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PKC δ regulates the stimulation of vascular endothelial factor mRNA translation by angiotensin II through hnRNP K

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Abstract

Angiotensin II (Ang II)-induced renal injury is partly mediated by growth factors such as VEGF. We have previously shown that Ang II rapidly increases VEGF protein synthesis in proximal tubular epithelial (MCT) cells by augmenting mRNA translation, which is partly dependent on activation and binding of hnRNP K to 3' untranslated region (UTR) of VEGF mRNA. Regulation of hnRNP K activation by PKC δ was studied in MCT cells. Transfection with a PKC δ siRNA inhibited hnRNP K Ser302 phosphorylation and activation, and reduced Ang II stimulation of VEGF synthesis. Inhibition of PKC δ with rottlerin also prevented binding of hnRNP K to VEGF mRNA and reduced the efficiency of VEGF mRNA translation. In db/db mice at 2 weeks of type 2 diabetes, VEGF expression was increased, which was due not to increase in transcription but to augmented translation of VEGF mRNA. Augmented VEGF expression was associated with increased binding of hnRNP K to VEGF mRNA. c-src and PKC δ activities and hnRNP K phosphorylation on Ser302 in renal cortex of db/db mice were increased compared to control mice. We conclude: Ang II induced VEGF mRNA translation is associated with activation of hnRNP K in MCT cells. In the signaling pathway leading to hnRNP K activation induced by Ang II, PKC δ is downstream of c-src. PKC δ -mediated phosphorylation of hnRNP K is required for Ang II stimulation of VEGF mRNA translation. In mice with type 2 diabetes, src and PKC δ activation and hnRNP K phosphorylation correlate with increased VEGF mRNA translation and kidney hypertrophy. 3' UTR events are important in regulation of VEGF expression in models of renal injury.

Introduction

Angiotensin II is a mediator of renal injury in diabetic nephropathy and other chronic kidney diseases [1]. Treatment of proximal tubular epithelial cells (MCT cells) with 1 nM of angiotensin II (Ang II) stimulates synthesis of vascular endothelial growth factor (VEGF) through increased translation of its mRNA [2]. This was shown to be due to stimulation of cap-dependent translation initiation, which takes place at the 5' untranslated region (UTR) of the mRNA [2]. Additional studies showed that VEGF mRNA translation was positively regulated by binding of heterogeneous ribonucleoprotein K (hnRNP K) to the 3' UTR of the VEGF mRNA [3]. Activation of c-src by Ang II is critical to the binding of hnRNP K to VEGF mRNA, through phosphorylation of the latter on Ser302, which is a target of PKC δ [3].

There are six known tyrosine residues in hnRNP K that undergo phosphorylation by members of the c-src family [4]. Phosphorylation of hnRNP K on tyrosine residues by c-src is believed to generate several c-src homology domain 2 (SH2)-docking sites, which allow the protein to interact with various SH2-containing proteins, such as Lck and PKC δ . Although the tyrosine

residues affected in hnRNP K lie outside the KH domains responsible for RNA binding, tyrosine phosphorylation of hnRNP K is generally considered to inhibit its binding to specific RNAs. For instance, Ostareck-Lederer et al. have shown that hnRNP K and the c-src kinase specifically interact with each other, leading to c-src-mediated tyrosine phosphorylation of hnRNP K in vivo and in vitro [4]. c-src-mediated phosphorylation reversibly inhibits the binding of hnRNP K to the differentiation control element (DICE) of the LOX mRNA 3' untranslated region in vitro and specifically de-represses the translation of DICE-bearing mRNAs in vivo [4]. Similarly, Ostrowski et al have shown that hnRNP K protein is constitutively bound to many mRNAs in vivo, and that many hnRNP K-mRNA complexes are disrupted by tyrosine phosphorylation [5]. Interestingly, states of enhanced cell proliferation were associated with increased levels of hnRNP K tyrosine phosphorylation [6].

