

Overproduction of phosphoprotein enriched in diabetes (PED) induces mesangial expansion and upregulates protein kinase C- β activity and *TGF- β 1* expression

F. Oriente · S. Iovino · A. Cassese · C. Romano · C. Miele · G. Troncone · M. Balletta · A. Perfetti · G. Santulli · G. Iaccarino · R. Valentino · F. Beguinot · P. Formisano

Received: 18 May 2009 / Accepted: 5 August 2009 / Published online: 30 September 2009
© Springer-Verlag 2009

Abstract

Aims/hypothesis Overproduction of phosphoprotein enriched in diabetes (PED, also known as phosphoprotein enriched in astrocytes-15 [PEA-15]) is a common feature of type 2 diabetes and impairs insulin action in cultured cells and in mice. Nevertheless, the potential role of PED in diabetic complications is still unknown.

F. Oriente and S. Iovino contributed equally to this study.

Electronic supplementary material The online version of this article (doi:10.1007/s00125-009-1528-z) contains supplementary material, which is available to authorised users.

F. Oriente · S. Iovino · C. Romano · A. Perfetti · F. Beguinot · P. Formisano (✉)
Department of Cellular and Molecular Biology and Pathology,
Federico II University of Naples,
Via Pansini 5,
80131 Naples, Italy
e-mail: fpietro@unina.it

A. Cassese · C. Miele · R. Valentino · F. Beguinot · P. Formisano
Istituto di Endocrinologia ed Oncologia Sperimentale del C.N.R.,
Federico II University of Naples,
Naples, Italy

G. Troncone
Department of Biomorphological and Functional Sciences,
Federico II University of Naples,
Naples, Italy

M. Balletta
Department of Nephrology, Federico II University of Naples,
Naples, Italy

G. Santulli · G. Iaccarino
Department of Clinical Medicine,
Cardiovascular and Immunological Sciences,
Federico II University of Naples,
Naples, Italy

Methods We studied the effect of PED overproduction and depletion on kidney function in animal and cellular models. **Results** Transgenic mice overexpressing *PED* (PEDTg) featured age-dependent increases of plasma creatinine levels and urinary volume, accompanied by expansion of the mesangial area, compared with wild-type littermates. Serum and kidney levels of TGF- β 1 were also higher in 6- and 9-month-old PEDTg. Overexpression of *PED* in human kidney 2 cells significantly increased TGF- β 1 levels, SMAD family members (SMAD)2/3 phosphorylation and fibronectin production. Opposite results were obtained following genetic silencing of *PED* in human kidney 2 cells by antisense oligonucleotides. Inhibition of phospholipase D and protein kinase C- β by 2-butanol and LY373196 respectively reduced TGF- β 1, SMAD2/3 phosphorylation and fibronectin production. Moreover, inhibition of TGF- β 1 receptor activity and SMAD2/3 production by SB431542 and antisense oligonucleotides respectively reduced fibronectin secretion by about 50%. TGF- β 1 circulating levels were significantly reduced in *Ped* knock-out mice and positively correlated with PED content in peripheral blood leucocytes of type 2 diabetic patients. **Conclusions/interpretation** These data indicate that PED regulates fibronectin production via phospholipase D/protein kinase C- β and TGF- β 1/SMAD pathways in kidney cells. Raised PED levels may therefore contribute to the abnormal accumulation of extracellular matrix and renal dysfunction in diabetes.

Keywords Diabetic nephropathy · PEA-15 · PED · PKC · TGF- β 1

Abbreviations

BIM Bisindolylmaleimide
ECL Enhanced chemiluminescence
ECM Extracellular matrix

HK2 cells	Human kidney 2 cells
PBL	Peripheral blood leucocyte
PED	Phosphoprotein enriched in diabetes/ phosphoprotein enriched in astrocytes-15
<i>Ped</i> -KO	<i>Ped/Pea-15</i> knockout mice
PEDTg	Transgenic mice overexpressing <i>PED</i>
PKC	Protein kinase C
PLD	Phospholipase D
SMAD	SMAD family member

