Protein kinase C α inhibition prevents peritoneal damage in a mouse model of chronic peritoneal exposure to high-glucose dialysate

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Chronic exposure to commercial glucose-based peritoneal dialysis fluids during peritoneal dialysis induces peritoneal membrane damage leading to ultrafiltration failure. In this study the role of protein kinase C (PKC) α in peritoneal membrane damage was investigated in a mouse model of peritoneal dialysis. We used 2 different approaches: blockade of biological activity of PKCα by intraperitoneal application of the conventional PKC inhibitor Go6976 in C57BL/6 wild-type mice and PKCα-deficient mice on a 129/Sv genetic background. Daily administration of peritoneal dialysis fluid for 5 weeks induced peritoneal upregulation and activation of PKCα accompanied by epithelial-to-mesenchymal transition of peritoneal mesothelial cells, peritoneal membrane fibrosis, neoangiogenesis, and macrophage and T cell infiltration, paralleled by reduced ultrafiltration capacity. All pathological changes were prevented by PKCα blockade or deficiency. Moreover, treatment with Go6976 and PKCα deficiency resulted in strong reduction of proinflammatory, profibrotic, and proangiogenic mediators. In cell culture experiments, both treatment with Go6976 and PKCα deficiency prevented peritoneal dialysis fluid–induced release of MCP-1 from mouse peritoneal mesothelial cells and ameliorated transforming growth factor-β1–induced epithelial-to-mesenchymal transition and peritoneal dialysis fluid–induced MCP-1 release in human peritoneal mesothelial cells. Thus, PKCα plays a crucial role in the pathophysiology of peritoneal membrane dysfunction induced by peritoneal dialysis fluids, and we suggest that its therapeutic inhibition might be a valuable treatment option for peritoneal dialysis patients.

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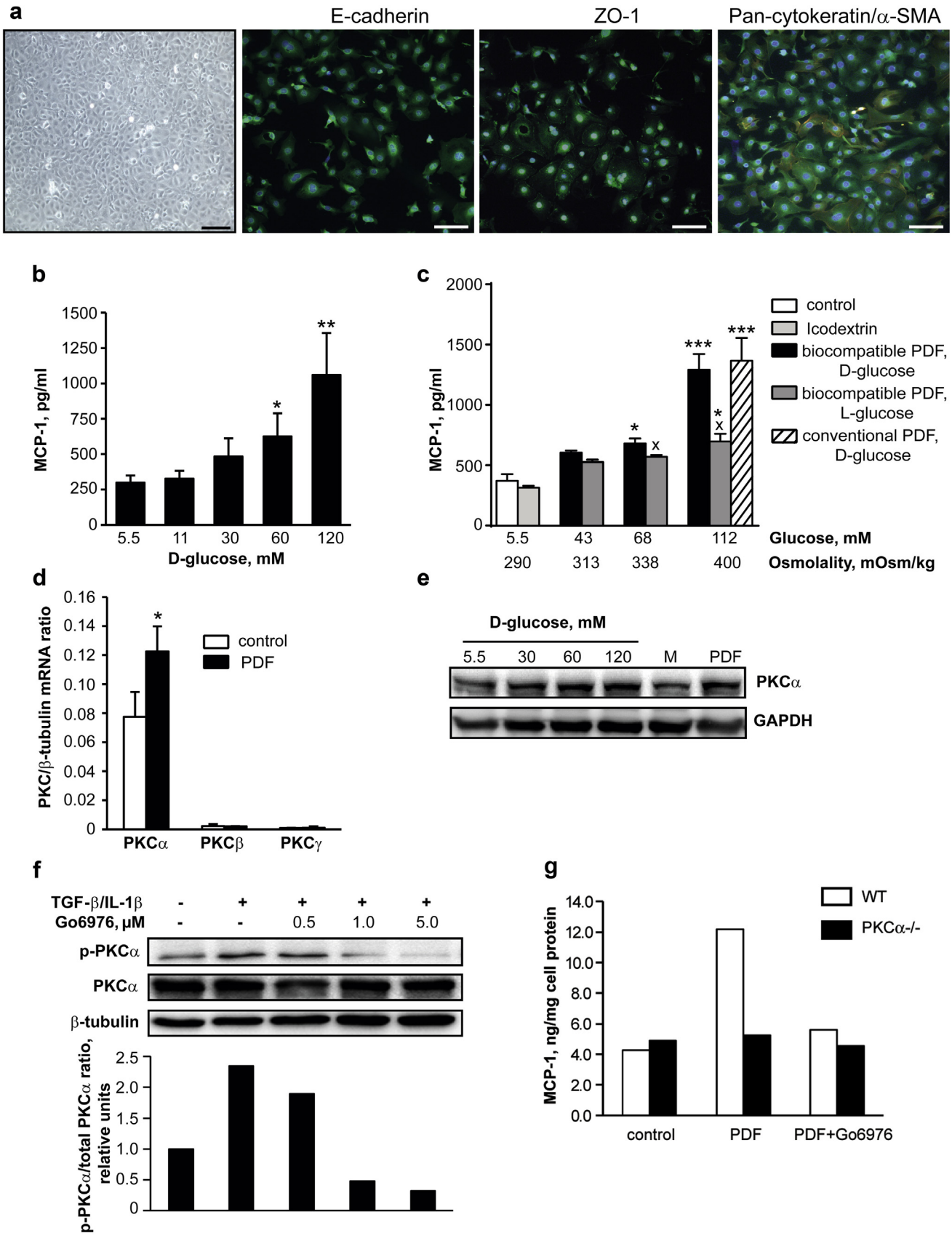
Peritoneal dialysis (PD) is an important treatment option for a worldwide growing number of patients with end-stage renal disease requiring renal replacement therapy. PD can be regarded as a safe and cost-effective renal replacement therapy within an integrated care concept,[1] and, especially in developing countries, it often is the only feasible form of renal replacement therapy.[2,3] However, chronic exposure to glucose-containing PD fluids (PDFs) trigger inflammatory, neoangiogenic, and profibrotic processes[4] leading to progressive structural and functional changes of the peritoneum, ultimately resulting in technique failure and discontinuation of PD.[5] Therapies ameliorating high-glucose–induced detrimental changes and enhancing peritoneal membrane (PM) function are desperately needed.

The PKC superfamily constitutes several homologous serine/threonine kinases divided into 3 groups depending on their biochemical properties of activation: the conventional/classical (α, βI, βII, γ) PKC isoforms have DAG- and Ca^{2+}-binding domains; the novel PKCs (ε, δ, θ, η) have DAG- but not Ca^{2+}-binding domains; and the atypical (ζ, λ/ι) isoforms have neither Ca^{2+}- nor DAG-binding domains.[6] Some PKC isoforms, such as PKCα, βI and II, δ, ε, and ζ, are ubiquitously expressed in different cells and tissues, whereas the expression of others, such as PKCγ or PKCθ, is restricted to distinct cell and tissue types.[7] On the other hand, multiple PKC isozymes can be present in the same cell, and can translocate to different subcellular localizations in response to the same stimuli mediating different cellular responses.[6–8]

Using PKCα isoform knockout animals as well as PKC inhibitors, our group previously demonstrated isoform-specific PKCα-mediated "glucotoxic" effects in a rodent model of diabetic nephropathy.[9–12] Since during PD the peritoneum is exposed to high amounts of glucose on a daily basis, we hypothesized that at least part of the PD-induced detrimental peritoneal changes might be mediated in a similar fashion by PKCα. Along these lines, others have previously shown that exposure of human peritoneal mesothelial cells


a
E-cadherin
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MCP-1, pg/ml
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g
MCP-1, ng/mg cell protein
WT
PKCα-/-
control
PDF
PDF+Go6976

(HPMCs) to high glucose induces chemokines like monocyte chemoattractant protein 1 (MCP-1) as well as reactive oxygen species synthesis in a PKC-dependent fashion.[13–16] However, previously used inhibitors Ro-31-8820[11] and calpostin C[14] are pan-PKC inhibitors,[8] and there are no studies on the distinct role of the PKCα isoform in PD. In this study we used an experimental PD mouse model to test our hypothesis. PKCα-deficient ($PKC\alpha^{-/-}$) mice and C57BL/6 wild-type (WT) mice treated with Go6976, an inhibitor of conventional PKC isoforms, were investigated. The results of *in vivo* studies were supported by *in vitro* experiments in immortalized mouse peritoneal mesothelial cells (MPMCs) and in primary HPMCs.

RESULTS

PKCα is the predominant conventional PKC isoform in MPMCs and its activation *in vitro* has physiological relevance under conditions simulating PD

For *in vitro* experiments, immortalized MPMC cell lines were generated from WT and $PKC\alpha^{-/-}$ mice. The cultured cells demonstrated typical cobblestone morphology and expressed MPMC markers E-cadherin, pan-cytokeratin, and zonula occludens protein 1 (ZO-1) as well as low levels of α-smooth muscle actin (α-SMA) (Figure 1a). Incubation of WT MPMCs with high glucose results in increased MCP-1 release in a concentration-dependent manner (Figure 1b). To distinguish between glucose-specific effects and effects induced by osmolality, glycation, or oxidation, WT MPMCs were incubated with PDFs diluted 1:1 with culture medium. We compared conventional PDF with biocompatible PD solutions containing different glucose concentrations and icodextrin. Locally produced sterile filtered biocompatible PD solutions containing the same concentrations of L-glucose were used as osmotic controls. In all cases pH was adjusted to 7.4 by addition of 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES). The constitutive MCP-1 secretion (under 5.5 mM D-glucose) was significantly increased under hyperosmotic conditions for both D- and L-glucose starting from 68 mM and 112 mM, respectively. At the 68 and 112 mM concentration the effect was significantly higher for D-glucose than for L-glucose and was similar in both biocompatible and conventional PDFs. Icodextrin (5.5 mM D-glucose) did not enhance MCP-1 release over basal level (Figure 1c). These results indicate the D-glucose as a culprit in the PDF-induced MCP-1 release.