There are four serine residues in hnRNPK that undergo phosphorylation by extracellular signal-regulated kinase (ERK), c-Jun N terminal kinase (JNK), and members of the protein kinase C (PKC) family. It has been shown that serine phosphorylation of hnRNP K is RNA- and DNA-dependent, i.e., RNA- or DNA-bound hnRNP K is a preferred substrate for protein kinases [7]. Serine phosphorylation of hnRNP K is believed to alter its intracellular distribution, leading to cytoplasmic accumulation [8,9]. Phosphorylation of hnRNP K by ERK also leads to inhibition of mRNA translation [8].

hnRNP K has been shown to act as a docking platform that allows Lck, a member of the c-src family, to interact with a member of another kinase cascade, PKC δ to control the activity of a translation eukaryotic elongation factor, eEF1A (EF-1 α) [4,5,7,10]. While bound to RNA, hnRNP K is prevented from direct interaction with PKC δ [10]. The binding of Lck to hnRNP K enhances its activity resulting in phosphorylation of hnRNP K on additional tyrosine residues, causing dissociation of hnRNP K protein from RNA [4,5] and allowing the recruitment of PKC δ [10]. Following binding, the DAG-primed PKC δ is further induced through tyrosine phosphorylation by the activated Lck docked next to it [10]. Activated PKC δ not only targets Ser302 on hnRNP K [10] but also phosphorylates effectors either bound to K protein or present in its microenvironment through imposed proximity. EF-1 α binds hnRNP K [11] and is a substrate of PKC δ [12]. Therefore, PKC δ -mediated phosphorylation of EF-1 α could occur in the context of hnRNP K, and contribute to the activation of the translational machine.

Although the importance of hnRNP K phosphorylation by PKC δ has been established [3,10], the functional consequences of phosphorylation of hnRNP K by PKC δ are not known. Also unknown is whether similar association between hnRNP K and PKC δ occurs in vivo in the kidney in a disease state. In this study, we sought to investigate the role of PKC δ -mediated phosphorylation of hnRNP K in the regulation of VEGF mRNA translation by Ang II in MCT cells and assess the association between the two proteins in vivo in the kidney tissue of mice with type 2 diabetes.

Materials & Methods

Cell Culture

SV40-immortalized murine proximal tubular epithelial cells (MCT) were provided by Dr. Eric Neilson, Vanderbilt University, Nashville, TN. MCT cells in culture express in vivo characteristics of proximal tubular epithelial cells. [13]. The cells were grown in Dulbecco's minimal essential medium (DMEM) containing 5 mM glucose and 10% FBS [2,3]. Confluent monolayers of cells were serum-deprived in DMEM for 18 h before treatment.

Animal experiments

C57BLKsJ *lepr*^{-/-} db/db mice and their lean littermate controls (db/m) were purchased from Jackson Lab (Bar Harbor, ME). The db/db mice develop kidney disease related to type 2 diabetes resembling human disease, i.e., hypertrophy, extracellular matrix accumulation and albuminuria [14,15]. Experiments were initiated at 2 weeks following the appearance of hyperglycemia in db/db mice, when the mice are approximately 8–10 weeks old. Blood glucose was measured by an Accucheck instrument (Bayer Diagnostics, NY). Kidney weight and blood glucose levels were measured at the time of sacrifice. The Institutional Animal Care and Use Committee of the University of Texas Health Science Center at San Antonio approved these studies.

Transfection experiments were carried out using Superfect transfection reagent (Qiagen, Valencia, CA), according to the manufacturer's instructions using 100 nM siRNA per well in 6-well plates. PKC δ and c-src siRNAs were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA).

Immunoblotting experiments were performed as previously described [3]. MCT cells or kidney cortices were homogenized in lysis buffer (50 mM Tris.HCl, pH 7.4, 150 mM KCl, 1 mM EDTA, 50 mM β -glycerophosphate, 0.1 mM sodium orthovanadate, 1 mM EGTA, 0.5% Nonidet P40, and protease inhibitor mix [Sigma, St Louis, MO]). Protein concentration was measured and indicated amounts of homogenates were separated on SDS-PAGE, transferred to nitro-cellulose membranes and probed with various primary antibodies, and IRDye800- or IRDye700-coupled secondary antibodies were used for detection using Odyssey Infrared Imaging System (LiCor Biosciences, Lincoln, NE).

Immunoprecipitation experiments were carried out as previously described [3] on cell lysates or kidney homogenates to which relevant antibodies were added overnight at 4°C with rotation. Protein A/G agarose slurry was added for 1 h at 4°C with rotation. Agarose beads were pelleted and washed 3 times with lysis buffer and twice with PBS, and suspended in Laemmli sample buffer and boiled for 5 min.

Pull-down assays were performed as described in [3]. Polycytidylic acid-immobilized on cross-linked 4% beaded agarose (poly(C)-agarose [Sigma, St Louis, MO]) was added to an equal amount (500 μ g protein) of cell lysates. After a 2-h incubation at 4°C with constant rotation, the agarose beads were extensively washed and suspended in Laemmli sample buffer and boiled for 5 min.