Introduction

Diabetic nephropathy is a frequent complication of type 1 and type 2 diabetes mellitus and is currently considered the leading cause of end-stage renal disease [1]. While thickening of the glomerular basement membrane, glomerular hypertrophy and mesangial expansion are well known features of diabetic nephropathy, the pathogenesis of these alterations is not very clear yet. The DCCT, UK Prospective Diabetes Study (UKPDS) and Action in Diabetes and Vascular Disease: Preterax and Diamicon MR Controlled Evaluation (ADVANCE) studies have shown the role of hyperglycaemia as a causative factor in the development and the progression of diabetic nephropathy [2–5]. Nevertheless, hyperglycaemia alone is clearly not sufficient to account for the heterogeneity and variability of the clinical appearance of the disorder. Indeed, accumulating evidence points to critical genetic factors predisposing only a subset of patients with diabetes to nephropathy [6–8].

Several studies have shown an important involvement of growth factors and cytokines [9, 10]. In particular, the TGF- β 1 is a key factor in experimental models of diabetic kidney disease as well as in patients with diabetic nephropathy [11–15]. In fact, TGF- β 1 levels are increased in diabetic patients and high glucose levels upregulate expression and bioactivity of TGF- β 1 in almost all renal cell types [11, 12, 16, 17]. Upon binding to its receptor, TGF- β 1 phosphorylates the receptor-regulated SMAD family members (SMAD)2 and 3, which form oligomeric complexes with the common SMAD (SMAD 4). These complexes then translocate into the nucleus, thereby regulating transcription of target genes, including those encoding type I and type IV collagen, laminin and fibronectin [14, 16, 18, 19].

Phosphoprotein enriched in diabetes (PED, also known as phosphoprotein enriched in astrocytes-15 [PEA-15]) is a scaffold cytosolic protein originally identified as a major astrocyte phosphoprotein and found to be widely present in different tissues [20, 21]. PED plays an important role in mitogenic and metabolic signalling [21–23]. Gene profiling studies have shown that *PED* (also known as *PEA15*) is

commonly overexpressed in individuals with type 2 diabetes [21, 24]. In cultured muscle and adipose cells and in peripheral tissues from transgenic mice, forced production of PED impairs insulin-stimulated GLUT4 translocation and glucose transport, suggesting that PED may contribute to insulin resistance in type 2 diabetes [25]. Further studies have shown that PED stabilises phospholipase D (PLD) [26] and induces activation of classical protein kinase C (PKC) isoforms, including PKC α and PKC β [23, 27]. The induction of classical PKCs, in turn, inhibits insulin signalling, at least in skeletal muscle, adipose and liver cells [28–31]. Nevertheless, the role of PED in diabetic complications is not known.

In the present work we addressed the question of whether PED overproduction determines abnormalities in kidney function and whether it may represent an initial defect in the progression toward diabetic nephropathy.

Methods

Materials Media, sera, antibiotics for cell culture and the lipofectamine reagent were from Invitrogen (Grand Island, NY, USA). The anti-PED polyclonal rabbit antibody and pcDNA3PED vector have been previously described [21]. pSMAD2/3 (Ser433/435), SMAD2, PKC α , PKC β , fibronectin, laminin and collagen I and IV antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). SMAD3 was purchased from Calbiochem (San Diego, CA, USA). Phosphorothioate oligonucleotides antisense sequences used were as follows: *SMAD2* AS: 5'-GCAC GATGGACGACAT-3'; *SMAD2* S: 5'-CAATGCGAGTACG CGA-3'; *SMAD3* AS: 5'-GCAGGATGGACGACAT-3'; *SMAD3* S: 5'-GGAGTCAGACTGACGA-3'; *PED* antisense oligonucleotides were as previously described [25]. Protein electrophoresis reagents were purchased from Bio-Rad (Richmond, VA, USA), and western blotting and enhanced chemiluminescence (ECL) reagents from Amersham Biosciences (Arlington Heights, IL, USA). All other chemicals were from Sigma Aldrich (St Louis, MO, USA).

