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M. H. A Baccora
Henry Ford Health

P. Cortes
Henry Ford Health

C. Hassett
Henry Ford Health

D. W. Taube
Henry Ford Health

Jerry Yee
Henry Ford Health, JYEE1@hfhs.org

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Effects of long-term elevated glucose on collagen formation by mesangial cells

MHA Baccora[1], P Cortes[1], C Hassett[1], DW Taube[1] and J Yee[1]

[1]Division of Nephrology, Department of Medicine, Henry Ford Hospital, Detroit, Michigan, USA

Glomerulosclerosis is one of the complications of diabetes that occurs after many years of uncontrolled hyperglycemia. Mesangial cells (MCs) exposed to high glucose (HG) for short periods have shown that transforming growth factor-β (TGF-β) and activated diacylglycerol-dependent protein kinase C (PKC) mediate increased collagen formation. Our study examined collagen formation by MCs exposed to HG for 8 weeks. Exposure to HG in overnight culture resulted in the activation of all PKC isoforms. In contrast, 8-week exposure to HG resulted in the persistent activation of PKC-δ, did not change PKC-α or -β activity, and decreased PKC-ε activity while increasing collagen I and IV gene and protein expression. Collagen IV accumulation was reversed by specific PKC-δ inhibition. Collagen IV gene expression was completely normalized by TGF-β neutralization; however, this was associated with plasminogen activator inhibitor-1 (PAI-1) overexpression and a modest reduction in collagen protein. Our studies suggest that prolonged exposure to HG results in PKC-δ-driven collagen accumulation by MCs mediated by PAI-1 but independent of TGF-β.

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KEYWORDS: diabetic nephropathy; mesangial cells; extracellular matrix; TGF-β; hyperglycemia; PKC

Correspondence: *P Cortes, Division of Nephrology, Department of Nephrology, CFP-5, Henry Ford Hospital, 2799 West Grand Boulevard, Detroit, Michigan 48202-2689, USA. E-mail: cortes.pedro@usa.net*

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Our group and others have emphasized the altered metabolic activity of mesangial cells (MCs) in the pathobiology of diabetic glomerulosclerosis. The initial, most characteristic glomerular lesion of diabetes is mesangial extracellular matrix (ECM) deposition, which precedes interstitial disease.[1–4] This lesion may progress despite the absence of microalbuminuria in humans and experimental animals.[5,6] Thus, although podocyte injury and progressive interstitial alterations may be significant,[7–9] the heightened ECM formation/deposition has generally been considered central to the pathogenesis of diabetic nephropathy.

MCs and other cells in tissue culture, exposed for 60 min to 4 weeks to high glucose (HG) concentrations, synthesize, independent of associated osmolarity changes, increased ECM that accumulates in the conditioned medium and cell layer.[10–14]

Consequently, hyperglycemia is considered a critical factor for diabetic mesangial expansion with the attendant augmentation of ECM. Intuitively, because glomerulosclerosis occurs only after long-standing diabetes, MC chronically exposed to HG is a more relevant *in vitro* correlate to the *in vivo* circumstance. However, a single previous study of MCs incubated in HG for 8 weeks documented a gradual decrease in collagen formation, and it was concluded that HG does not persistently stimulate MC collagen synthesis.[15] This postulate contradicts observations of progressive MC-induced glomerular collagen accumulation *in vivo*.

Numerous studies have demonstrated increased protein kinase C (PKC) activity in MCs exposed to HG for short periods of time (6 h to 8 days) and implicated the activation of PKC isoforms as a major contributor to signaling events that led to increased ECM synthesis.[12,16–21] Isolated glomeruli and MCs in culture express specific PKC isoforms including those represented in diacylglycerol (DAG)-sensitive conventional (PKC-α, -βI, -βII) and novel groups (PKC-δ, -ε), and those in the DAG-insensitive atypical group (PKC-ζ).[22–24] The differential overexpression and discrete subcellular localization of PKC has been found in isolated glomeruli of streptozotocin-diabetic rats studied within 2–4 weeks after disease initiation.[22,25–28] HG-induced PKC activation is intimately linked to multiple alterations that convene to enhance ECM formation and accumulation, including NADPH oxidase upregulation and reactive oxygen species formation,[29,30] activation of extracellular signal-regulated

protein kinase (ERK1/2) signaling,[31] stimulation of angiotensin II production,[18] enhancement of TGF-β expression,[32,33] and suppression of ECM catabolism by activation of plasminogen activator inhibitor-1 (PAI-1) transcription.[34]

PKC activity has been assessed by two different approaches, previously. In the first approach, overall or isoform-specific immunoprecipitate PKC activity has been determined, according to PKC-specific substrate phosphorylating activity.[27,28,35–38] In the second approach, specific PKC isoform activity has been evaluated according to the degree of kinase membrane association.[36,39,40] However, this second approach may be problematic. It has been shown that newly synthesized, catalytically inactive, unphosphorylated PKC is localized exclusively to the membrane fraction.[41] Phosphorylation of this newly synthesized PKC is initiated by 3-phosphoinositide-dependent kinase-1 (PDK)-1, followed by autophosphorylation and release of the mature, catalytically competent enzyme into the cytosol and maintained in an inactive state by binding to the autoinhibitory pseudosubstrate.[42] Enzymatic activation of pPKC occurs by DAG-mediated membrane translocation and phosphatidylserine generation, Ca^{2+}-dependent binding of anionic phospholipids, and release of the pseudosubstrate.[43] Therefore, not all pPKC is membrane-bound and active and, conversely, not all membrane-bound PKC is phosphorylated or active and all membrane-associated pPKC is believed to be active.[42,43]

It is not unequivocally determined whether altered ECM accretion is still present in MCs after long-term HG exposure or what mechanisms maintain enhanced ECM accumulation under such conditions. Therefore, we quantified MC collagen metabolism after long-term HG exposure and the role of specific PKC isoforms and TGF-β in regulating this activity. PKC activity has been assessed according to the membrane-associated content of DAG-sensitive pPKC. The results indicate that MCs exposed to HG for long periods continue to accumulate excessive amounts of collagen and that the mechanisms maintaining this chronically enhanced collagen accretion differ from those during a short HG exposure.

