tubulointerstitial fibrosis in the serum in these patients relative to the placebo-treated group [85]. We therefore also studied mice lacking PKC- β (*Prkcb*^{−/−} mice)

with streptozotocin-induced diabetes and appropriate 129/Sv wild-type controls [78]. After 8 weeks of diabetes mellitus the renal and glomerular hypertrophy and increased levels of ECM proteins such as collagen III and IV and fibronectin induced by high glucose were reduced in *Prkcb*^{-/-} mice [78]. Furthermore, the high-glucose-induced production of the profibrotic cytokine TGF- β 1 and connective tissue growth factor was significantly diminished in the diabetic *Prkcb*^{-/-} mice compared with the diabetic WT mice, suggesting a role of the PKC- β isoform in the regulation of renal hypertrophy [78]. Notably, increased urinary ACR persisted in the diabetic 129/Sv mice even when the gene encoding PKC- β , was deleted in vivo [78]. Furthermore, loss of the basement membrane proteoglycan perlecan and the podocyte protein nephrin in the diabetic state was not prevented in the *Prkcb*^{-/-} mice, as previously demonstrated in the non-albuminuric diabetic *Prkca*^{-/-} mice [76–78]. Another group has reported similar data [86], demonstrating in *Prkcb*^{-/-} mice that a lack of PKC- β can protect against diabetes-induced renal hypertrophy, glomerular hyperfiltration and increased production of profibrotic growth factors and reactive oxygen species [86]. Although in this study less proteinuria was observed (though not abolished) [86], it should be noted that these mice were on a different background (129 $\times$ C57BL6) [86] than our PKC- β and - α mice 129/Sv strains [76–78], which may explain the diverse results [63]. Interestingly, Langham et al. have suggested that, in addition to activation, diabetes may lead to increased production of the PKC- β isoform in late human diabetic nephropathy [21].

The promising results regarding a specific role of both classical PKC isoforms in the development of diabetic nephropathy have raised more questions about the role of high-glucose-induced activation of novel and atypical PKC isoforms as suggested by others [20]. Whiteside and Dlugosz have demonstrated the presence of PKC- α , PKC- β 2, PKC- δ , PKC- ϵ and PKC- ζ in glomeruli isolated from normal and streptozotocin-induced diabetic rats and the expression of genes encoding these PKC isozymes, plus PKC- β 1, in primary cultured rat mesangial cells [20].

Our analysis of the renal phenotype of mice with genetic deletion of PKC- ϵ (*Prkce*^{-/-} mice) with regard to renal hypertrophy and fibrosis revealed that the kidney weight: body weight ratio, a marker of renal hypertrophy, was similar in diabetic mice to that in wild-type controls [79]. The urinary ACR remained normal in wild-type rodents, whereas PKC- ϵ -null mice showed elevated albuminuria at 6 and 16 weeks of age [79]. Masson–Goldner staining revealed that tubulointerstitial fibrosis and mesangial expansion was significantly increased in the *Prkce*^{-/-} mice even in the non-diabetic state [79]. However, this profibrotic phenotype was not observed in other organs such as liver and lung [79]. Immunohistochemistry of the kidneys

from *Prkce*^{-/-} mice showed increased renal fibronectin and collagen IV levels, which was further aggravated in the streptozotocin-induced diabetic stress model [79]. Furthermore, TGF- β 1 production and activation, measured as phospho-Smad2 and phospho-p38MAPK levels was increased in the *Prkcb*^{-/-} mice, suggesting a suppressive role for PKC- ϵ in the TGF- β 1 signalling pathway in the (diabetic) kidney [79]. Therefore, activated PKC- ϵ in the diabetic state may represent a protective response to injury rather than a mediator of renal injury. Based on these latest in vivo studies we postulate that hyperglycaemia leads to activation of the common intracellular PKC signalling pathway and its different isoforms, which will induce distinct pathophysiological changes in the intrarenal milieu leading to the development and progression of diabetic kidney disease (Fig. 4).

Methods have recently been developed to control the cell type, timing and reversibility of target gene production. In recent years, the emergence of site-specific recombinases such as Cre-loxP, Flp-FRT and phi C31-att as tools to engineer mammalian genomes has opened new avenues into the design of genetically modified mouse models. The original Cre and FLP recombinases have demonstrated their utility in developing conditional gene targeting while

Fig. 4 Activation of PKC in the diabetic kidney by hyperglycaemia leads to multiple pathological changes in diabetic nephropathy. Although initiated by the same external stimulus, namely hyperglycaemia, these intracellular cascades activate distinct kinase isoforms, each with its own specific role. Thus, PKC isoform specificity and cellular diversity seem to be responsible for the divergent outcomes leading to albuminuria and/or renal fibrosis

permitting cell-specific KO of genes not only in the kidney [87]. The properties of site-specific recombinases in combination with other biotechnological tools (tet on/off system, small interfering RNA-mediated gene silencing, fluorescent proteins, etc.) make them useful tools for the induction of precise mutations in specific cells or tissues in a time-controlled manner [87]. Temporally regulated gene expression, particularly using doxycycline- and tamoxifen-inducible systems, holds great promise as a method avoiding developmental effects of gene mutations, as well as facilitating comparison of the same animal's phenotype before and after gene modification [87]. Furthermore, the field of RNA interference is undergoing tremendous growth and has great potential for achieving gene knockdown quickly and reversibly [87]. Finally, new gene-targeting tools are in development that may substantially simplify the generation of transgenic animals. To date, however, the effectiveness of these systems for modifying renal function in transgenic mice remains unproven. However, we are sure that they will lead us to a more complete understanding of the functions of individual PKC isoforms in the kidney. The ability of isoform-specific *Prkc*^{-/-} mice to antagonise the development and progression of various features of diabetic kidney diseases has already provided new avenues for the treatment of this fatal diabetic complication. Further development of isoform-specific inhibitors or gene therapy that otherwise modulates levels and activation of specific isoforms will be necessary to apply this knowledge not only to the treatment of diabetic renal disease but also cellular dysregulation in general.

Conclusion

This review presents new and exciting evidence indicating that PKC isoforms are necessary for the development of diabetic vascular complications and suggests that efforts should be made to identify novel isoform-selective PKC inhibitors. Although initiated by the same external stimuli, namely hyperglycaemia, PKC isoform specificity and cellular diversity seem to determine whether albuminuria and/or renal fibrosis occur. Clinical trials currently underway will continue to unravel the impact of the different PKC isoforms, which should lead to the emergence of useful therapeutics in the near future.

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