Mouse phenotyping Generation of transgenic mice overexpressing *PED* (PEDTg) [25] and of *Ped/Pea-15* knockout mice (*Ped*-KO) bearing ubiquitous ablation of the gene [32] has been previously described. Homozygous *Ped*-KO were used for the study. Both PEDTg and *Ped*-KO mice were fertile. Body weight of PEDTg was comparable to that of wild-type littermates, while, as previously described [32], *Ped*-KO displayed a slightly lower body weight. All procedures described below were approved by the Institutional Animal Care and Utilisation Committee. Animals were kept in a 12-h dark–light cycle and had free access to standard diet. Mice chosen for experimentation were

randomly selected from each box of mice housed in groups of three to four. Mice were habituated in individual metabolism cages (Lenderking Caging Products, Millersville, MD, USA) for 24 h. Then, the following variables were analysed: food intake (g/24 h), water intake (ml/24 h), urine volume excretion (ml/24 h), urine specific gravity, urine pH, urine glucose (mg/24 h) and albumin excretion ($\mu\text{g}/24\text{ h}$). The measurements of blood pressure and heart rate were performed partially modifying previously described methods [33]. Briefly, a 1.0-Fr polyimide pressure catheter (SPR 1000/2; Millar Instruments, Houston, TX, USA) was inserted into the left carotid artery and advanced into the ascending aorta of anaesthetised mice (2% isoflurane, 98% oxygen). Blood pressure and heart rate were recorded for 15 min after suspending isoflurane administration with an 8 channel recorder (Gould Instruments Systems, Cleveland, OH, USA). Data were analysed using Powerlab and Chart 5 software (AD Instruments, Sydney, NSW, Australia).

Renal histology and morphometric analysis Kidney sections were fixed by immersion in Carnoy solution followed by 4% buffered formaldehyde (vol./vol.) and embedded in paraffin. The fixed, embedded kidneys were cut into 2 μm sections and stained with periodic acid–Schiff's reagent. To quantify mesangial expansion, sections were coded and examined by light microscopy by two observers unaware of the experimental protocol applied. According to previous reports [34, 35], measurement of the mesangial area of 30 glomeruli randomly selected in each mouse by scanning of the outer cortex was performed with a computer-aided manipulator (KS-400; Carl Zeiss Vision, Munich, Germany)

Measurement of serum TGF- β 1 and urine albumin Blood samples of mice were collected from the orbital sinus under anaesthesia. After centrifugation ($800\times g$) of the blood samples, TGF- β 1 levels in the supernatant fractions were measured using ELISA kits (R&D System, Minneapolis, MN, USA). The same kits were used to measure TGF- β 1 levels in the media of the cells. Samples of urine collected through metabolism cages (five samples for each animal; ten animals/group) were briefly centrifuged at $500\times g$ and then albumin concentration was determined using ELISA kits (Bethyl Laboratories, Montgomery, TX, USA).

Tissue collection and primary mouse tubular kidney cell cultures Tissue samples (kidney) were collected rapidly after mice were killed by pentobarbitone overdose. Tissues were snap-frozen in liquid nitrogen and stored at -80°C for subsequent western blot analysis. Mouse tubular epithelial cells were isolated as previously described [36]. Cells were grown until confluent (8 to 12 days) in RPMI 1640 medium supplemented with 20% fetal calf serum, 2% (wt/vol.) L-

glutamine, 20,000 units/ml penicillin, 20,000 $\mu\text{g}/\text{ml}$ streptomycin at 37°C in 5% CO_2 .