Association hnRNP K with VEGF mRNA

MCT cells or kidney cortices were homogenized in resuspension buffer containing: 10 mM Tris (pH 7.5), 250 mM KCl, and 2 mM MgCl₂. After 5 min incubation on ice, 60 μ l of a 10% Tween 80–5% deoxycholate mix was added to the lysate. Lysates were kept on ice for 15 min and centrifuged for 10 min at 14,000 rpm. The supernatants were used for immunoprecipitation using anti hnRNP K antibody as described above. RNA was extracted from pellets using TRIzol (Invitrogen, Carlsbad, CA). RT-PCR amplification of VEGF or GAPDH transcript was performed using the Superscript One-Step RT-PCR kit from Invitrogen (Carlsbad, CA) and employing the specific primers described in [2]. PCR products were analyzed by electrophoresis on agarose gel.

Polysome assay was performed as described in [3]. Samples (MCT cells or kidney cortices) were prepared as described above. The supernatants were laid on top of 15–40% sucrose gradient, and centrifuged for 90 min at 200,000 $\times$ g. After centrifugation, the gradients were separated into 4 fractions, from which RNA was extracted and used for RT-PCR, as described above. PCR products were analyzed by electrophoresis on agarose gel.

In-vitro kinase assays

PKC δ and c-src were immunoprecipitated from cell or kidney lysates as described above. Pelleted agarose beads were resuspended in kinase buffer containing 25 mM Tris.HCl (pH 7.4), 10 mM MgCl₂, 25 mM β -glycerophosphate, 2 mM dithiothreitol, 0.1 mM sodium orthovanadate, 50 μ M ATP and 5 μ g myelin basic protein (MBP) for PKC δ . For c-src kinase assay, no exogenous substrate was added, as the kinase activity was assessed by c-src autophosphorylation. Reactions were carried out in the presence of 1 μ Ci of γ [³²P]-ATP for 20 min at 30 °C, and stopped by the addition of Laemmli buffer and boiling for 5 min. An aliquot of reaction mixture was separated by electrophoresis. Radioactivity incorporated in MBP (for PKC δ) or in c-src was detected by autoradiography and quantified by densitometry.

Immunofluorescence microscopy

MCT cells were seeded in 4-well chamber slides. Semiconfluent cells were serum-starved for 18 h and treated with Ang II (1 nM) for the indicated times. Cells were washed with phosphate-buffered saline, fixed, and incubated with rabbit anti-PKC δ and mouse anti-hnRNP K antibody followed by staining with FLUOR-conjugated donkey anti-rabbit and Cy3-conjugated donkey anti-mouse secondary antibody (Chemicon International, Temecula, CA), respectively. The slides were visualized with a confocal laser microscopy system (Olympus Fluoview 500)

Statistics

Data from a minimum of three experiments were expressed as mean $\pm$ SEM and analyzed by ANOVA for comparison among multiple groups using Bonferroni post-test analysis (GraphPad Prism®) and t-test for comparison between two groups; $p < 0.05$ was considered significant.

Results

Ang II stimulates PKC δ activity in MCT cells with a time-course corresponding to that of increased VEGF synthesis [3]. Phosphorylation of hnRNP K on Ser302, a known target of PKC δ [10], positively correlated with its binding to VEGF mRNA, and knockdown of hnRNP K expression by RNA interference significantly reduced Ang II stimulation of VEGF synthesis [3]. However, the requirement for PKC δ for either hnRNP K phosphorylation on Ser302 or for Ang II-induction of VEGF synthesis has not been explored. We investigated the role of PKC δ in Ang II stimulation of VEGF synthesis by RNA interference. MCT cells were transfected with control or PKC δ -specific siRNA 48 h before treatment with Ang II for 30 min. Figure 1 shows that Ang II stimulated VEGF synthesis in control siRNA-transfected cells ($293 \pm 37\%$, $p < 0.001$ vs con by ANOVA). This stimulation was attenuated in PKC δ -specific siRNA-transfected cells ($194 \pm 21\%$, $p < 0.01$ vs Ang II-control siRNA by ANOVA), but still significantly higher than in control cells transfected with control siRNA and not treated with Ang II ($p < 0.01$ by ANOVA). These data show that PKC δ contributes to Ang II stimulation of VEGF synthesis. Because we have previously shown that c-src is also required for VEGF synthesis, PKC δ activation and hnRNP K activation, hnRNP K could serve as a scaffold to bring together c-src and PKC δ .