The expression of 3 classical PKC isoforms (PKCα, PKCβ, and PKCγ) was investigated in WT MPMCs by TaqMan technology. Whereas PKCα mRNA was expressed on the high level in unstimulated cells and further upregulated in cells stimulated with PDF, only very low levels of both PKCβ and PKCγ mRNAs were found under normal glucose and were not changed by PDF stimulation (Figure 1d). The basal expression of PKCα (Figure 1e) but not PKCβ and PKCγ (data not shown) protein was found in WT MPMCs under normal glucose, and it was upregulated after stimulation with high glucose or with PDF but not with mannitol used as osmotic control. Based on these results indicating PKCα as a predominant classical PKC isoform in MPMCs, we further investigated its role in physiological responses under conditions mimicking PD. PKCα phosphorylation was increased

Figure 1 | Physiological relevance of PKCα in MPMCs stimulated with TGF-β/IL-1β combination or with PDF. (**a**) Characteristics of immortalized MPMCs cultured for 7 days at 37 °C without interferon gamma (nonpermissive conditions). Phase-contrast microscopy shows typical cobblestone morphology of MPMCs; bar = 100 μm. Immunofluorescence staining demonstrates the expression of typical markers of PMCs: E-cadherin, zonula occludens protein 1 (ZO-1), and pan-cytokeratin (green) as well as a low level of α-smooth muscle actin (α-SMA) expression (red). Bar = 50 μm. (**b**) Effect of different concentrations of D-glucose on monocyte chemoattractant protein 1 (MCP-1) release from wild-type (WT) MPMCs. Quiescent WT MPMCs were incubated with cell culture medium containing different D-glucose concentrations for 24 hours. The MCP-1 protein levels were analyzed in conditioned medium by bead-based flow cytometry assay. The results are presented as mean ± SD for 3 independent experiments (*$P < 0.05$ and **$P < 0.01$ vs. 5.5 mM D-glucose). (**c**) Effect of D-glucose and osmolality on peritoneal dialysis fluid (PDF)–induced release of MCP-1 from MPMCs. Quiescent WT MPMCs were incubated for 24 hours with different PDFs (biocompatible and conventional) containing different concentrations of D- or L-glucose and diluted 1:2 with cell culture medium. The control cells were incubated for 24 hours with medium diluted 1:2 with the same PDF solutions without glucose or with icodextrin. The results are presented as mean ± SD for 3 independent experiments (*$P < 0.05$ and ***$P < 0.001$ vs. 5.5 mM D-glucose; $^{x}P < 0.05$ vs. D-glucose). (**d**) Expression of mRNA of 3 classical protein kinase C (PKC) isoforms (α, β, γ) under normal glucose condition (control, 5.5 mM glucose) or after 48 hours of incubation with conventional PDF diluted 1:2 with cell culture medium. The control cells were incubated for 48 hours with medium diluted 1:2 with sterile saline. Only PKCα mRNA was highly expressed and upregulated by PDF in WT MPMCs. The results are presented as mean ± SEM for 3 independent experiments performed in duplicates (*$P < 0.05$ vs. control). (**e**) Western blot analysis for PKCα protein demonstrates that PKCα protein expression increased after 48 hours of incubation with high glucose or with conventional PDF. This effect was not observed in cells incubated with 120 mM mannitol (M) used as osmotic control. The results are representative of 4 independent experiments with similar results. (**f**) Effect of different concentrations of Go6976 on PKCα activation induced by transforming growth factor-β (TGF-β)/interleukin-1β (IL-1β) stimulation. Quiescent WT MPMCs were preincubated for 1 hour with different concentrations of Go6976 (0.5–1 μM) and thereafter stimulated with TGF-β/IL-1β for 48 hours in the presence of Go6976 or left unstimulated (control). Western blot analysis performed for p-PKCα and total PKCα demonstrated that TGF-β/IL-1–induced activation of PKCα can be successfully blocked by Go6976 in a concentration-dependent manner. Quantification by densitometry is shown below. The results are representative of 4 independent experiments with similar results performed with 4 different WT MPMC clones. (**g**) Effect of PKCα deficiency and PKCa blockade on PDF-induced release of MCP-1 from MPMCs. Quiescent WT and $PKC\alpha^{-/-}$ MPMCs were preincubated or not for 1 hour with 5 μM Go6976 and thereafter stimulated for 24 hours with 50% conventional PDF containing or not containing 5 μM Go6976. The control cells were incubated for 24 hours with medium diluted 1:2 with sterile saline. PDF-induced release of MCP-1 from WT MPMCs was completely abrogated by Go6976 treatment and was absent in $PKC\alpha^{-/-}$ MPMCs. The results are representative of 3 independent experiments with similar results. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MPMC, mouse PMC; PMC, peritoneal mesothelial cells; p-PKC, phospo-PKC.

by incubation of cells with a combination of 10 ng/ml transforming growth factor-β1 (TGF-β1) and 2 ng/ml interleukin-1β (IL-1β). Preincubation with Go6976 blocked TGF-β1/IL-1β–induced PKCα activation in a dose-dependent manner (Figure 1f). Incubation with PDF increased the MCP-1 release from WT but not from PKCα$^{-/-}$ MPMCs. Preincubation with 5 μM Go6976 completely abrogated PDF-induced MCP-1 release from WT MPMCs but had no additional effect on PKCα$^{-/-}$ MPMCs (Figure 1g). These results demonstrate that PKCα is not only expressed in MPMCs, but also has a physiological relevance under conditions mimicking PD *in vitro*.

PKCα is overexpressed, activated, and inhibitable during PD in mice

The presence of PKCα in peritoneum and its possible activation during PD was investigated using an established mouse model for PD[17] in C57BL/6 WT mice. After PD catheter implantation the mice were treated daily over 5 weeks with 1.5 ml of saline or standard PDF (Stay Safe, Fresenius, Bad Homburg, Germany). Peritoneal PKCα expression and activation was assessed by immunofluorescence (Figure 2). In saline-instilled mice only very weak expression of total PKCα protein and no positive signal for phospho-PKCα (p-PKCα) could be seen in MPMC monolayer. In contrast, after PDF treatment peritoneal upregulation of both total and p-PKCα was detected. Adding of PKCα inhibitor Go6976 to the PDF at a final concentration 1 μM reduced the upregulation of total PKCα in peritoneum and abrogated its activation by inhibiting PKCα phosphorylation completely.

Inhibition of PKCα activity diminishes PDF-induced peritoneal changes in mice

To analyze the role of PKCα in PDF-induced peritoneal fibrosis, inflammation, and neoangiogenesis *in vivo*, PKCα biological activity was blocked by addition of 1 μM Go6976 to the PDF in the same model using C57BL/6 mice. Functional analysis of peritoneal transport capacity was performed by an ultrafiltration test after 5 weeks of PD. In PDF-instilled mice the volumes recoverable from the peritoneal cavity were significantly reduced compared to the saline-instilled group, suggesting a malfunction of the peritoneum (Figure 3a). Adding of Go6976 to the PDF prevented this loss of functional capacity. PDF-induced morphologic alterations of PM were evaluated in Masson trichrome–stained parietal peritoneum. PDF-instilled mice demonstrated thickening of PM, which was mainly due to matrix collagen deposition and hypercellularity. These changes were abolished by adding Go6976 to the