RESULTS

Effects of different periods of HG exposure on MC collagen formation

As described by others, collagen I was the predominant form synthesized by MCs in tissue culture.[44,45] In normal glucose (NG) concentration, collagen I formation was 40-fold that of collagen IV. Notably, the amount of collagen formed by control, NG-incubated cells was not significantly different between 2 and 8 weeks in tissue culture (Figure 1).

Short-term HG induced significant increases in collagen accumulation in the conditioned media that were more greatly accentuated for collagen IV than collagen I. The collagen IV increase was already significant by 48 h (NG, 0.852 ± 0.080 ng/ng DNA; HG, 0.959 ± 0.096 ng/ng DNA, $n = 11$, $P = 0.012$). This HG-induced change was augmented at 2 weeks with respective increases for collagen IV and I of 13- and 2-fold over controls (Figure 1). After long-term HG exposure, the magnitude of the HG-induced stimulation of collagen accumulation was less than in short-term HG-exposed cells, yet was still significantly increased (Figure 1). Under these conditions, collagen IV and I gene expressions were increased by 24 and 35%, respectively (Figure 2).

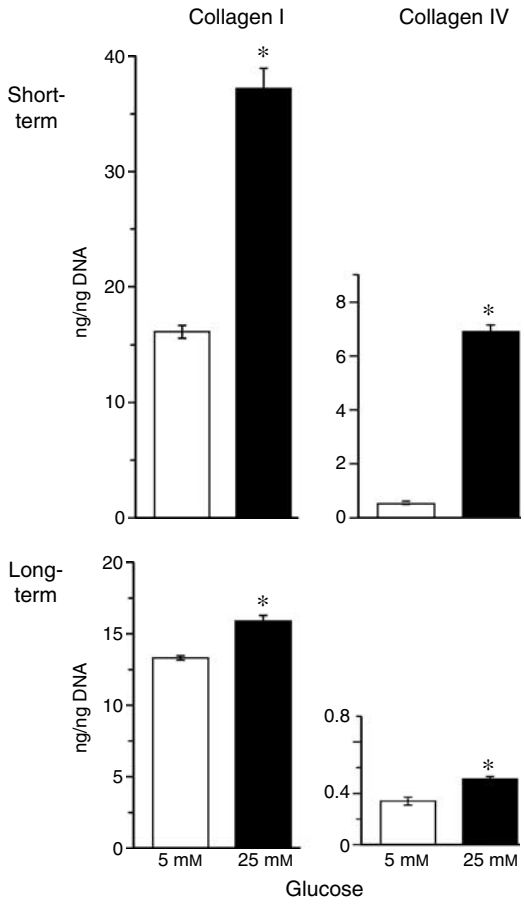

Figure 1 | Collagen media accumulation in MCs after a 2- or 8-week incubation in HG concentration. Primary rat MCs were cultured in a 5 or 25 mM glucose medium containing 15% fetal bovine serum for 2 weeks (short-term) or 8 weeks (long-term). Cultures were studied at 80–90% confluence after 48 h of serum deprivation (1%). Conditioned media were analyzed for collagen I and IV content by ELISA and results adjusted per DNA content in the cell layer. Results are mean $\pm$ s.e.m., $n = 11$–12 per group (*$P < 0.001$).

Effects of different periods of HG exposure on MC PKC isoform activity

The fraction of pPKC that associated with the membrane preparation was considered an index of PKC activation. Accordingly, a wide variation in total PKC phosphorylation vs activity was observed in different isoforms in NG (5 mM) and HG conditions. For example, in NG-incubated MCs, approximately 25% of total pPKC-α was membrane-associated whereas 70% of detectable pPKC-δ was contained in the membrane fraction (Figures 3 and 4).

Long-term MC HG exposure did not alter total PKC, but HG statistically decreased the total content of pPKC-α, -βII, -δ, and -ε by 13.0–19.5% (Figure 3). This reduced

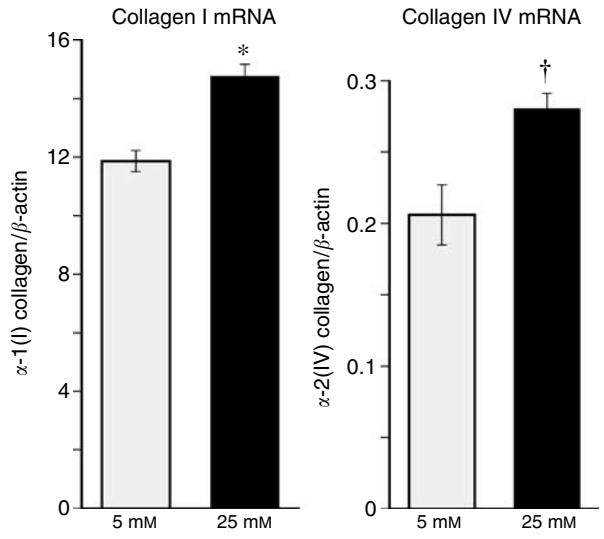

Figure 2 | Collagen gene expression in MCs after an 8-week incubation in HG concentration. Primary rat MCs were cultured as in Figure 1. Total RNA was extracted from the cell layer and collagen I and IV mRNA expression quantified by multiplex, real-time RT-PCR. Results were adjusted for the expression of *β-actin*. Results are mean ± s.e.m., $n = 10$ per group (*$P < 0.001$, †$P = 0.007$).

phosphorylation occurred concurrent with unchanged membrane-associated and membrane/cytosolic distribution of pPKC-α and pPKC-βII isoforms and a decreased membrane-associated pPKC-ε (Figures 3–5). Only pPKC-δ demonstrated significant increases of 49 and 36% in the membrane-associated and membrane/cytoplasm fractions, respectively (Figures 3–5). Thus, by membrane pPKC content, long-term HG either did not alter or reduce isoform activities, except for PKC-δ, which was activated over NG controls.