To study this possibility, we treated MCT cells with Ang II for up to 15 min, immunoprecipitated hnRNP K and measured presence of c-src and PKC δ in the immunoprecipitates. Figure 2A (top panel) shows that c-src association with hnRNP K was maximum after 1 min of treatment with Ang II ($205.0 \pm 10\%$ of control, $p < 0.001$ by ANOVA) and decreased thereafter. Figure 2A (middle panel) shows that PKC δ association with hnRNP K was evident after 2 min and was sustained for at least 15 min ($161.0 \pm 13.1\%$ of control, $p < 0.001$ by ANOVA). These data suggest that hnRNP K serves as a scaffold that brings together c-src and PKC δ as they are activated by Ang II. Association between c-src and PKC δ could

not be detected by immunoprecipitation/immunoblot experiment (data not shown), indicating that either this association is weak and disrupted by cell homogenization or that they interact only indirectly through hnRNP K or some other protein in the macromolecular complex containing hnRNPK.

As we have previously shown that c-src is required for PKC δ activation by Ang II in MCT cells [3], we investigated the possibility that c-src activity is required for PKC δ forming a macro-molecular complex with hnRNP K. Quiescent MCT cells were pre-treated with 5 μ M PP2, a specific inhibitor of c-src, before treatment with Ang II for 5 and 15 min. Association of PKC δ and hnRNP K was evaluated by tandem immunoprecipitation and immunoblot assays. Figure 2B shows that Ang II-induced hnRNP K/PKC δ complex formation was prevented by PP2, indicating that c-src activity is required for PKC δ association to hnRNP K in Ang II-treated cells.

We next examined the subcellular localization of hnRNP K and PKC δ in MCT cells by immunofluorescence. Figure 2C shows that in untreated cells, hnRNP K (red signal) is present in the nucleus and in discrete spots in the cytoplasm, and PKC δ (green signal) is cytoplasmic. After a 15 minute incubation with 1 nM Ang II, a fraction of PKC δ translocates to the plasma membrane and into the nucleus where it colocalizes with hnRNP K (orange signal). A strong colocalization is observed in discrete spots inside the nucleus (yellow signal). Pre-incubation with PP2 prevented both the membrane and the nuclear translocation of PKC δ and its colocalization with hnRNP K. Together, these data indicate that the interaction between hnRNP K and PKC δ occurs in the nucleus.

We investigated the mechanism of PKC δ activation by c-src. Previous studies have shown that Tyr311, located near the hinge region of PKC δ , is a target of members of the c-src family of tyrosine kinases and that phosphorylation of this site leads to activation of PKC δ [16,17]. Figure 2D shows that Ang II stimulated phosphorylation of PKC δ on Tyr311 at 15 minutes in cells transfected with a control siRNA but failed to do so in cells transfected with a siRNA specific for c-src, in which c-src expression was reduced by 65%. These data show that c-src phosphorylates PKC δ on Tyr311 and activates it.

Having explored the role of c-src, we next evaluated that of PKC δ in Ang II-induction of VEGF synthesis by RNA interference. MCT cells were transfected with siRNA (control or specific for PKC δ) 48 h before a 15 min treatment with 1 nM Ang II. hnRNP K activation was measured by its binding to poly(C)-agarose. Transfection of MCT cells with a siRNA specific for PKC δ , but not a control siRNA, reduced its expression by ~80% (Fig. 3A, third panel, lanes 3–4 vs 1–2). Ang II activated hnRNP K (245% of control, $p < 0.01$ by ANOVA) in cells transfected with a control siRNA but not in cells transfected with a siRNA specific for PKC δ (107% of control, ns by ANOVA). We next determined whether PKC δ , which is required for hnRNP K activation, plays a role in recruitment of hnRNP K to VEGF mRNA. Quiescent MCT cells were pretreated with 5 μ M röttlerin, a specific inhibitor of PKC δ [18], before treatment with Ang II for 30 min. RNA was extracted from hnRNP K immunoprecipitates and used for RT-PCR using primers specific for murine VEGF and GAPDH mRNAs. Figure 3B shows that Ang II increased recruitment of hnRNP K to VEGF mRNA, that was prevented by röttlerin. Together, these data show that PKC δ is required for hnRNP K activation and binding to VEGF mRNA.

Binding of hnRNP K to VEGF mRNA plays a regulatory role in the stimulation of VEGF mRNA translation by Ang II [3]. Since PKC δ is required for recruitment of hnRNP K to VEGF mRNA, we studied the role of PKC δ in VEGF mRNA translation. Polysome assay was performed on MCT cells treated with Ang II for 30 min, with or without pre-incubation with 5 μ M röttlerin. Figure 3C shows that in untreated cells, VEGF mRNA is present throughout

the sucrose gradient and that treatment with Ang II provoked a shift in the distribution of VEGF mRNA toward the heaviest sucrose fractions (#3–4) that contain the polysomes. Pre-treatment with röttlerin induced a partial reversal of that shift. These data indicate that inhibition of PKC δ decreases the efficiency of VEGF mRNA translation, but does not totally inhibit it.