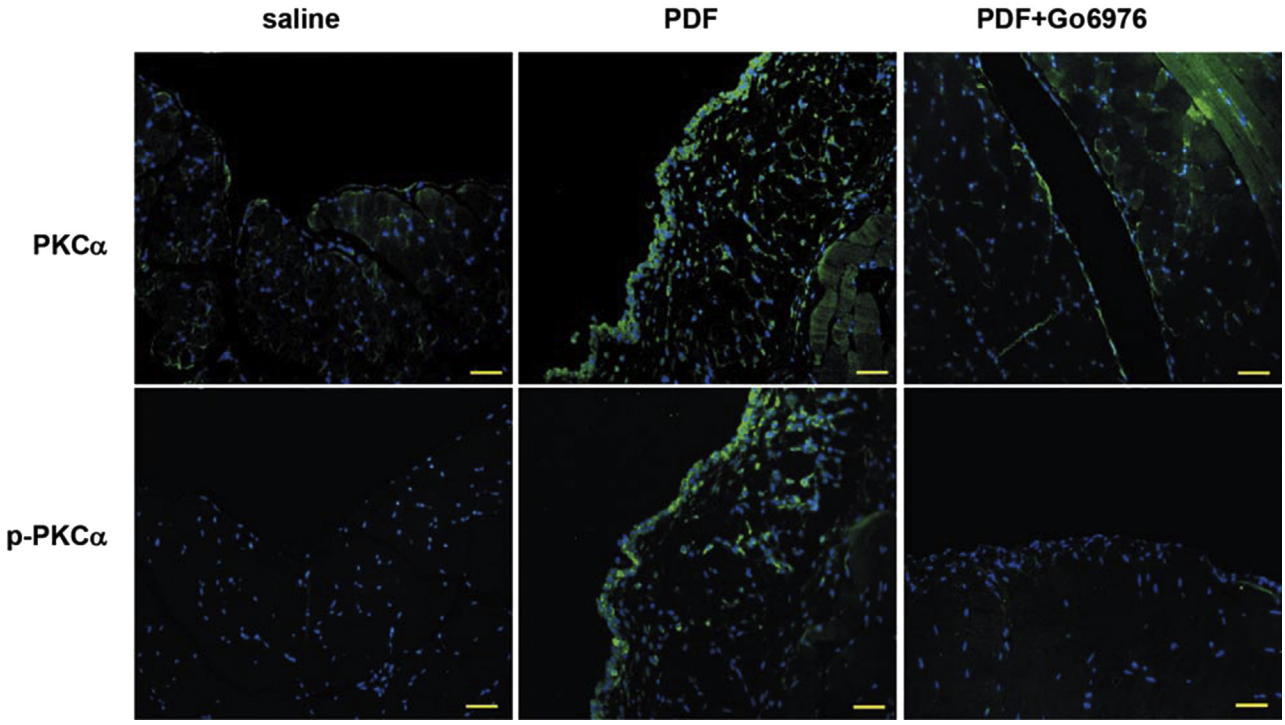


Figure 2 | PKCα is upregulated and activated during PD in WT C57BL/6 mice. Immunofluorescence analysis of total protein kinase C α (PKCα) expression (upper row) and its phosphorylation (lower row) was performed in the parietal peritoneum of WT C57BL/6 mice instilled for 5 weeks with saline (left panel), with peritoneal dialysis fluid (PDF, middle panel), or with PDF containing 1 μM Go6976 (right panel). Bar = 50 μm. Whereas only a very weak expression of nonphosphorylated PKCα could be detected in PMCs of saline-instilled mice, the upregulation of PKCα expression and phosphorylation has been detected in PDF-instilled mice. Go6976 treatment reduced PDF-induced upregulation of PKCα total protein expression and blocked completely its phosphorylation. The representative pictures are shown for each group (n = 8, 12, and 7 for mice instilled with saline, with PDF, and with PDF containing 1 μM Go6976, respectively). PKC, protein kinase C; p-PKC, phospo-PKC; WT, wild type.

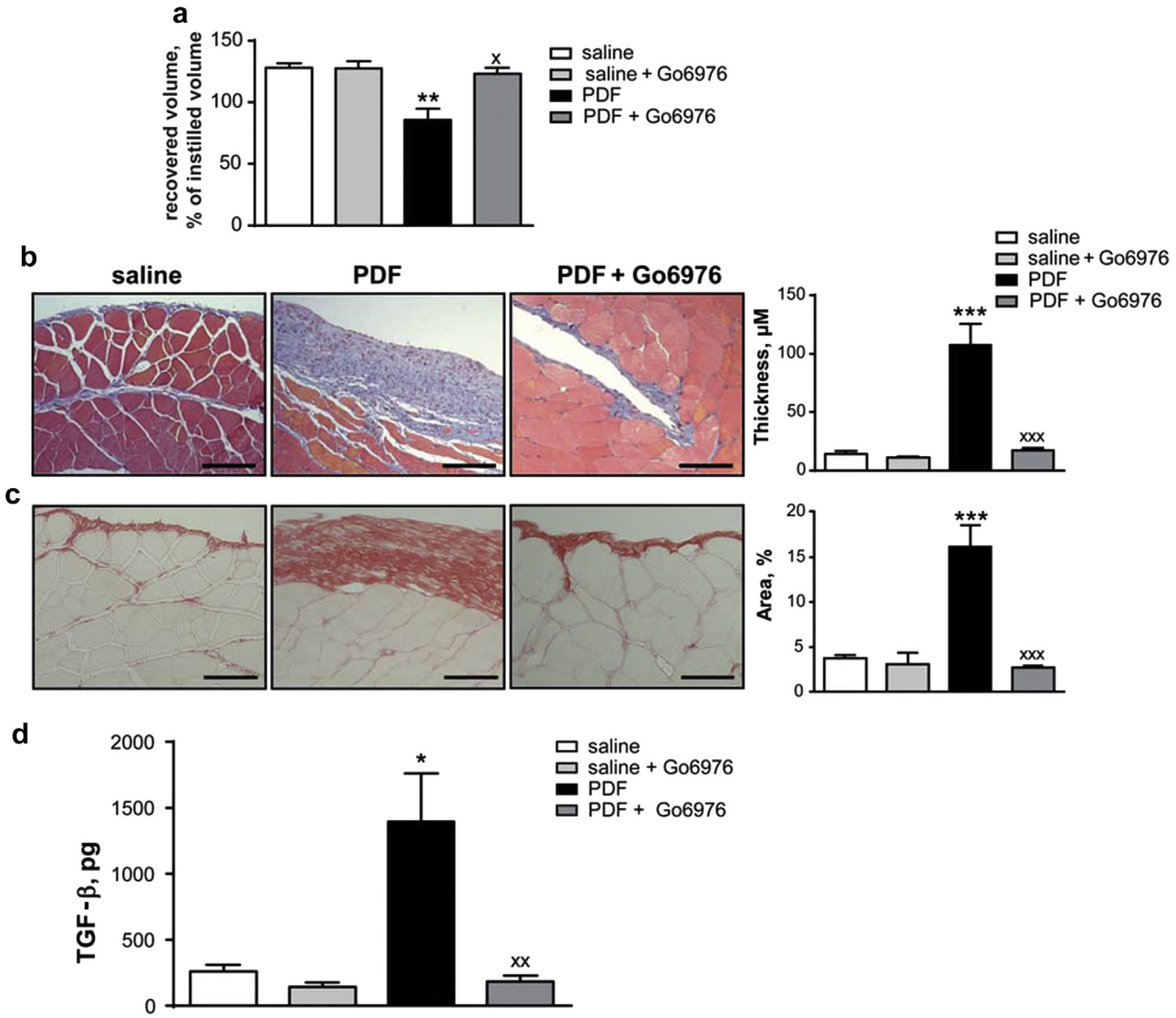


Figure 3 | Blockade of physiological activity of PKCα with Go6976 ameliorates functional and structural changes of PM during PD. (**a**) Ultrafiltration capacity of PM was tested after 5 weeks of dialysis and was determined as the percentage of peritoneal effluent recovered 90 minutes after instillation of 3 ml of peritoneal dialysis fluid (PDF). The volume recovery from mice exposed to PDF was less than from control mice instilled with saline. Treatment with Go6976 had no effect on ultrafiltration capacity of PM in saline-instilled mice, and a recovery of net ultrafiltration has been observed in mice exposed to PDF containing 1 μM Go6976. Data are presented as mean ± SEM ($n = 8$, 3, 12, and 7 for mice instilled with saline, saline with 1 μM Go6976, PDF alone, and PDF with 1 μM Go6976, respectively). $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ versus saline-instilled mice; $^{X}P < 0.05$, $^{XX}P < 0.01$, and $^{XXX}P < 0.01$ versus PDF-instilled mice. (**b**) PD-induced structural changes of PM were evaluated in peritoneal biopsies collected after 5 weeks of fluid instillation and stained with Masson's trichrome stain. Representative images of the saline-instilled, PDF-instilled, and PDF/Go6976–instilled groups are shown (bar = 50 μm). Chronic exposure to PDF resulted in increased deposition of extracellular matrix, cell numbers, and thickness of PM, and these changes were strongly reduced by Go6976 treatment. Graph represents quantification of PM thickness. Data are presented as mean ± SEM. Significance was determined as described in **a**. (**c**) PD-induced fibrosis was evaluated in peritoneal biopsies using picrosirius red staining specific for collagen I and III. Representative images of the saline-instilled, PDF-instilled, and PDF/Go6976–instilled groups are shown (bar = 50 μm). Mice exposed to PDF for 5 weeks showed a prominent deposition of fibrous tissue compared to saline-instilled controls. Treatment with Go6976 prevented fibrosis development in PDF-instilled mice. Quantification of picrosirius red staining was done by ImageJ software (right panel). Data are presented as mean ± SEM. Significance was determined as described in (**a**). (**d**) Transforming growth factor-β1 (TGF-β1) levels were measured in effluents obtained on the last day of experiments by enzyme-linked immunosorbent assay (ELISA). Chronic exposure to PDF results in local accumulation of TGF-β1 in PDF-instilled mice. Treatment with Go6976 completely abrogated this effect. Data are presented as mean ± SEM. $^{*}P < 0.05$ versus saline-instilled mice; $^{XX}P < 0.01$ versus PDF-instilled mice. PD, peritoneal dialysis; PKCα, protein kinase Cα; PM, peritoneal membrane.

PDF (Figure 3b). Submesothelial PDF-induced fibrosis evaluated by picrosirius red staining was observed only in PDF-instilled but not in saline-treated mice and not in mice instilled with PDF containing Go6976 (Figure 3c). The concentrations of TGF-β1, a well-known profibrotic mediator,[18,19] were significantly higher in effluents from PDF-instilled mice compared to the saline-instilled group. Adding of Go6976 to PDF normalized TGF-β1 levels (Figure 3d).