Cell culture procedures and transfection Human kidney 2 (HK2) proximal tubular cells were cultured in RPMI 1640 medium (Invitrogen), containing 11.2 mmol/l glucose supplemented with 10% fetal calf serum, 2% (wt/vol.) L-glutamine, 10,000 units/ml penicillin, 10,000 $\mu\text{g}/\text{ml}$ streptomycin at 37°C in 5% (vol./vol.) CO_2 . Stable transfection of *PED* cDNA and transient transfection of antisense oligonucleotides [37] were performed by the lipofectamine method according to the manufacturer's instructions (Invitrogen). For these studies, 60 to 80% confluent cells were washed twice with Optimem (Invitrogen) and incubated for 8 h with 5 μg of plasmid construct or antisense oligonucleotides and 45 to 60 μl of lipofectamine reagent. The medium was then replaced with DMEM with 10% (vol./vol.) fetal calf serum and cells further incubated for 15 h before being assayed. Transfection efficiency for antisense oligonucleotides was estimated as $45\pm 10\%$ by co-transfection with green fluorescent protein.

Tissue and cell lysates and immunoblotting Tissue samples were homogenised in a Polytron (Brinkman Instruments, Westbury, NY, USA) in 20 ml T-PER reagent (Pierce, Rockford, IL, USA) per gram of tissue according to manufacturer's instructions. After centrifugation at $5000\times g$ for 5 min, supernatant fraction was collected. Cells were solubilised in lysis buffer (50 mmol/l HEPES, pH 7.5, 150 mmol/l NaCl, 10 mmol/l EDTA, 10 mmol/l $\text{Na}_4\text{P}_2\text{O}_7$, 2 mmol/l Na_3VO_4 , 100 mmol/l NaF, 10% (vol./vol.) glycerol, 1% (vol./vol.) Triton X-100, 1 mmol/l PMSF, 10 mg/ml aprotinin) for 1 h at 4°C and lysates were centrifuged at $5,000\times g$ for 20 min. Total homogenates were separated by SDS-PAGE and transferred on to 0.45 μm Immobilon-P membranes as previously described [38]. Upon incubation with primary and secondary antibodies, immunoreactive bands were detected by ECL according to the manufacturer's instructions.

For peripheral blood leucocyte (PBL) separation, EDTA-treated whole-blood samples were first centrifuged for 10 min at $300\times g$ and the plasma removed. PBLs were separated using a 6% (vol./vol.) dextran gradient in filtered PBS, pH 7.4, as previously described [24], washed three times in PBS, counted and resuspended in 1 ml of PBS for subsequent use.

Real-time RT-PCR analysis Total cellular RNA was isolated from whole kidneys of wild-type and PEDTg mice using a kit (RNeasy; Qiagen, Hilden, Germany) according to manufacturer's instructions. Tissue or cell RNA (1 μg) was reverse-transcribed using Superscript II Reverse Transcriptase (Invitrogen). PCR reactions were analysed using

SYBR Green mix (Invitrogen). Reactions were performed using Platinum SYBR Green qPCR Super-UDG and a multicolour real-time PCR detection system (Cycler IQ; Bio-Rad). All reactions were performed in triplicate and β -actin was used as an internal standard. Primer sequences used were as follows: *TGF- β 1* (forward) 5'-TGCGC TTGCAGAGATTA AAA-3', (reverse) 5'-CTGCCGTACA ACTCCAGTGA-3'; β -actin (forward) 5'-GCGTGACATC AAAGAGAAG-3, (reverse) 5'-ACTGTGTTGGCATAG AGG-3'.

PKC assay For this assay, HK2 cells were deprived of serum and PKC activity then measured in immunoprecipitates with anti PKC α or PKC β antibodies, as previously described [28].

Statistical methods Data were analysed with Statview software (Abacus Concepts, Piscataway, NJ, USA) by one-factor analysis of variance. *p* values of less than 0.05 were considered statistically significant.