We next sought confirmation of in vitro studies in a rodent model of type 2 diabetes, because Ang II is a prime mediator of kidney injury in diabetes [19]. We studied the db/db mice, a commonly employed animal model of type 2 diabetes [14,15], at 2 weeks after onset of hyperglycemia. These mice demonstrated severe hyperglycemia and significant kidney hypertrophy, accompanied with increased VEGF expression [20]. We measured VEGF expression at the protein level by immunoblot and at the mRNA level by RT-PCR. Figure 4A shows that VEGF protein expression is significantly increased in the kidney cortex from db/db mice ($188 \pm 18\%$ of db/m, $p < 0.01$ by t-test); however, VEGF mRNA level was unchanged in these kidney cortices ($108 \pm 5\%$ of db/m, not significant by t-test). These data show that at this time point in evolution of type 2 diabetes in db/db mice the increase in VEGF expression is not due to increased transcription of the *veg*f gene but could be due to increased translation of its mRNA. To test this hypothesis, we performed polysome assay on kidney cortex from db/m and db/db mice. Figure 4B shows that in kidney cortex from db/m mice, VEGF mRNA is equally distributed in all sucrose fractions (~25% of total in each fraction), but in the kidney cortex from db/db mice, VEGF mRNA is present only in the heaviest sucrose fractions that contain the polysomes (~50% of total in fractions # 3–4). In contrast, the distribution of GAPDH mRNA was not changed in kidney cortex from db/db mice as compared to the control db/m mice. These data provide strong evidence for increased translation of VEGF mRNA in kidney cortex in early phase of type 2 diabetes. Next, we examined recruitment of hnRNP K to VEGF mRNA, because it participates in VEGF mRNA translation in MCT cells [3]. hnRNP K was immunoprecipitated from the same amount of kidney cortex of db/m and db/db mice. RNA was extracted from these immunoprecipitates and used for RT-PCR using primers specific for murine VEGF and GAPDH. Figure 4C shows that hnRNP K binds to VEGF mRNA in non-diabetic db/m animals, and that this binding significantly increased in the kidney cortex from db/db mice ($295 \pm 40\%$ of db/m, $p < 0.05$ by t-test). Together, these data suggest that hnRNP K binding to VEGF mRNA could participate in increased translation in diabetic kidney cortices.

As hnRNP K is activated by c-src and PKC δ , we measured their activity by immunokinase assay in kidney cortices. PKC δ and c-src were sequentially immunoprecipitated from the same kidney cortex homogenates. PKC δ activity was measured using MBP as an exogenous substrate and c-src activity was measured using autophosphorylation of the kinase. Figure 5A shows that PKC δ activity was significantly higher in kidney cortex from diabetic mice ($175 \pm 4\%$ of db/m, $p < 0.01$ by t-test). Figure 5B shows that c-src activity was higher in the kidney cortex from db/db mice ($178 \pm 9\%$ of db/m, $p < 0.01$ by t-test).

We next examined phosphorylation of PKC δ on Tyr311 in kidney cortices by immunoprecipitation of PKC δ followed by immunoblot with an antibody specific for PKC δ phosphorylated on Tyr311. Figure 5C shows that phosphorylation of this residue was weak in control kidney and was markedly increased in diabetic kidneys ($198 \pm 5\%$ of db/m, $p < 0.01$ by t-test). This suggests that increased PKC δ activity in diabetic renal cortex is due to c-src-dependent phosphorylation of Tyr311.

Recruitment of PKC δ to hnRNP K was assessed by immunoprecipitation of PKC δ followed by immunoblot for hnRNP K. Figure 5D shows that PKC δ was associated with hnRNP K in control kidneys and that this association was significantly increased in diabetic kidneys ($160 \pm 14\%$ of db/m, $p < 0.05$ by t-test). Finally, we assessed PKC δ -dependent phosphorylation of hnRNP K in renal cortex by measuring the amount of phosphorylated Ser302 in hnRNP K.

immunoprecipitates. Figure 5E shows that hnRNP K was basally phosphorylated on Ser302 in control kidneys and that this phosphorylation was significantly increased in diabetic kidneys ($185 \pm 14\%$ of db/m, $p < 0.01$ by t-test). Together, these data suggest that the c-src-PKC δ -hnRNP K signaling pathway is activated in the kidney cortex from db/db mice 2 weeks after onset of hyperglycemia.