Blockade of PKCα activity ameliorates epithelial-to-mesenchymal transition of MPMCs, peritoneal neoangiogenesis, and inflammation

Epithelial-to-mesenchymal transition (EMT) plays an active role in PM dysfunction. To investigate the possible role of PKCα in EMT of MPMCs, we stained the peritoneal sections for cytokeratin and α-SMA. In saline-instilled mice MPMCs were presented as a continuous monolayer of cytokeratin-positive and α-SMA–negative flattened cells. In PDF-instilled mice a loss of MPMC monolayer integrity

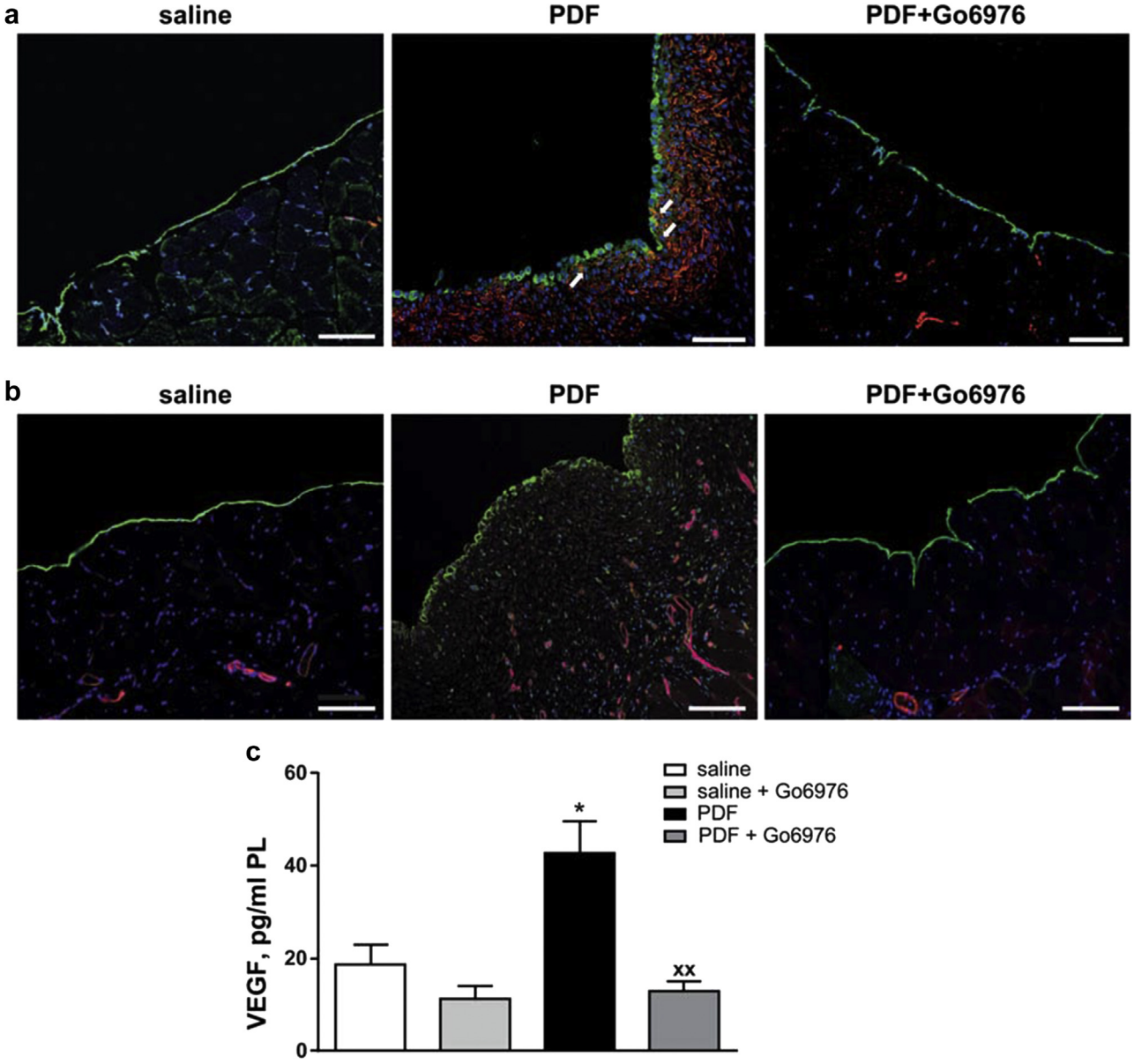


Figure 4 | Blockade of PKCα activity ameliorates EMT of MPMCs and neoangiogenesis during PD. (**a**) Immunofluorescence microscopy analysis of peritoneal biopsy sections stained for cytokeratin (green) and α-SMA (red) and counterstained with 4′,6-diamidino-2-phenylindole (blue). A single layer of flat peritoneal mesothelial cells was observed in saline-instilled mice (left panel). Peritoneal dialysis fluid (PDF) exposure resulted in the loss of mesothelial cell monolayer integrity and altered morphology of mesothelial cells as well as in a strong accumulation of α-SMA–positive fibroblastic cells in the submesothelial space (middle panel). Some transdifferentiated cytokeratin- and α-SMA–positive mesothelial cells have been detected in this group (arrows). Treatment with Go6976 completely abrogates this effect of PDF. Representative images of the saline-instilled, PDF-instilled, and PDF/Go–instilled groups are shown (bar = 50 μm). (**b**) Immunofluorescence microscopy analysis of peritoneal biopsy sections stained with lectin (green) and CD31 (red) and counterstained with 4′,6-diamidino-2-phenylindole (blue) showed an increase in vasculature in PDF-instilled mice (middle panel) compared to saline-instilled controls (left panel) but not in mice instilled with PDF containing Go6976 (right panel). The representative pictures are shown for saline-instilled, PDF-instilled, and PDF/Go6976–instilled groups (bar = 50 μm). (**c**) Vascular endothelial growth factor (VEGF) levels were measured in effluents (peritoneal lavage, PL) obtained at the end point after 5 weeks of dialysis by ELISA. Chronic exposure to PDF resulted in local accumulation of VEGF. Treatment with Go6976 completely abrogated this effect. Data are presented as mean ± SEM. *$P < 0.05$ versus saline-instilled mice; $^{xx}P < 0.01$ versus PDF-instilled mice. α-SMA, α smooth muscle actin; ELISA, enzyme-linked immunosorbent assay; EMT, epithelial-to-mesenchymal transition; MPMC, mouse PMC; PD, peritoneal dialysis.

was accompanied by morphologic changes of remaining cells. Moreover, some transdifferentiated cytokeratin-positive MPMCs showed expression of α-SMA, and a strong accumulation of α-SMA–positive fibroblastic cells was observed in the submesothelial space. Adding of Go6976 to PDF preserved the normal MPMC morphology and prevented their transdifferentiating (Figure 4a), suggesting involvement of PKCα in peritoneal EMT.

To analyze the role of PKCα in PDF-induced neoangiogenesis, parietal peritoneum samples were double-stained for lectin and anti-CD31 antibody. Whereas only a few vessels could be seen between the muscles in saline-instilled mice, PDF exposure resulted in pronounced new vessel formation in parietal peritoneum. Treatment with Go6976 completely abrogated submesothelial neoangiogenesis and MPMC monolayer disturbances (Figure 4b). Since the role of vascular endothelial growth factor (VEGF) in neoangiogenesis during PD is well known,[20] we analyzed VEGF levels in effluents after 5 weeks of treatment. VEGF levels were significantly increased in samples from the PDF group compared to the saline group, but addition of Go6976 completely abolished this effect (Figure 4c).

We next investigated the possible role of PKCα in the PDF-induced local inflammation. The measurement of proinflammatory mediators IL-6, tumor necrosis factor-α, IL-17, and MCP-1 in the effluents revealed that the levels of all 4 cytokines were strongly increased in the PDF group compared to the saline group. In contrast, none of these mediators was increased if Go6976 was added to PDF (Figure 5a). We further analyzed inflammatory cell influx into the peritoneal cavity (Figure 5b). CD11b-positive myeloid cell numbers were significantly higher in PDF-instilled mice compared with saline-instilled mice. The majority of these cells were F4/80-positive macrophages. There also appeared to be an increase of Gr1-positive polymorphonuclear leukocyte influx in PDF-instilled mice, although this was not statistically significant. Additionally, conventional T cell counts were significantly increased in this group. Treatment with Go6976 significantly blocked PDF-induced leucocyte accumulation. We also detected a strong accumulation of CD45-positive leucocytes in the peritoneum of the PDF group (Figure 5c, upper row, left panel). The majority of these cells were macrophages (Figure 5c, upper row, middle panel) with a small population of neutrophils (Figure 5c, upper row, right panel). We could also detect CD4-positive and, to a lesser extent, CD8-positive T cells in the submesothelial zone (Figure 5c, lower row). This inflammatory cell infiltration was found neither in the saline group nor in mice treated with Go6976, suggesting the important role of PKCα in PD-induced inflammation.

PKCα deficiency ameliorates PDF-induced functional and structural PM alteration and abrogates TGF-β, VEGF, and MCP-1 upregulation

We next used PKCα$^{-/-}$ mice on a 129/Sv genetic background and corresponding WT 129/Sv mice in the same PD model. It is noteworthy that PD-induced changes were much less pronounced in this strain than in C57BL/6 mice used in experiments with Go6976. However, as demonstrated by ultrafiltration testing, chronic instillation of PDF induced alteration of membrane permeability in WT mice but not in PKCα$^{-/-}$ mice compared to saline-treated animals (Figure 6a). In line with the functional impairment, morphologic alterations of the peritoneum were observed in the PDF-instilled WT but not in PKCα$^{-/-}$ mice. PDF-induced PM thickening in WT mice was mainly due to extracellular matrix deposition (Figure 6b and c). Collagen I and III deposition representing fibrosis was observed only in the peritoneum of WT mice exposed to PDF but not to saline. The PDF-induced collagen deposition was completely prevented in PKCα$^{-/-}$ mice (Figure 7). These results confirm the important role of PKCα in the pathophysiology of PDF-induced functional and structural alterations of the peritoneum.

The measurements of profibrotic, proangiogenic, and proinflammatory mediators revealed that TGF-β1, VEGF, and MCP-1 effluent concentrations were significantly elevated in PDF-instilled WT mice compared to saline-instilled mice, whereas PKCα$^{-/-}$ mice were protected (Figure 8).