Results

Kidney phenotype in PEDTg mice PED was present at comparable levels in renal cortex and medulla of C57/BL6 mice (Fig. 1a). A 4.5-fold overproduction was detected in PEDTg mice compared with their wild-type littermates. In 6- and 9-month-old PEDTg mice, urine volume was increased by about two- and threefold, respectively, compared with controls, accompanied by a similarly sized increase of daily urinary albumin excretion (Table 1). No significant differences were detected in younger animals (3 months). In addition, at 9 months, plasma creatinine levels were significantly elevated only in PEDTg. Specific gravity, urinary pH and glucose and kidney weight, as well as food and water intake, blood glucose, HbA_{1c}, systolic and diastolic blood pressure, and heart rate did not show significant differences at 3, 6 and 9 months of age. Moreover, consistent with previous reports [25], fasting insulin, NEFA and triacylglycerol were higher in PEDTg than in wild-type mice, independently of age. In 9-month-old PEDTg mice, histological examination of the kidney, followed by morphometrical analysis, revealed a 2.3-fold increase of mesangial area compared with their wild-type littermates (Fig. 1b). Again, no change was detected in 3- and 6-month-old mice (data not shown). Thus, as well as deranging glucose tolerance [25], overproduction of PED induces age-dependent mesangial expansion and affects renal function in transgenic mice.

TGF- β 1 is a cytokine that has been extensively studied as a major mediator of kidney damage in diabetic

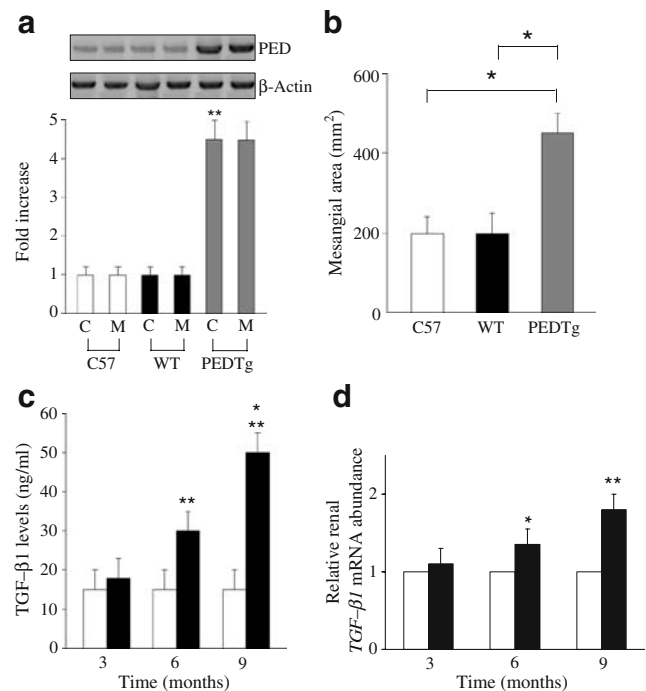


Fig. 1 PED levels, mesangial expansion and TGF- β 1 levels/expression in PEDTg. **a** Cortex (C) and medulla (M) of the kidneys of wild-type (WT) and PEDTg were dissected, solubilised and western blotted with anti-PED antibodies. Blots were revealed by ECL and autoradiography, and quantified by laser densitometry, as shown in bar graph. Values were normalised for actin. Each bar represents the mean \pm SD of duplicate determinations in ten mice per group. **b** Mesangial area from 9-month-old WT and PEDTg mice was measured as described. Bars represent mean \pm SD of determinations in ten mice per group. **c** Serum TGF- β 1 levels were determined by ELISA in PEDTg (black bars) and control mice (white bars) at 3, 6 and 9 months, $n=15$ /group. **d** *Ped* mRNA was determined by real-time RT-PCR analysis of total RNA isolated from kidneys of the PEDTg (black bars) and control mice (white bars), using β -actin as internal standard. Each bar represents the mean \pm SD of four independent experiments in each of which reactions were performed in triplicate using the pooled total RNAs obtained from seven mice per genotype. * $p<0.05$, ** $p<0.01$, *** $p<0.001$

nephropathy [15]. We therefore measured plasma and renal TGF- β 1 levels in PEDTg animals. In 9-month-old PEDTg mice we saw a greater than twofold increase of circulating TGF- β 1 levels compared with wild-type littermates (Fig. 1c). Moreover, *Tgf- β 1* (also known as *Tgfb1*) mRNA content was increased by about twofold in kidney extracts of PEDTg mice (Fig. 1d). Less evident, although significant, changes were detected in 6-month-old but not in 3-month-old PEDTg mice.