Discussion

Our data demonstrate the following: (1) Inhibition of PKC δ expression reduces Ang II stimulation of VEGF synthesis, similar to inhibition of hnRNP K expression. (2) hnRNP K recruits both c-src and PKC δ activated by Ang II that allows phosphorylation and activation of PKC δ by c-src. (3) PKC δ , in turn, phosphorylates and activates hnRNP K, allowing its binding to VEGF mRNA (4) Following Ang II stimulation, both hnRNP K binding to VEGF mRNA and its translation are PKC δ dependent. (5) In kidney cortex from mice at 2 weeks type 2 diabetes, VEGF synthesis is increased, due to increased translation of its mRNA, that is paralleled by increased binding of hnRNP K to VEGF mRNA. (6) c-src and PKC δ activities are increased in diabetic kidneys, leading to phosphorylation of hnRNP K. Our data demonstrate that PKC δ positively regulates VEGF mRNA translation through activation of hnRNP K.

Activation of PKC δ by Ang II

The involvement of PKC δ in Ang II signaling is complex. In vascular smooth muscle cells, PKC δ contributes positively to Ang II signaling. Through AT1 receptor Ang II activates PKC δ which in turn activates JAK2 [21], PKD [22], PAK1 [23] and the Rho-ROCK axis [24], all of which are involved in vascular remodeling. In hepatic C9 cells, Ang II activation of ERK is dependent on c-src and PKC δ [25]. However, role of PKC δ in Ang II signaling is cell-specific. For instance, in cardiomyocytes and myocardium, however, PKC δ exerts a negative effect on Ang II signaling: in these cells and tissue, Ang II stimulates production of CTGF through activation of PKC α , PKC ϵ and PKC ζ whereas PKC δ exerts a tonic inhibition of this effect, keeping connective tissue growth factor (CTGF) expression moderate [26]. In neurons in culture, the effect of PKC δ in Ang II signaling depends on the physiologic status: in normoxic conditions, Ang II activates PKC δ which does not induce DNA fragmentation; however, treatment of hypoxic neurons with Ang II results in activation and limited proteolysis of PKC δ which then induces DNA fragmentation [27]. To our knowledge, this is the first report of activation of PKC δ by Ang II in renal epithelial cells.

Mechanisms of PKC δ activation

PKC δ is unique among PKC isoforms in that it can be activated by tyrosine phosphorylation in the absence of diacylglycerol (DAG) production [16]. Phosphorylation of Tyr311 located in the hinge region of PKC δ is mediated by members of the c-src family [16], and induces a conformational change in the enzyme similar to the one induced by DAG binding. In hydrogen peroxide-treated cardio-myocytes, PKC δ phosphorylated on Tyr311 accumulates in both soluble and particulate fraction [16] and exhibits lipid-independent kinase activity [28]. In this instance, PKC δ can be activated in the absence of membrane translocation. As Ang II can activate other PKC isoforms (such as PKC β) through increased DAG production [29], and PKC δ can be activated by DAG [30], it appears that Ang II could activate PKC δ in two independent ways: through increased DAG production which would lead to membrane translocation and through phosphorylation of Tyr311, without membrane translocation. As subcellular localization of activated PKC δ is very important in determining its substrate specificity [28], this could lead to PKC δ phosphorylating two different sets of substrates after activation by Ang II.

Activation of PKC δ in diabetes and hyperglycemic conditions

Activation of PKC δ , measured by translocation into membrane fraction, is significantly increased in diabetic glomeruli, that is prevented by the antioxidant taurine [31], suggesting that oxidative stress is an activator of PKC δ in vivo in diabetic kidneys.