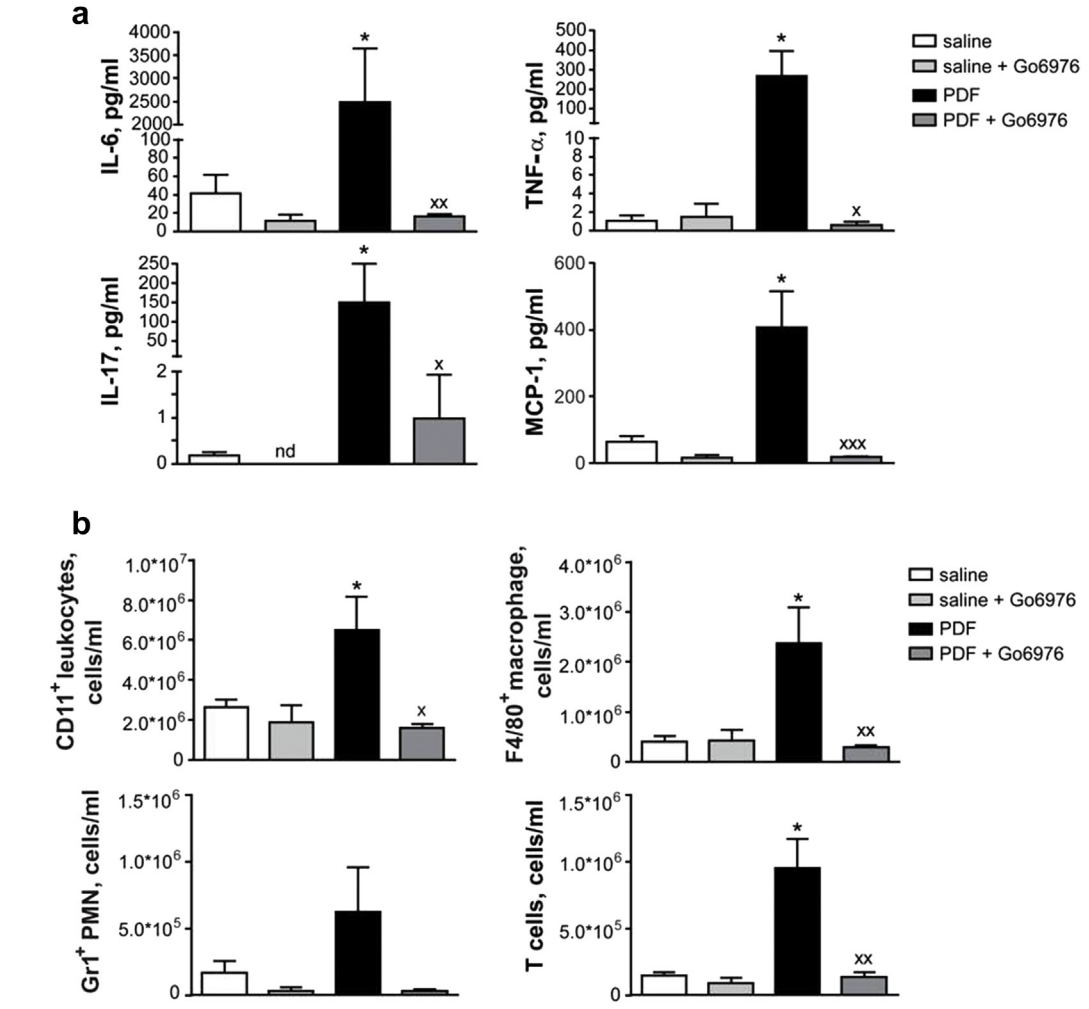
PKCα inhibition abrogates EMT and MCP-1 release in HPMCs *in vitro*

We further tested whether inhibition of PKCα would have a similar protective effect in human cells. Omentum-derived HPMCs showed basal levels of PKCα, and its phosphorylation was upregulated by incubation with 10 ng/ml human recombinant TGF-β1 for 48 hours. Preincubation of cells with Go6976 prevented the TGF-β1–induced activation of PKCα in a dose-dependent manner (Figure 9a). Incubation of HPMCs with PDF also resulted in activation of PKCα, which was abrogated by Go6976 pretreatment (Figure 9b). Moreover, TGF-β1/PDF–induced downregulation of E-cadherin and upregulation of α-SMA (Figure 9a and b) as well as TGF-β1–induced EMT-associated morphologic changes (Figure 9c) were ameliorated by preincubation with Go6976. Go6976 pretreatment also significantly decreased PDF-induced MCP-1 release from HPMCs (Figure 9d).

DISCUSSION

PD has been a successful modality for chronic renal replacement therapy for over 30 years. Nevertheless, glucose as the main osmotic agent in PDF and its degradation products induce a local diabetic environment within the peritoneal cavity that is associated with long-term mesothelial deterioration. Using a PD mouse model in PKCα$^{-/-}$ mice as well as an activity neutralization approach in WT mice, in this study we demonstrate the importance of PKCα in the PD-induced disturbances of PM. The underlying mechanisms involve PKCα-mediated development of PM fibrosis, neovascularization, EMT of peritoneal mesothelial cells (PMCs), and inflammation.

We demonstrate that PKCα is the only classical PKC isoform expressed in MPMCs. PKCα protein expression and activation is upregulated in MPMCs *in vitro* under PD-simulating conditions and *in vivo* after prolonged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exposure to PDF. Whereas in WT mice chronic PDF administration leads to severe morphologic, structural, and functional changes of the PM, the functional blockade of PKCα activity with Go6976 completely protected PM structure and function. PKCα deficiency showed a comparable benefit, suggesting a crucial role of PKCα in PM disturbances. It should be noted that PDF-induced alterations were much more pronounced in C57BL/6 mice compared to 129/Sv mice. Significant differences in mouse strain susceptibility to TGF-β1–induced peritoneal fibrosis were recently described.[21] The authors demonstrated the strongest responses, such as fibrosis, angiogenesis, and evidence of EMT, in C57BL/6 mice compared to other strains; however, 129/Sv mice were not included. Previously we have shown that C57BL/6 mice are much more sensitive to damage caused by renal ischemia–reperfusion injury than 129/Sv mice as reflected by increased fibrotic and inflammatory responses.[22] In line with this previous observation, the findings of our current study confirm significant differences between the C57BL/6 and 129/SV mouse strains in their susceptibility to profibrotic stimuli.

Fibrosis and TGF-β1 are key determinants of ultrafiltration dysfunction.[18,21,23] It has been described that high-glucose-induced activation of PKC in HPMCs results in upregulation of TGF-β1 and fibronectin synthesis;[24] however, the role of distinct PKC isoforms was not investigated. Previously we and others demonstrated a causative role of glucose-mediated PKCα activation in the development of diabetic nephropathy[12] and fibrosis.[9,25,26] PKCα has also been shown to mediate high-glucose-induced TGF-β1 and TGF-β receptor-1 expression in vascular smooth muscle cells.[27] Our data demonstrate that PKCα activation is crucial for the increased TGF-β1 production *in vivo* and *in vitro*. In line with these observations, PDF-induced fibrosis in WT mice was absent in PKCα$^{-/-}$ mice and could be successfully abrogated by local blockade of PKCα activity with Go6976. PKCα might be involved in fibrosis development during PD not only via PDF-induced increase of TGF-β1 level but also via modulation of its signaling pathways.[11] Moreover, PKCα might contribute directly to extracellular matrix production as was demonstrated for different cell types.[28–30]

Fibrogenic cells in PD comprise activated resident stromal fibroblasts, fibroblast-like cells derived from the mesothelium and endothelium by EMT or endothelial-to-mesenchymal transition, and inflammatory cells.[23,31,32] Besides of EMT of PMCs, which was observed *in vitro*[33] and *in vivo* for both human[34,35] and mouse,[31,36,37] type I collagen–producing submesothelial fibroblasts have been shown to be another important source of α-SMA–positive myofibroblasts progressively accumulating in the submesothelial space.[38] A crucial role of PKCα was demonstrated in EMT of renal tubular epithelial cells and pancreatic cancer cells[28,39] and in TGF-β1–induced transdifferentiating of resident adventitial fibroblasts to α-SMA–positive myofibroblasts.[40] PKCα is also expressed in endothelial cells,[41] and exposure of these cells to high glucose significantly increased PKCα activation[42] and results in increased TGF-β1 production,[43] which, in turn, plays an important role in regulating of endothelial-to-mesenchymal transition.[44] Since PKCα is ubiquitously expressed in different cell types including epithelial, endothelial, and inflammatory cells and resident fibroblasts, we hypothesized that PKCα blockade would result in reduced amount of activated myofibroblasts independently of their origin. Indeed, in our study submesothelial accumulation of α-SMA–positive myofibroblasts and transdifferentiated MPMCs was prevented by PKCα blockade, suggesting an important role of PKCα in generation of fibrogenic cells. The data showing that PKCα blockade successfully prevents proliferation of activated myofibroblasts in the submesothelial space[45] support this conclusion.