The effects of rottlerin, a specific PKC-δ inhibitor at low concentrations, were studied in HG-exposed MCs. Treatment with 0.5 μM rottlerin induced a significant decrease in collagen IV accumulation to levels equivalent to NG controls. Increases in rottlerin concentration were associated with additional decrements in collagen IV accumulation (Figure 6). In addition, 0.5 μM rottlerin had no effect on collagen IV accumulation in NG controls (Figure 6).

PKC inactivation of most isoforms by long-term HG exposure contradicts prior findings recorded in MCs after short-term HG incubation. The possibility that these opposing responses resulted from differential methodological approaches was explored. To reproduce findings by others, studies were repeated after 24 h of HG incubation, using the same membrane pPKC parameter as an indicator of activation. This period of HG was selected because this interval has been the most frequently used in former studies.[17,20,21,23] Short-term HG incubation significantly increased the amount of membrane-associated pPKC-α, pPKC-βII, pPKC-δ, and PKC-ε by 23.3, 21.1, 30.4, and 37.9%, respectively, as well as the membrane/cytoplasmic distributions of pPKC-βII and pPKC-δ (Figures 4, 5, and 7).

Role of TGF-β in the enhanced collagen formation after long-term HG exposure

Cultured MCs house a completely functional TGF-β/Smad signaling pathway, which exerts substantial regulatory control over collagen synthesis.[46–48] Additionally, there is a synergistic profibrogenic relationship between TGF-β/Smad and PKC signaling. TGF-β activates PKC-δ-dependent enhancement of collagen gene expression that is not observed with the α- or βI-isoform.[37] The observation that only the δ-isoform was HG-activated implies that TGF-β-enhanced collagen formation may proceed through this isoform. Therefore, the role of TGF-β was studied by quantifying MC collagen expression following TGF-β neutralization in cells maintained under long-term HG conditions.

Long-term HG MC exposure elevated TGF-β formation twofold over NG control levels (Figure 8), and antibody-mediated TGF-β neutralization produced significant decreases of TGF-β1 concentration in NG and HG (Figure 8). In HG, the cytokine increase was abrogated completely to NG control values by antibody neutralization and also inducing a complete reversal of HG-induced collagen IV mRNA overexpression (Figure 8). However, normalizing TGF-β levels resulted only in modest (17%) amelioration of the exaggerated accumulation of collagen, with collagen IV quantities still fourfold greater than NG controls (Figure 8). This observation suggests a reduction of protein catabolism, independent of TGF-β overexpression. Furthermore, TGF-β-mediated PKC-δ activation in MCs unlikely represents a principal pathway of HG-associated collagen accumulation. Lastly, under NG conditions, TGF-β neutralization to subnormal levels did not alter collagen IV mRNA or protein expression (Figure 8).

PAI-1 activity is an important determinant of MC ECM degradation and turnover *in vitro*.[49] HG and glycated proteins induce MC PAI-1 transcriptional activation via PKC-δ and TGF-β/Smad-dependent mechanisms.[50,51] Accordingly, MC PAI-1 expression was studied in HG and during TGF-β inhibition. PAI-1 mRNA and protein expressions were increased 1.5- and 3-fold, respectively, by HG compared to NG controls (Figure 9). TGF-β neutralization increased gene expression, but did not alter PAI-1 protein formation in NG. However, in HG, TGF-β inhibition caused an unanticipated significant increase of MC PAI-1 mRNA and protein overexpression (Figure 9). Taken collectively, the results suggest that, after TGF-β neutralization and normalization of collagen IV gene overexpression, the continual collagen IV accrual stems from a PAI-1-dependent decrease in protein degradation (Figures 8 and 9).

DISCUSSION

MCs in tissue culture characteristically demonstrate a rapid turnover of collagen. Work by us and others has shown brisk synthesis and breakdown of newly formed collagen, achieving steady-state conditioned medium concentrations within 24 h, with only 3% of the total synthesized collagen deposited in the cell layer.[47,52] Therefore, data presented here on collagen