The role of different PKC isoforms in diabetic nephropathy has been studied using specific knockout mice. Study of PKC α -null mice showed that this isoform plays a role in up-regulation of VEGF and its receptor in diabetic glomeruli, as well as later development of albuminuria [32]. Study of PKC β II-null mice has shown that this isoform is involved in renal fibrosis in diabetic kidneys, as increased expression of TGF- β 1 and CTGF, as well as extracellular matrix proteins induced by diabetes were significantly reduced in the glomeruli of these mice as compared with their wild-type littermates. This was due to reduced oxidative stress [33]. In addition, the administration of a specific and selective PKC β inhibitor, ruboxistaurin was able to prevent both mesangial expansion and basement membrane thickening in diabetic db/db mice [34]. Ruboxistaurin has also been shown to ameliorate diabetic nephropathy in human subjects [35]. PKC δ knockout mice have been generated and are viable; they exhibit an expansion of the B-lymphocyte population with formation of numerous germinal centers but no obvious kidney abnormalities have been reported [36]. However, studies have suggested that PKC δ may be important in diabetic nephropathy. In mesangial cells exposed to high glucose for 24 hours, total PKC δ expression is significantly increased [37], accompanied by increased activity [38]. In glomeruli from db/db mice studied after 2 weeks of hyperglycemia, there was a significant translocation of PKC δ in the membrane fraction, which was interpreted to reflect its activation [39]. In kidney cortices, made of both glomeruli and tubules, from db/db mice after 2 weeks of hyperglycemia, we did not detect increase in total amount of PKC δ but its activity was significantly increased as compared to kidney cortices from db/m mice (Figure 5A). We also found that PKC δ activation was paralleled by its phosphorylation on Tyr311, a residue targeted by c-src [3], but we haven't ascertained that this phosphorylation activates PKC δ in vivo, as it does in MCT cells [3]).

Our data indicate that hnRNP K acts as a scaffold protein that binds both c-src and PKC δ . c-src phosphorylates PKC δ on Tyr311 and activates it; activated PKC δ phosphorylates hnRNP K on Ser302, which promotes binding of hnRNP K to VEGF mRNA and stimulates its translation (Fig. 6).

Together, these studies suggest that PKC δ could play a role in the pathogenesis of diabetic nephropathy. Our data show that such a role could be the regulation of VEGF synthesis, an important pathogenic factor in the development of long-term renal changes in type 2 diabetes [40].

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Figure 1. Effect of PKC δ siRNA on Ang II stimulation of VEGF synthesis

Immunoblots for VEGF, PKC δ and actin were carried out on lysates from MCT cells transfected with siRNA (control or specific for PKC δ , 100 nM each) for 48 h, and incubated with or without Ang II (1 nM) for 30 min. PKC δ immunoreactivity was measured to assess efficiency of RNA interference. The lower panel represents quantitation of 3 independent experiments, expressed as percent (mean $\pm$ se) of cells transfected with control siRNA and untreated. ** p < 0.01, *** p < 0.001 by ANOVA.

A

B

D

Figure 2. c-src is required for PKCδ activation and association with hnRNP K

A. c-src and PKCδ associate with hnRNP K. PKCδ and c-src immunoreactivity was measured in hnRNP K immunoprecipitates from MCT cells treated with Ang II for the indicated times. Note the presence of the immunoglobulin heavy chain (IgG-H) in the c-src immunoblot. The figure presents blots representative of 3 independent experiments. The lower panel represents quantitation of 3 independent experiments, expressed as percent (mean ± se) of total VEGF mRNA (in all 4 fractions). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ by ANOVA.

B. c-src inhibition prevents PKCδ association with hnRNP K. PKCδ immunoreactivity was measured in hnRNP K immunoprecipitates from MCT cells preincubated with PP2 for 30 min before treatment with Ang II (1 nM) for 5 and 15 min. The figure presents blots representative of 3 independent experiments. The lower panel represents quantitation of 3 independent experiments, expressed as percent (mean ± se) of total VEGF mRNA (in all 4 fractions). * $p < 0.05$, ** $p < 0.01$ by ANOVA.

C. hnRNP K and PKCδ colocalize in the nucleus of MCT cells treated with Ang II. MCT cells were seeded in 4-well chamber slides. Quiescent cells were treated with 1 nM Ang II for 15

min in the absence or presence of PP2. PKC δ was detected with FLUOR-conjugated antibody (green) and hnRNPk with Cy3-conjugated antibody (red). Orange and yellow colors represent weak and strong co-localization, respectively. Phase contrast was used to visualize the cells. Shown are pictures representative from two independent experiments.

D. *c-src* inhibition prevents PKC δ tyrosine phosphorylation. Immunoblots for phospho-Tyr311-PKC δ , PKC δ and actin were carried out on lysates from MCT cells transfected with siRNA (control or specific for c-src) for 24 h, and incubated with or without Ang II (1 nM) for 15 min. C-src immunoreactivity was measured to assess efficiency of RNA interference. The lower panel represents quantitation of 3 independent experiments, expressed as percent (mean $\pm$ se) of cells transfected with control siRNA and untreated. ** $p < 0.01$, ns - not significant by ANOVA.