Peritoneal vascularization is an essential component of PM deterioration during PD.[15,23] VEGF is an important mediator of neoangiogenesis, and its upregulation is associated with PM dysfunction in PD patients.[46–48] Previously we have shown that PKCα mediates the hyperglycemia-induced expression of VEGF and Flk-1 in kidneys of diabetic mice.[12] Here we demonstrate that PKCα blockade or deficiency prevented the upregulation of VEGF and neoangiogenesis in PD model. PKCα promotes angiogenesis not only by induction of VEGF[41] but also mediates its signaling via an autocrine feedback loop in which PKCα enhances VEGF,

Figure 5 | Blockade of PKCα activity ameliorates PD-induced inflammation. (**a**) The levels of inflammatory mediators interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), IL-17, and monocyte chemoattractant protein 1 (MCP-1) were measured in effluents obtained on the last day of experiments by bead-based flow cytometry assay. Chronic exposure to peritoneal dialysis fluid (PDF) resulted in local accumulation of all 4 mediators in PDF-instilled mice. Treatment with Go6976 completely abrogated this effect. Data are presented as mean ± SEM. $^{*}P < 0.05$ versus saline-instilled mice; $^{X}P < 0.05$, $^{XX}P < 0.01$, and $^{XXX}P < 0.01$ versus PDF-instilled mice. (**b**) PD-induced inflammatory cell influx into peritoneal cavity was evaluated by FACS method in effluents obtained on the last day of experiments. A strong accumulation of CD11b-positive leukocytes observed in peritoneal cavity of PDF-instilled mice compared with saline-instilled control mice was due to an increase in F4/80-positive macrophages. Also, conventional T-cell counts in the effluents were significantly increased in this group compared to saline-instilled controls. An increase of Gr1-positive PMN influx was not statistically significant compared with saline-instilled controls. No differences could be detected for total CD11b-positive leukocyte, macrophage, and T cell counts between saline-instilled controls and saline- or PDF-instilled mice treated with Go6976. Data are presented as mean ± SEM. $^{*}P < 0.05$ versus saline-instilled mice; $^{X}P < 0.05$ and $^{XX}P < 0.01$ versus PDF-instilled mice. (**c**) Immunofluorescence microscopy analysis of parietal biopsy sections obtained from PDF-instilled mice was performed for CD45-positive leukocytes, F4/80-positive macrophages, Gr1-positive PMNs, and CD4- and CD8-positive T cells. The representative pictures are shown for the PDF-instilled group (bar = 100 μm). FACS, fluorescence-activated cell sorting; nd, not detectable; PD, peritoneal dialysis; PKCα, protein kinase Cα, PMN, polymorphonuclear leukocyte.

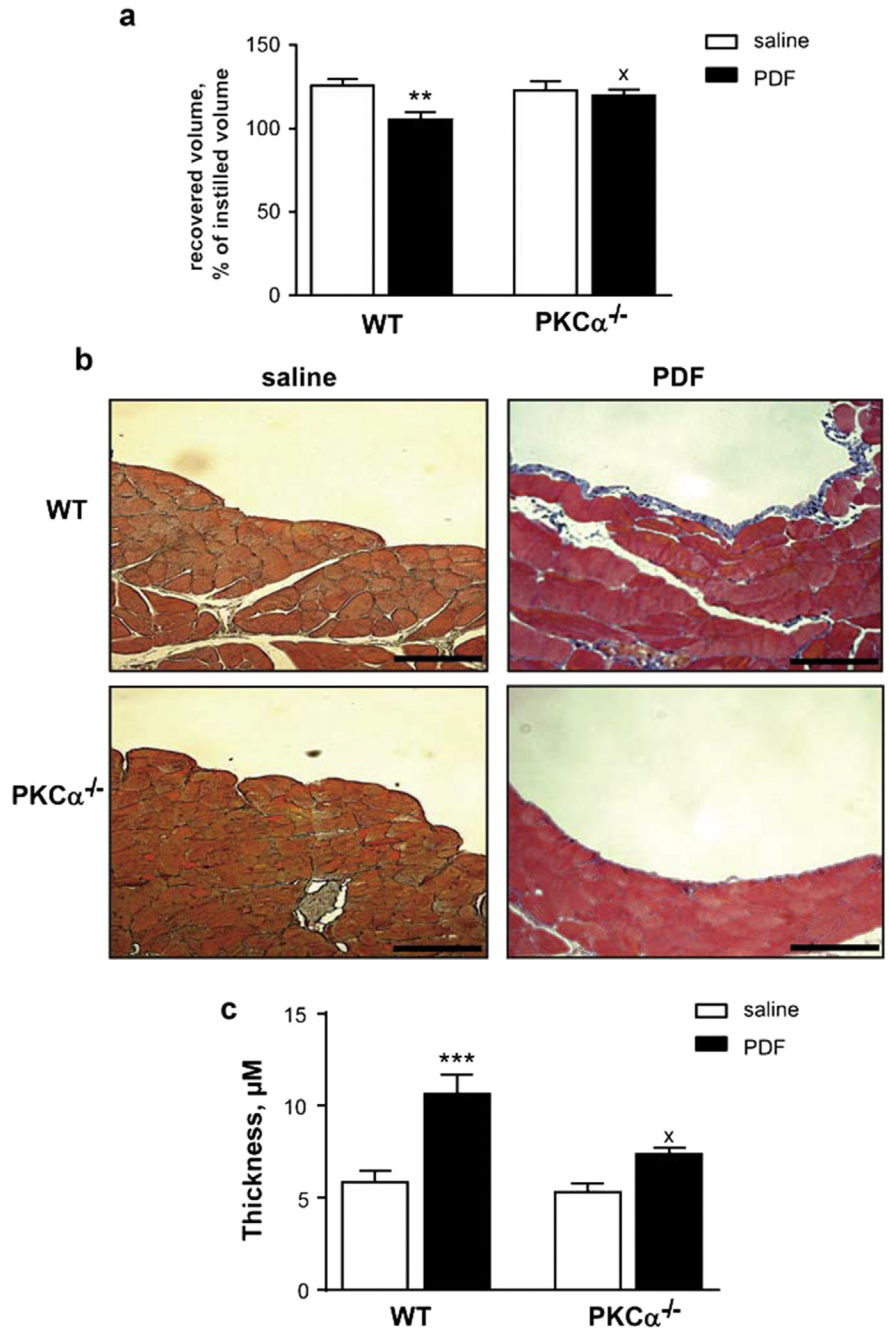

Figure 6 | PKCα deficiency ameliorates functional and structural changes of PM during PD. (**a**) The functional analysis of the PM was performed in 129/Sv wild-type (WT) and PKCα$^{-/-}$ mice by ultrafiltration test after 5 weeks of dialysis. The volume recovery from peritoneal dialysis fluid (PDF)–instilled WT 129/Sv mice was less than from WT mice instilled with saline, and a recovery of net ultrafiltration has been observed in PKCα$^{-/-}$ mice exposed to PDF. Data are expressed as mean ± SEM (n = 8–11 mice per group). $^{**}P < 0.01$ versus corresponding saline-instilled mice; $^{X}P < 0.05$ versus PDF-instilled WT mice. (**b**) Masson's trichrome staining of peritoneal biopsies demonstrated that PDF exposure results in enhanced thickness of the PM membrane in WT mice, while PKCα deficiency ameliorated these changes almost completely. The representative pictures are shown for each group (n = 8–11 mice per group; bar = 50 µm). (**c**) The results of the quantitative analysis of PM thickness are presented as mean ± SEM (n = 8–11 mice per group). $^{***}P < 0.001$ versus corresponding saline-instilled mice; $^{X}P < 0.05$ versus PDF-instilled WT mice. PD, peritoneal dialysis; PM, peritoneal membrane; PKCα, protein kinase Cα.

and VEGF in turn stimulates PKCα activity,[41] leading to PKCα-mediated activation of a cellular transcription factor, cAMP response element–binding protein (CREB), and secondary expression of the proangiogenic enzyme cyclooxygenase-2.[49] These mechanisms might be responsible for the observed beneficial effect of PKCα blockade on the peritoneal vasculature.

Low-grade PDF-induced chronic inflammation contributes significantly to progressive structural alterations of PM.[23,50] Under high-glucose conditions, inflammatory

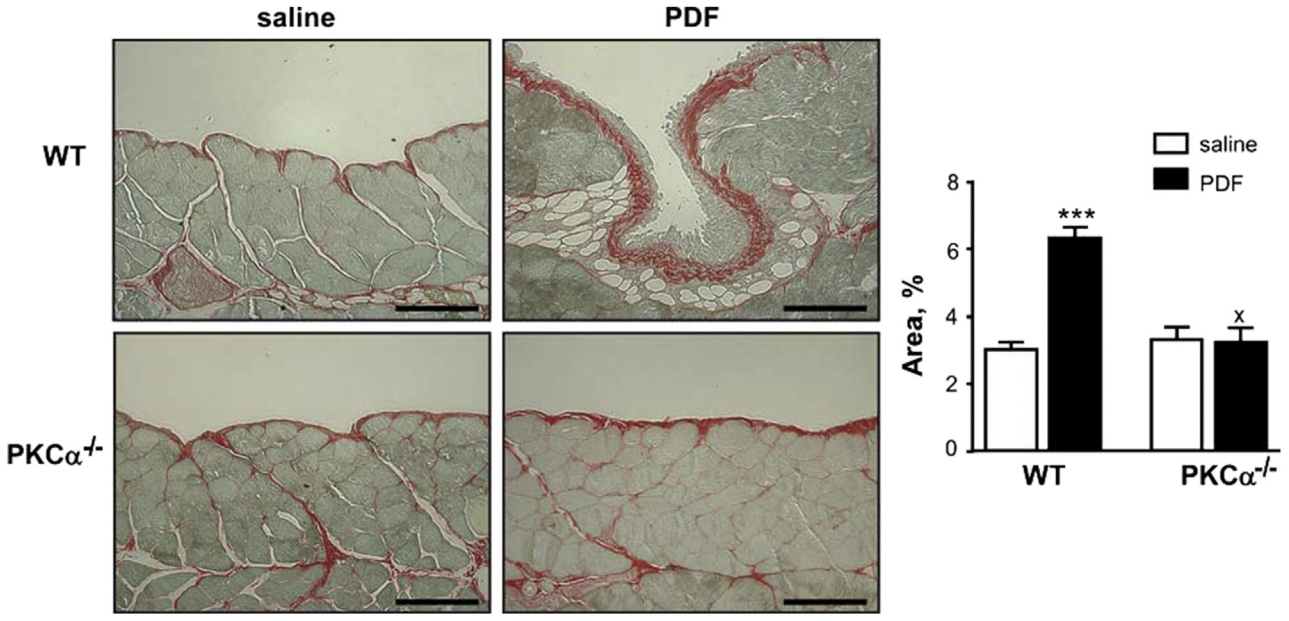

Figure 7 | PKCα deficiency ameliorates collagen I and III accumulation during PD. PD-induced fibrosis was evaluated in peritoneal samples using picrosirius red staining specific for collagen I and III. Wild-type (WT) 129/Sv mice exposed to peritoneal dialysis fluid (PDF) for 5 weeks showed an increased collagen I and III deposition compared to saline-instilled WT mice, which was not observed in the PKCα$^{-/-}$ mice exposed to PDF. The representative pictures are shown for each group (n = 8–11 mice per group, bar = 50 μm). Quantification of picrosirius red staining was performed using ImageJ software (right panel). Data are presented as mean ± SEM. ***$P < 0.001$ versus corresponding saline-instilled mice; $^{x}P < 0.05$ versus PDF-instilled WT mice.