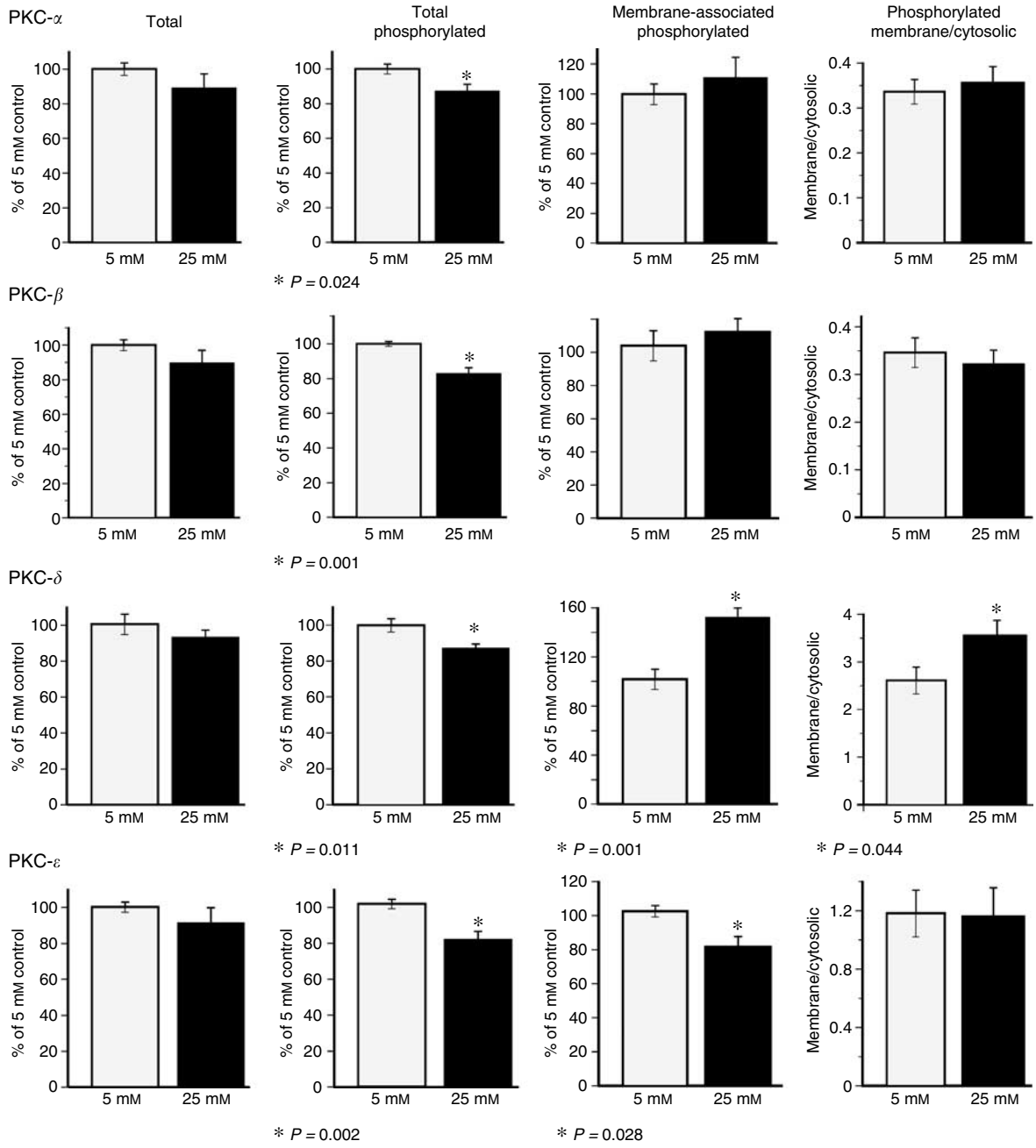

Figure 3 | PKC isoform activity in MCs after an 8-week incubation in HG concentration. Primary rat MCs were cultured as in Figure 1. Cell layer hydrolysates were analyzed for total PKC isoform or phosphorylated isoform by immunoblotting. Phosphorylated isoforms were also quantified in crude membrane preparations and in the corresponding cytosolic fractions to determine their membrane-associated amount and the membrane/cytosolic ratio. Data were normalized by the amount of protein loaded and expressed relative to control values (total amount, membrane-associated) or as an unadjusted ratio (membrane/cytosolic). Results are mean ± s.e.m., $n = 6$ individual samples per group, analyzed in duplicate.

medium accumulation reflect the major part of collagen turnover activity under steady-state conditions.

Experimentally induced mesangial expansion in the insulin-deficient rat involves excessive ECM deposition, including collagen IV. This is evident at 8 weeks and definitively established by 9–12 months.[53–55] Accordingly, the 8-week exposure of rat MCs in tissue culture utilized to test our hypothesis mimics the *in vivo* model.

There are no studies available of MCs in tissue culture comparable to the present investigation. A single previous investigation of long-term effects of HG on MCs with a different experimental approach did not address PKC activity and revealed a paradoxical decrease in collagen synthesis, with unchanged gene expressions of collagens I and IV.[15] Regarding short-term HG incubation, the present study confirms the previously acknowledged increases in collagens I

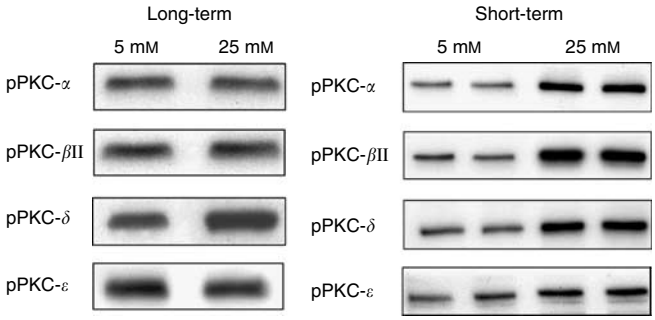

Figure 4 | Membrane-associated content of phosphorylated PKC isoforms in MCs after short- and long-term incubation in HG concentration. Examples of immunoblots of proteins contained in detergent extracts of MC membrane preparations are presented. Cells were exposed to 25 mM glucose medium for 24 h (short-term) or 8 weeks (long-term).

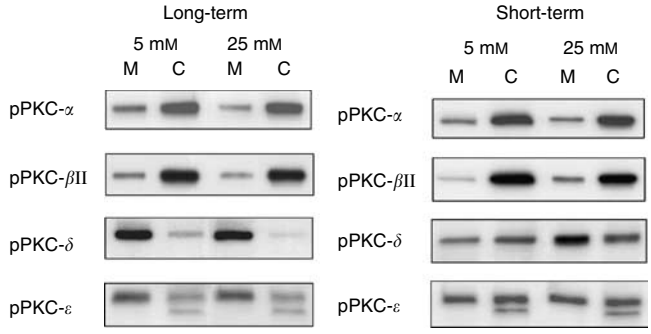

Figure 5 | Membrane/cytoplasm distribution of phosphorylated PKC isoforms in MCs after short- and long-term incubation in HG concentration. Presented are examples of immunoblots of proteins contained in the membrane (M) and cytosolic (C) fractions corresponding to the same sample of MC protein. Cells were exposed to 25 mM glucose medium for 24 h (short-term) or 8 weeks (long-term).