Figure 3. PKC δ is required for hnRNP K activation and binding to VEGF mRNA and regulates VEGF mRNA translation

A. PKC δ and hnRNP K activation. Poly(C)-agarose pull-down assay was carried out on lysates from MCT cells transfected with siRNA (control or specific for PKC δ) for 24 h, and incubated with or without Ang II (1 nM) for 30 min. PKC δ and hnRNP K immunoreactivity was measured in cell lysates to assess efficiency of RNA interference and total amount of hnRNP K, respectively. The lower panel represents quantitation of 3 independent experiments, expressed as percent (mean $\pm$ se) of cells transfected with control siRNA and untreated. ** $p < 0.01$, ns - not significant by ANOVA. **B. hnRNP K binding to VEGF mRNA.** Presence of VEGF mRNA in hnRNP K immunoprecipitates from MCT cells pre-treated with röttlerin (10 μ M, 30 min) before treatment with Ang II (1 nM) for 30 min was assessed by RT-PCR. The lower panel represents quantitation of 3 independent experiments, expressed as percent (mean $\pm$ se) of untreated cells. ** $p < 0.01$ by ANOVA. **C. PKC δ and VEGF mRNA translation.** Polysome assay was performed on lysates from MCT cells treated with Ang II with or without pre-incubation with röttlerin. The lower panel represents quantitation of 3 independent experiments, expressed as percent (mean $\pm$ se) of total VEGF mRNA (in all 4 fractions). ** $p < 0.01$ by ANOVA.

C **Figure 4. VEGF synthesis in diabetic kidneys**

A. VEGF expression. VEGF protein expression was measured by immunoblot on kidney cortex homogenates, and VEGF mRNA expression was measured by RT-PCR on RNA extracted from kidney cortex from control (db/m) and diabetic (db/db) mice. The lower panel represents quantitation of data obtained on kidney cortices from 5 individual mice, expressed as percent (mean $\pm$ se) of db/m mice. ** $p < 0.01$ by t-test. **B. VEGF mRNA translation.** Polysome assay was performed on homogenates of kidney cortex from control (db/m) and diabetic (db/db) mice. The lower panel represents quantitation of 3 independent experiments, expressed as percent (mean $\pm$ se) of total VEGF mRNA (in all 4 fractions). ** $p < 0.01$ by ANOVA. **C. hnRNP K binding to VEGF mRNA.** Presence of VEGF mRNA in hnRNP K immunoprecipitates

from kidney cortex from control (db/m) and diabetic (db/db) mice was assessed by RT-PCR. The lower panel represents quantitation of data obtained on 5 individual mice, expressed as percent (mean $\pm$ se) of db/m mice. * $p < 0.05$ by t-test.

Figure 5. signaling pathway leading to hnRNP K activation in diabetic kidneys

The lower panels represent quantitation of data obtained on 5 individual mice for each group, expressed as percent (mean $\pm$ se) of db/m mice. * $p < 0.05$, ** $p < 0.01$ by t-test. A. *PKC δ* activity was measured by an in vitro kinase assay, using MBP as a substrate in kidney cortex homogenates. Total *PKC δ* in the corresponding lysates was measured by immunoblot. Incorporation of [32 P]-ATP into MBP was measured by autoradiography and quantitated by densitometry. B. *c-src* activity was measured by an in vitro kinase assay in the same kidney cortex homogenates used in Fig. 5A. Total *c-src* in the corresponding lysates was measured by immunoblot. *c-src* autophosphorylation was measured by autoradiography and quantitated by densitometry. C. Phosphorylation of *PKC δ* on Tyr311 was assessed by immunoprecipitation of *PKC δ* followed by immunoblot using an antibody specific for phospho-Tyr311. D. Association of *PKC δ* with hnRNP K was assessed by immunoprecipitation of *PKC δ* from kidney cortex homogenates followed by immunoblot for hnRNP K. E. Phosphorylation of hnRNP K on Ser302 was assessed by immunoprecipitation of hnRNP K from kidney cortex homogenates followed by immunoblot with an antibody specific for phospho-Ser302.

Figure 6. sequential phosphorylation by c-src and PKCδ leads to hnRNP K activation
 basal state: minimal association of hnRNP K with c-src and PKCδ. 5 min treatment with Ang II: c-src binds hnRNP K and phosphorylates it; 15–30 min of stimulation: PKCδ binds to hnRNP K and is phosphorylated by c-src on Tyr311; activated PKCδ phosphorylates hnRNP K on Ser302, which promotes binding of hnRNP K to VEGF mRNA and stimulates its translation.