conditions, or both, PMCs produce proinflammatory mediators such as IL-6, MCP-1, IL-17, and tumor necrosis factor-α,[16,51–54] which are important for peritoneal recruitment of inflammatory cells and fibrosis progression.[51] Since total PKC activity has been shown to be involved in this response,[16] we investigated whether PKCα is the PKC isoform responsible for proinflammatory mediator upregulation and for inflammatory cell influx. Our data suggest that this is indeed the case as reflected by drastically reduced levels of inflammatory mediators and decreased inflammatory cell influx in states of PKCα inhibition or deficiency. Further *in vitro* studies confirmed that PKCα inhibition or deficiency prevents PDF-induced MCP-1 release from MPMCs. The underlying molecular mechanisms may include both direct involvement of PKCα in nuclear factor-κB signaling pathway leading to tumor necrosis factor-α[55] or MCP-1 release[16] and its indirect action via upregulation of some other upstream mediators. For example, TGF-β1–induced IL-17A synthesis is critically dependent on PKCα directly regulating the kinase activity of TGFβRI, which itself activates SMAD2-3, and maintains effective IL-17A responses *in vitro* and *in vivo*.[56] This locally produced IL-17A could, in turn, activate IL-17 receptor–expressing PMCs[57] and fibroblasts to release MCP-1, which is a downstream target of IL-17.[53]

Our study has some limitations. Although we clearly demonstrate the pivotal role of the classical PKCα isoform in PD-induced PM injury and inflammation, we cannot rule out that in addition other PKC isoforms play a role. In preliminary experiments we found PDF-induced upregulation of a novel PKC (PKCδ) and downregulation of an atypical PKC (PKCζ) isoform in MPMCs and in peritoneum of PDF-instilled WT mice. The possible role of these changes in PM injury during PD remains to be investigated.

Involvement of different PKC isoforms has been shown to participate in various pathological processes, and specific PKC inhibitors could exert a wide range of therapeutically beneficial pharmacological activities. However, the pharmacological inhibition of different PKC isoforms has significant limitations. Practically all known PKC inhibitors are either pan-PKC inhibitors or of limited specificity due to the high homology of PKC isoforms.[8] Moreover, many of these inhibitors, that were initially thought to be highly specific, were subsequently found to affect multiple kinases from a variety of protein kinase families and usually have other pharmacological actions leading to different effects *in vitro* and *in vivo* independently from PKC inhibition.[7] Up to now, generating inhibitors specific for one PKC isoform remains a challenge. New approaches such as designing peptide inhibitors specific for protein–protein interaction between different PKC isoforms and their downstream targets or agents modulating adenosine triphosphate–independent activation of PKC by posttranslational modification may be helpful.[58]

Taken together, these data indicate that PKCα contributes significantly to PDF-induced peritoneal damage and suggest that PKCα may be a novel therapeutic target to preserve the PM integrity in PD patients.

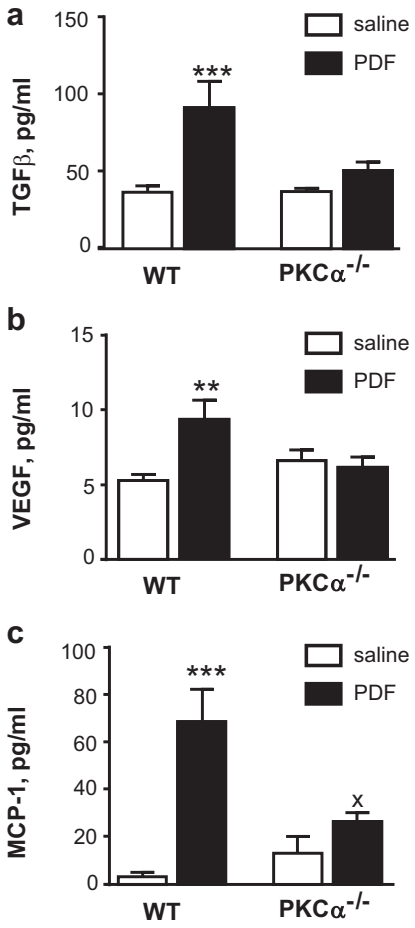

Figure 8 | PKCα deficiency reduces TGF-β1, VEGF, and MCP-1 upregulation in a mouse model of PDF exposure. The levels of transforming growth factor-β1 (TGF-β1) (**a**), vascular endothelial growth factor (VEGF) (**b**), and monocyte chemoattractant protein 1 (MCP-1) (**c**) were measured in effluents obtained on the last day of experiments by ELISA or by bead-based flow cytometry assay for TGF-β1, VEGF, and MCP-1, respectively. Chronic exposure to peritoneal dialysis fluid (PDF) resulted in local accumulation of all 3 mediators in PDF-instilled wild-type (WT) mice. PKCα deficiency completely abrogated this effect. Data are presented as mean ± SEM. $^{**}P < 0.01$, $^{***}P < 0.001$ versus corresponding saline-instilled controls; $^{x}P < 0.05$ versus PDF-instilled WT mice. ELISA, enzyme-linked immunosorbent assay; PKCα, protein kinase Cα.

MATERIALS AND METHODS

PDF exposure model in mice

The animal protection committee of the local authorities (Lower Saxony state department for food safety and animal welfare [LAVES]) approved all experiments (approval: 33.9-42502-04-12/0847). 1.5 ml of standard PDF composed of 4.25% glucose and buffered with lactate (CAPD/DPCA3, Stay Safe, Fresenius, Bad Homburg, Germany) or saline solution was instilled daily via a peritoneal catheter connected to an implanted subcutaneous mini-access port (Access Technologies, Skokie, IL) for 5 weeks as described.[17] Pharmacological studies with a cell-permeable, reversible, and adenosine triphosphate–competitive PKC inhibitor, Go6976 (Calbiochem, Darmstadt, Germany), were performed in 10- to 12-week-old female C57BL/6 mice (Charles River Laboratories, Sulzfeld, Germany). Another set of experiments was performed with female 129/Sv WT and PKCα-deficient ($PKC\alpha^{-/-}$) mice on 129/Sv background generated as previously described.[59] For details, see the Supplementary Methods online. On the last day of experimentation the functional analysis of PM was performed by ultrafiltration test as described in the Supplementary Methods. The recovered peritoneal fluids were used for the fluorescence-activated cell sorting (FACS) analysis of the peritoneal influx of inflammatory cells and for the measurements of inflammatory mediators. Thereafter, tissue samples were collected from the anterior abdominal wall for histologic analysis.

Flow cytometry and enzyme-linked immunosorbent assay measurements in effluents

The inflammatory cell populations in the effluents were analyzed by flow cytometry using a FACS Canto II cytometer (BD Biosciences, Heidelberg, Germany) as described in the Supplementary Methods.

The levels of the proinflammatory cytokines tumor necrosis factor-α, IL-6, IL-17, and MCP-1 were quantified in effluents by bead-based flow cytometry assay (CBA Kit, BD Biosciences, Heidelberg, Germany) according to the manufacturer's instructions. The concentrations of TGF-β1 and VEGF were measured with the specific enzyme-linked immunosorbent assay kits (R&D Systems, Wiesbaden, Germany) according to the manufacturer's instructions.

Morphologic analysis of peritoneal samples

The thickness of the submesothelial layer was determined on 5-μm paraffin-embedded tissue sections stained with Masson's trichrome stain (Sigma-Aldrich, Munich, Germany) by blinded microscope analysis (Leica DM IL with a Leica DC 300F, Leica Microsystems, Wetzlar, Germany) using Leica IM500 software and expressed as the mean of 40 independent measurements for each animal. To quantify fibrosis, tissue sections were stained with picrosirius red stain (Sigma-Aldrich). Images were analyzed by computerized digital image analysis (ImageJ software, National Institute of Health, Bethesda, MD, USA). The positive area for collagens I and III was calculated as a percentage of the total area of the tissue.

Immunofluorescence analysis of peritoneal samples

2.5-μm paraffin-embedded tissue sections were stained for p-PKCα, total PKCα, α-SMA, pan-cytokeratin, endothelial cell marker CD31, macrophages, T4 and T8 cells, and granulocytes as described in the Supplementary Methods.