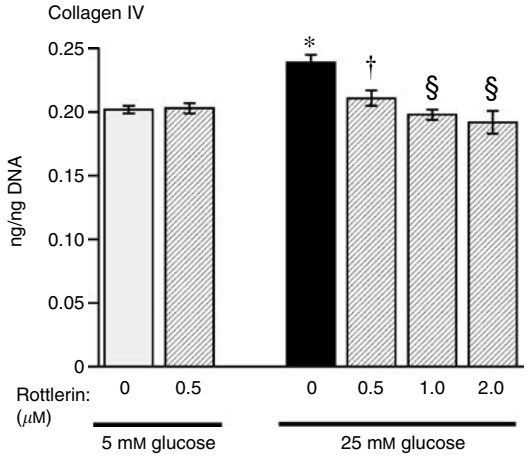

Figure 6 | Collagen media accumulation during PKC-δ inhibition in MCs incubated in HG concentration for 8 weeks. MCs were cultured as in Figure 1. Cultures were treated with the low concentrations of rottlerin noted during the last 48 h of incubation. Conditioned media were analyzed for collagen IV content by ELISA and results adjusted per DNA content in the cell layer. Results are mean ± s.e.m., $n = 12$ per group (*$P < 0.001$ vs 5 mM glucose, †$P = 0.005$ vs 25 mM glucose, §$P < 0.001$ vs 25 mM glucose).

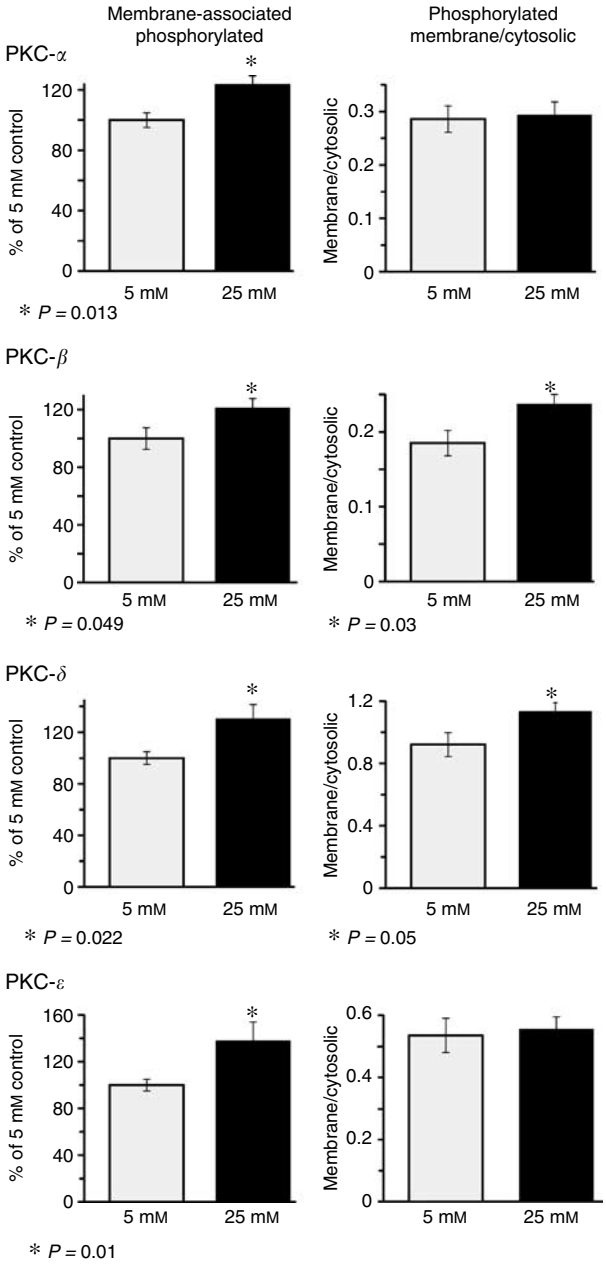

Figure 7 | PKC isoform activity in MCs after a 24-h incubation in HG concentration. MCs were cultured and cell lysates analyzed for PKC isoform-specific activation as in Figure 3. Results are mean ± s.e.m., $n = 10$ individual samples per group, analyzed in duplicate.

and IV expression.[10,56] In addition, these changes endure with prolonged HG exposure, signifying their importance in the pathogenesis of glomerular mesangial expansion. Subsequently, we sought to identify factors responsible for maintaining protracted collagen accrual, and potential pathobiological mechanisms of PKC and TGF-β activation were studied.

Short-term exposure of MCs to HG activated DAG-sensitive PKC isoforms, and this result was most evident for pPKC-βII and -δ, the latter of which demonstrated increased

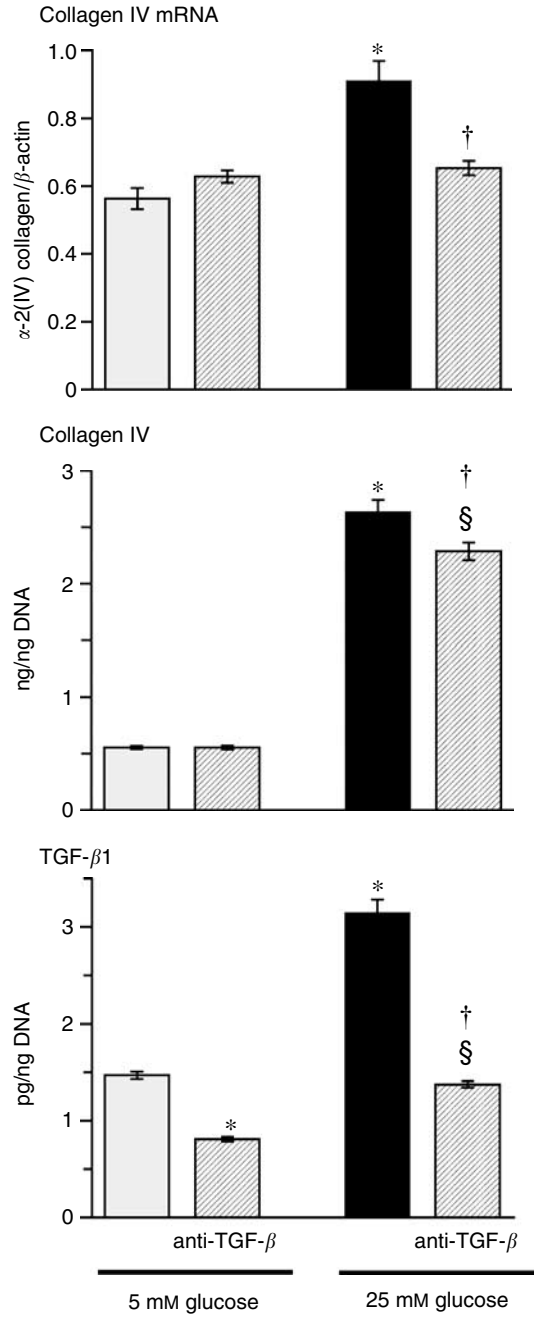

Figure 8 | Collagen IV gene and protein expression in MCs incubated for 8 weeks in HG concentration and following TGF-β neutralization. Primary rat MCs were cultured as in Figure 1. Growth factor neutralization was achieved by addition of mouse monoclonal anti-TGF-β antibody or nonspecific mouse IgG as control during the last 48 h of incubation. Collagen IV mRNA expression was quantified by multiplex, real-time RT-PCR. Collagen IV and TGF-β protein in 48-h conditioned media was quantified by ELISA. The ELISA method used for TGF-β does not recognize antibody-neutralized growth factor. Results were adjusted per DNA content in the cell layer. Results are mean $\pm$ s.e.m., $n = 12$ per group (*$P < 0.001$ vs 5 mM glucose, $^{\dagger}P < 0.001$ vs 25 mM glucose, $^{\S}P < 0.001$ vs 5 mM glucose, anti-TGF-β).

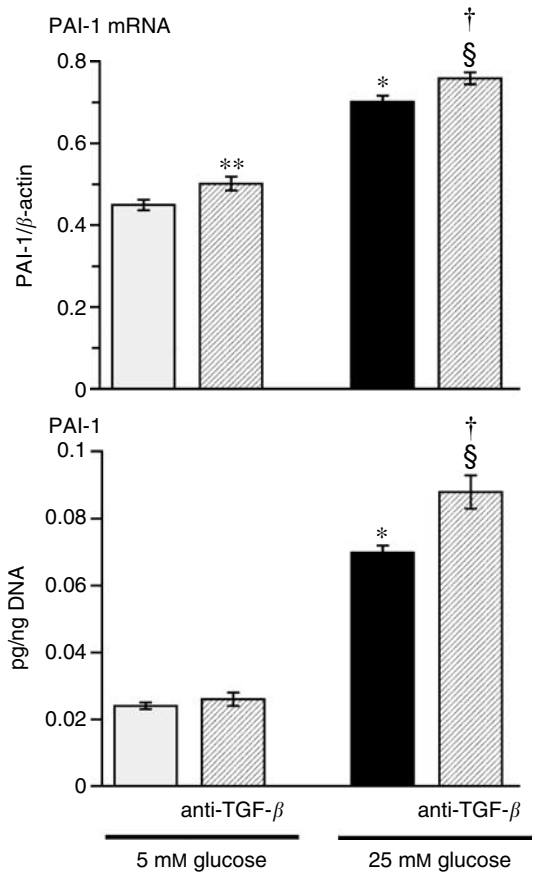

Figure 9 | PAI-1 gene and protein expression in MCs incubated for 8 weeks in HG concentration and following TGF-β neutralization. Primary MCs were cultured and treated with neutralizing anti-TGF-β antibody as in Figure 8. Total RNA was extracted from the cell layer and PAI-1 mRNA expression was quantified by multiplex, real-time RT-PCR with results adjusted for the expression of *β-actin*. PAI-1 protein was quantified by ELISA in 48-h conditioned media. Protein results were adjusted per DNA content in the cell layer. Results are mean $\pm$ s.e.m., $n = 12$ per group (*$P < 0.001$ vs 5 mM glucose, $^{\dagger}P < 0.011$ vs 25 mM glucose, $^{\S}P < 0.001$ vs 5 mM glucose, anti-TGF-β, **$P = 0.019$ vs 5 mM glucose).

membrane translocation. These data are in agreement with previous studies.[16,34,36] In particular, they are consistent with observed specific HG-induced increases in PKC-δ activity, detectable at 72 h as documented in isoform-specific immunoprecipitates by a kinase assay.[57]

PKC signaling activity is distinctly altered by chronic HG exposure that overall deactivates the DAG-sensitive PKC isoforms. Compared to NG-incubated controls, the total content of all pPKC isoforms was decreased by HG, and PKC-α and -β_{II} revealed no evidence of increased activity. Also, the amount of membrane-associated pPKC-ε was reduced, although PKC-δ clearly remained activated. Consequently, this activation is critical for persistent collagen accumulation as delineated by the rottlerin dose–response relationship between collagen accumulation and concentration of PKC-δ inhibitor. This effect was specifically evident in HG-exposed cells at only one-tenth the concentrations used previously for PKC-δ inhibition in NG-incubated MCs.[37,57] However, there has been controversy regarding the specificity of rottlerin. At $\geqslant 30\,\mu$M, it inhibits other PKC isoforms and is known to

inhibit calmodulin-dependent kinase III (IC_{50} 5.3 μM). Also, rottlerin may uncouple oxidative phosphorylation (3–10 μM).[58–61] Nevertheless, any of these effects are unlikely at the very low concentrations applied in our experiments (0.5–2.0 μM). Furthermore, recent studies have reinforced the effectiveness and specificity of rottlerin as a PKC-δ inhibitor in mesangial and neuronal cells.[38,57]

The level of PKC activity demonstrated in MCs exposed to HG for 8 weeks in this study differs from results in isolated glomeruli of long-term diabetic rats. Studies in 24-week diabetic animals have shown an increase in glomerular total PKC phosphorylating activity.[28,62] In addition, total content of pPKC-α and pPKC-βI and total (phosphorylated and non-phosphorylated) membrane-associated amounts of these isoforms are also increased in 12-week diabetic rats.[32] Other studies in glomeruli from 4-week diabetic rats have demonstrated increased membrane association of total PKC-α, -δ, and -ε isoforms, with decreased PKC-β.[22] One explanation for the discrepant results between glomeruli and MCs in this study is the differential assessment of PKC activation, that is, glomerular studies have not included the specific membrane association of phosphorylated isoforms. A more plausible reason is the heterogeneity of glomeruli, of which MCs represent a fraction of the total cell population.