Isolation of mouse PMCs and establishment of conditionally immortalized mouse PMC cell lines

For generation of immortalized mouse PMC (MPMC) lines, $PKC\alpha^{-/-}$ mice were crossed to Immorto mice (C57BL/6 background, Animal Facility Medical School, Hanover, Germany) harboring the tsSV40T gene. PMCs were isolated using a standard trypsin and ethylenediamine tetraacetic acid digestion method from omentum tissue. The cells were propagated in Roswell Park Memorial Institute 1640 cell culture medium containing 1% penicillin–streptomycin, 10% fetal calf serum, 1% insulin/transferrin/selenium A (all from Life Technologies, Carlsbad, CA), 0.4 μg/ml hydrocortisone (Sigma-Aldrich), and 10 U/ml recombinant mouse interferon gamma (Cell Sciences, Canton, MA) at 33 °C (permissive conditions). Six and five monoclonal cell lines were generated by limited dilution cultures of primary cells for WT and $PKC\alpha^{-/-}$ genotype, respectively. These cell lines were identified as MPMC by its typical cobblestone morphology of confluent monolayers and by positive staining for

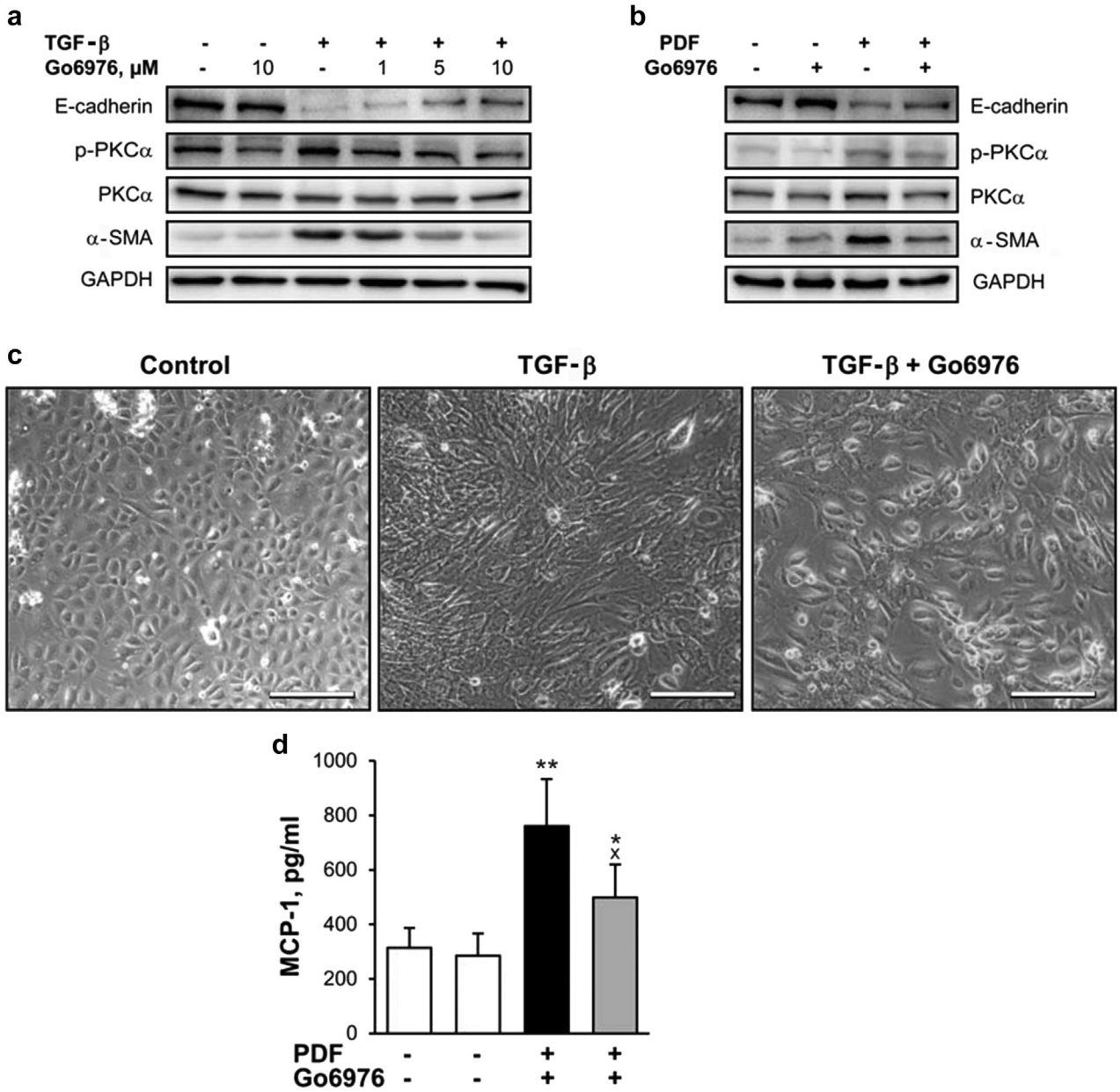

Figure 9 | Go6976 treatment affects the TGF-β1/PDF–induced EMT of HPMCs *in vitro* and reduces MCP-1 release after incubation with PDF. Omentum-derived HPMCs (passage 1–2) were preincubated or not with Go6976 for 1 hour and treated or not with 10 ng/ml of transforming growth factor-β1 (TGF-β1) or with 50% conventional peritoneal dialysis fluid (PDF) in the presence of Go6976 for 48 hours. Expression of p-PKCα (phospho–protein kinase C α), total PKCα, and EMT markers E-cadherin and α-smooth muscle actin (α-SMA) upon stimulation was assessed by Western blotting with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a loading control. (**a**) HPMCs express the basal level of PKCα, which was not affected by TGF-β1. However, TGF-β1 induced activation of PKCα, as demonstrated by increased p-PKCα level, and this activation can be successfully blocked by Go6976 in a concentration-dependent manner. Go6976 pretreatment also prevented TGF-β1–induced E-cadherin downregulation and α-SMA upregulation. (**b**) PKCα was activated by PDF on both phosphorylated and total protein levels. Preincubation with 10 μM Go6976 reduced PKCα activation as well as PDF-induced E-cadherin downregulation and α-SMA upregulation. (**c**) Phase-contrast microscopy demonstrated that Go6976 pretreatment (10 μM) prevented non-epithelioid phenotype acquisition of mesothelial cells. Bar = 100 μm. The results presented in **a**–**c** are representative of at least 4 independent experiments with similar results performed with different donors. (**d**) PDF-induced monocyte chemoattractant protein-1 (MCP-1) release was analyzed in conditioned medium by specific ELISA. Quiescent HPMCs were preincubated or not for 1 hour with 10 μM Go6976 and thereafter stimulated for 4 hours with 50% conventional PDF containing or not containing 10 μM Go6976. The control cells were incubated with medium diluted 1:2 with sterile saline. PDF-induced release of MCP-1 from HPMCs was not completely abrogated but was significantly reduced by Go6976 treatment. Data are presented as mean ± SEM of 4 independent experiments performed with HPMCs from different donors. $^{*}P < 0.05$ versus unstimulated cells; $^{**}P < 0.001$; $^{X}P < 0.05$ versus cells treated with PDF without Go6976. ELISA, enzyme-linked immunosorbent assay; EMT, epithelial-to-mesenchymal transition; HPMC, human peritoneal mesothelial cells.

MPMC markers E-cadherin, ZO-1, α-SMA, and pan-cytokeratin after 3-day culture at 37 °C without interferon gamma (non-permissive conditions). Every experimental setup and result was confirmed in 3 different clones of WT and PKCα$^{-/-}$ MPMCs.

Stimulation of conditionally immortalized MPMC cell lines with TGF-β1 and IL-1β and with PDF

MPMCs were grown on 60-mm plates until 80% confluence. Thereafter the cells were starved overnight in 1% fetal calf serum–containing medium and then stimulated for 48 hours with a combination of 10 ng/ml human recombinant TGF-β1 (R&D Systems) and 2 ng/ml mouse recombinant IL-1β (Sigma-Aldrich).[60] PKCα activity was inhibited by preincubation of cells for 1 hour with different concentrations of Go6976, and then the cells were stimulated with TGF-β1 and IL-1β in the presence of Go6976 for 48 hours. MPMCs incubated in medium without stimuli served as a control. After incubation the cell lysates were analyzed by Western blotting or mRNA was isolated and used for quantitative polymerase chain reaction (for details, see the Supplementary Methods).

In other experiments quiescent MPMCs were stimulated for 24 hours with cell culture medium containing different concentrations of glucose or with conventional PDF (CAPD/DPCA3, Stay Safe, 4.25% glucose, Fresenius), with icodextrin-containing PDF (Extraneal, Baxter, Deerfield, IL), or with locally produced sterile filtered PDFs identical to commercially available biocompatible PDF (Physioneal 40, Baxter) as described in the Supplementary Methods. After incubation, the conditioned media were used for the MCP-1 bead-based flow cytometry assay.

Human PMC culture

Human PMCs (HPMCs) were isolated using a standard trypsin and ethylenediamine tetraacetic acid digestion method from omentum tissue obtained from patients undergoing elective abdominal surgery. Informed consent was obtained for the use of omentum tissue, and the study was approved by the institutional ethics committee. The cells were grown in M199 medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 mg/ml streptomycin. For inhibition of PKCα activity the quiescent cells were preincubated for 1 hour with different concentrations of Go6976 and then stimulated with 10 ng/ml human recombinant TGF-β1 or with conventional PDF diluted one-half with culture medium in the presence of Go6976 for 48 hours. HPMCs incubated in medium without stimuli or with medium diluted 1:2 with sterile saline served as a control for TGF-β1–stimulated and PDF-stimulated cells, respectively. After incubation the cell lysates were analyzed by Western blotting. The level of MCP-1 was measured in the conditioned media after 4 hours of stimulation with PDF using specific MCP-1 enzyme-linked immunosorbent assay (R&D Systems).

Statistical analysis

Data are presented as means + SEM. Multiple comparisons were analyzed for significant differences using the Kruskal–Wallis nonparametric test with the Dunn as a *post hoc* test or by 1-way analysis of variance with the Tukey as a *post hoc* test for multiple comparisons. Significance was accepted at $P < 0.05$. GraphPad Prism version 6.0 (GraphPad Prism Software Inc., San Diego, CA) was used for data analysis.

DISCLOSURE

All the authors declared no competing interests.

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SUPPLEMENTARY MATERIAL

Supplementary Methods

Supplementary material is linked to the online version of the paper at www.kidney-international.org.

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