The PKC-β inhibitor LY333531 in 4-month diabetic animals reduces albuminuria and prevents mesangial expansion.[25,62] However, these data do not prove the pathogenetic importance of PKC-β activation. Inhibition of normal PKC-β activity to levels below those of basal conditions may be associated with suppression of mesangial collagen formation, irrespective of glycemia. That is, the inhibitor may be effective even when its therapeutic target is not relevant.

Activation of the TGF-β/Smad signaling pathway is considered an important component of HG-mediated stimulation of MC collagen synthesis, by virtue of short-term studies.[63,64] In addition, there is a close relationship between HG-induced alterations of PKC and TGF-β signaling pathways that has been defined in short-term investigations.[33,57,65] Relevant to this study are observations in MCs and other cells of TGF-β-induced activation of PKC-δ and PKC-δ-dependent TGF-β signaling.[37,57,66] In this study, complete abolition of TGF-β overexpression completely normalized HG-augmented collagen IV mRNA expression, whereas collagen IV protein accumulation was only moderately reduced. Under these circumstances, the vast part of collagen accumulation was attributed to TGF-β-independent PAI-1 upregulation with diminution of collagen breakdown that was accentuated by TGF-β neutralization. These results are in contrast to the findings of others though, where short-term treatment of MCs with TGF-β or oxidized low-density lipoprotein to induce TGF-β activation stimulated PAI-1 transcription.[67,68]

Therefore, the apparent main cause for collagen accumulation after long-term HG exposure is diminished collagen breakdown that is contingent on PKC-δ activation. Thus, TGF-β overactivity increases collagen synthesis but is only responsible for a minor fraction of the collagen accumulated. The results obtained in this study are in agreement with the demonstration of PAI-1 upregulation by HG in MCs *in vitro* and by diabetes *in vivo*,[69–71] and concordant with the observation that PAI-1 activity is a major pathogenetic component in the development of experimental diabetic nephropathy.[72] Notably, these observations are also in concert with the demonstration that PAI-1 gene expression is stimulated by HG in MCs through PKC-δ.[50]

The reasons for the differential behavior of MCs after short- and long-term HG exposure may be ascribed to differences in the stimuli (glucose concentration vs glycoxidative stress) and/or clonal selection of MC populations with specific phenotype.[73] In either case, we assume that the same occurs *in vivo*.

In conclusion, long-term HG exposure induces MC ECM accumulation via a PKC-δ-driven, PAI-1-mediated, and largely TGF-β-independent mechanism. Because the contribution of individual pathogenetic mechanisms maintaining this accumulation changes with time, the targets of therapeutic intervention should be primarily defined during chronic HG conditions.

MATERIALS AND METHODS

Tissue culture

Primary MCs were obtained from outgrowths of isolated glomeruli from Munich–Wistar rats.[74] Cells were grown in 75 cm^2 flasks (Corning Inc., Corning, NY, USA) or six-well plates (Becton Dickinson, Lincoln Park, NJ, USA) in RPMI medium 1640 (Gibco BRL, Grand Island, NY, USA), pH 7.4, containing, 2 mM L-glutamine, 23.8 mM $NaHCO_3$, 15% fetal bovine serum (Gibco BRL, Grand Island, NY, USA), 5 U/ml penicillin G, and 5 U/ml streptomycin. Cells were used at passage 3 and continuously grown in NG (5 mM) or in HG (25 mM) for 24, 48 h, 2 weeks (short-term), or 8 weeks (long-term). All studies were carried out in 48-h serum-deprived (1% fetal bovine serum) cells at 80–100% confluency. As previously shown by us, overall collagen accumulation rapidly decreases as cultures became more confluent.[75] This effect tends to blunt differences between groups, as results are expressed per total DNA. Neutralization of TGF-β activity was accomplished by adding 3 μg/ml of mouse monoclonal-1835 TGF-β antibody (R&D System Inc., Minneapolis, MN, USA) or a nonspecific mouse IgG to the incubation medium every 24 h during the last 48 h of the experiment. Rottlerin was purchased from Calbiochem® (EMD Biosciences Inc., La Jolla, CA, USA).

Harvesting and cell fractionation

Cell layers were obtained in lysis buffer containing 20 mM Tris-HCl, 150 mM NaCl, 2 mM EDTA, 5 mM EGTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 100 μM leupeptin, 30 μM pepstatin, 10 mM benzamidine, 50 mM sodium fluoride, 20 μM E-64, 50 μg/ml aprotinin, 1.2 mM sodium vanadate, 5 mM sodium pyrophosphate, and 1% Triton X-100. Samples were sonicated on ice using a CPX130PB sonicator (Cole Parmer Instruments, Vernon Hills, IL, USA) at 20% amplitude for 5 s and then centrifuged in a Beckman 22R centrifuge (Beckman Instruments, Palo Alto, CA, USA) at 2000 *g* and 4°C for 5 min to remove cell debris.

For membrane isolations, Triton X-100 was omitted from the lysis buffer. Crude membrane preparations were obtained as

described previously.[76] In these experiments, after the initial centrifugation, supernatants were centrifuged at 100 000 *g* at 4°C for 60 min in a Coulter LE-80 Series with Rotor SW 50.1 (Beckman). The resulting supernatants constituted cytosolic fractions and pellets were resuspended in 400 μl of the same lysis buffer containing 1% Triton X-100 by sonicating at 10% amplitude for 1 s and incubating on ice for 30 min with frequent vortexing. Resuspended pellets were re-centrifuged and supernatants collected as the membrane-associated fraction. Lack of cross-contamination between cytosolic and crude membrane preparations was documented by immunoblotting according to the restricted presence of Na^+-K^+-ATPase and sarcoendoplasmic reticulum Ca^{2+}-ATPase in the particulate fraction and of glyceraldehyde-3-phosphate dehydrogenase in the cytosolic supernatant.[77]

Immunoblotting

Protein measurement was assayed by a modified Lowry method.[78,79] Equal amounts of protein were loaded and separated on 4–15% SDS-polyacrylamide gels (Jule Biotechnology Inc., Milford, CT, USA), blotted onto polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA), and then blocked in Tris-buffered saline (Mediatech Inc., Herndon, VA, USA) containing 0.05% Tween 20 (TTBS) and 5% nonfat milk powder. pPKC/PKC isoforms were identified by reaction with the following isoform-specific antibodies: polyclonal rabbit anti-pPKC-α (Upstate Co., Lake Placid, NY, USA), polyclonal rabbit anti-pPKC-ε (Upstate Co.), polyclonal rabbit anti-pPKC-α/βII (Cell Signaling Technology®, Danvers, MA, USA), polyclonal goat anti-pPKC-δ (Santa Cruz Biotechnology, Santa Cruz, CA, USA), monoclonal mouse anti-PKC-α, -β, -δ, and -ε (BD Biosciences, Pharmingen, San Diego, CA, USA). After being washed, membranes were incubated with species-specific secondary horseradish peroxidase-conjugated antibodies: donkey anti-goat (Santa Cruz Biotechnology), donkey anti-rabbit and sheep anti-mouse antibodies (Amersham Bioscience, GE Healthcare, Buckinhamshire, UK). Immunoreactive bands were visualized with ECL™ detection reagent and exposure to Hyperfilm™ (Amersham Bioscience). Uniformity of loading and equal membrane transfer was determined in parallel membranes stained with Coomassie blue. Films and Commassie blue-stained membranes were scanned and bands analyzed using the National Institute of Health ImageJ v1.33U gel plotting software to obtain densitometry measurements. The sum of all protein bands contained in each sample lane in Coomassie blue-stained membranes was considered equivalent to the amount of loaded/membrane-transferred protein.

After first determining the variation between NG controls, the relative abundances of pPKC/PKC isoforms between groups were ascertained by comparison to values resulting from a pooled sample of NG control protein that was included in each electrophoretic separation. The ratio membrane/cytosolic fraction of individual pPKC isoforms was directly calculated from the results of membrane and cytosolic samples separated in the same gel. Final results were adjusted for differences in protein loading/transfer when indicated.

Enzyme-linked immunosorbent assay

The concentration of collagens I and IV in 48-h conditioned media was determined in 96-well plates (Microfluor® 2 black flat-bottom microtiter plates; Thermo Electron Co., Milford, MA, USA) by a high-sensitivity direct enzyme-linked immunosorbent assay (ELISA). Samples were diluted in coating buffer (51 mM $NaHCO_3$, 115 mM Na_2CO_3, pH 9.4) and incubated overnight. After being washed, plates were treated with 0.5% bovine serum albumin and 0.1% Tween 20 dissolved in blocking buffer (137 mM NaCl, 8.8 mM $Na_2HPO_4 \cdot 2H_2O$, 1.5 mM KH_2PO_4, and 2.7 mM KCl, pH 7.4) for 1 h and incubated overnight in the corresponding primary antibody (rabbit anti-rat collagen I, Chemicon Int., Temecula, CA, USA; affinity-purified rabbit anti-rat collagen IV, Rockland Inc., Gilbertsville, PA, USA). After being extensively washed, plates were treated with a horseradish peroxidase-conjugated goat anti-rabbit IgG (Invitrogen, Carlsbad, CA, USA) for 3 h. Finally, samples were incubated for 20 min with 0.05 mM Texas Red reagent (Invitrogen) and fluorescence intensity was determined in a Fluorskan Ascent FL (Thermo Electron Co.) at $530_{excitation}/590_{emission}$. Results were analyzed using a curve-fitting software (Interactive Microware, State College, PA, USA). Purified rat collagen type I (Upstate Biotechnology, Valerica, MA, USA) and type IV (Santa Cruz Biotechnology) were used as standards. Total TGF-β1 and PAI-1 proteins in the conditioned media were measured using ELISA Quantikine® (R&D Systems) and PAI-1 (Aniara, Mason, OH, USA) kits following the manufacturers' instructions. The ELISA assay for TGF-β reacts solely with free TGF-β1 and does not recognize the antibody-neutralized growth factor.

DNA content

Cell layers were obtained in a 10 mM sodium phosphate lysis buffer, pH 7.5, containing 150 mM NaCl, 2 mM EDTA, 1% Igepal, and 1% sodium deoxycholate. Samples were homogenized and centrifuged at 26 000 *g* for 20 min at 4°C. The resulting supernatants were used for DNA measurement by a fluorescence method using the PicoGreen™ dsDNA Kit (Molecular Probes Inc., Eugene, OR, USA) according to the manufacturer's directions.

Quantitative, real-time gene expression

Total RNA was extracted using the RNeasy mini Kit (Qiagen Inc., Valencia, CA, USA) after DNase treatment. The approach for the measurement of gene expression was a one-step reverse transcription (RT)-PCR with specific FRET probes using a multiplex mode (gene of interest and housekeeping gene expression measured simultaneously in the same reaction) in a LightCycler® (Roche Molecular Biochemicals, Manheim, Germany). Reactions were carried out in triplicate using Superscript™ III one-step QRT-PCR Platinum probe (Invitrogen) kit. As reference gene we used *β-actin*, because our recent studies have demonstrated this gene to be one of the most stable genes in MCs cultured in HG conditions.[80]

Statistical analysis

Protein medium accumulation was factored by the total DNA contained in the corresponding tissue culture well. Statistical analyses were carried out using the StatView® 5.0.1 software (Abacus Concepts Inc., Berkeley, CA, USA). Results were expressed as mean $\pm$ s.e.m. Results were analyzed by unpaired *t*-test with the level of significance calculated by a two-tailed test. In experiments containing more than two groups, significance was determined by analysis of variance and differences between groups determined by Fisher's protected least significant difference as *post hoc* test. Statistical significance was set at 5% level.

CONFLICT OF INTEREST

None of the authors have any current or past involvement, financial or otherwise, that could potentially bias the work presented.

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