Effect of PED overexpression on TGF- β 1 signalling in HK2 proximal tubular cells To address whether PED directly regulates TGF- β 1 expression in renal cells, HK2 proximal tubular cells were stably transfected with a *PED* full-length cDNA. Several clones were selected and characterised, of which two, namely those displaying lower (HK_{PED1}) and

Table 1 Characterisation of wild-type and PEDTg mice

Variable	At 3 months		At 6 months		At 9 months	
	Wild-type	PEDTg	Wild-type	PEDTg	Wild-type	PEDTg
Body weight (g)	26±1	25±2	28±1	28±3	30±2	31±2
Food intake (g/day)	2.8±1.1	2.7±1.1	3.0±0.8	3.1±0.4	3.3±0.6	3.5±0.2
Water intake (ml/day)	5.2±0.7	5.5±1.2	5.7±0.7	6.2±1.2	5.8±0.5	6.1±0.8
Urine excretion (ml/day)	1.0±0.05	1.5±0.5	1.1±0.7	2±0.7*	1.2±0.5	3.8±0.7**
Kidney weight (g)	0.23±0.2	0.24±0.2	0.32±0.4	0.31±0.3	0.38±0.2	0.37±0.2
Urine specific gravity	1030±3	1033±2	1032±3	1035±2	1030±3	1035±2
Urine pH	5±0.4	5±0.3	5±0.5	5±0.5	5±0.4	5±0.5
Urine glucose (mg/day)	0.5±0.2	0.5±0.1	0.5±0.4	0.5±0.2	0.5±0.1	0.5±0.3
Urinary albumin excretion (µg/day)	14±3	17±2	15±2	24±3*	14±3	33±3**
Plasma creatinine (µmol/l)	12.4±0.9	14.1±2.6	13.3±1.8	16.8±4.4	12.4±1.8	18.6±3.5***
Fasting blood glucose (mmol/l)	4.4±0.2	4.5±0.1	4.3±0.2	4.4±0.2	5.2±0.4	5.3±0.5
Fasting serum insulin (pmol/l)	63.7±6.8	191±13.8**	72.3±5.2	206.5±10.3**	122.2±8.6	309.8±5.5**
Systolic BP (mmHg)	116±1.4	112±2	118±5.3	117±4	119±3	116±1.7
Diastolic BP (mmHg)	80±0.6	80±0.5	81±1.3	82±0.8	80±1	81±1
Heart rate (beats/min)	384±17.7	377±11.6	361±9	358±14	370±13	374±10
HbA _{1c} (%)	4.5±0.2	4.5±0.3	4±0.4	4.2±0.2	5.2±0.4	5.5±0.3
NEFA (nmol/l)	0.58±0.08	0.82±0.02*	0.53±0.05	0.78±0.06*	0.61±0.05	0.93±0.09*
Triacylglycerol (mmol/l)	0.11±0.03	0.24±0.04*	0.15±0.02	0.26±0.03*	0.19±0.04	0.35±0.03*

Mice were analysed as described in the [Methods](#)

Data are the means ± SD of determinations in ten PEDTg and ten wild-type littermates

* $p<0.05$, ** $p<0.01$, *** $p<0.001$

higher (HK_{PED2}) PED levels, were studied in detail (Fig. 2a). *TGF-β1* mRNA content was increased by 1.5- and 2.1-fold, respectively in HK_{PED1} and HK_{PED2} cells (Fig. 2b), while connective tissue growth factor levels did not change (data not shown). Moreover, TGF-β1 levels were increased by 2.6- and 3.2-fold in the culture media of

HK_{PED1} and HK_{PED2}, respectively, compared with the parental HK2 cells (Fig. 2c). In addition, the amount of phosphorylated SMAD2/3 in the transfected clones was higher than in control cells, with no change in the cellular content of SMAD2 and SMAD3 proteins (Electronic supplementary material [ESM] Fig. 1), indicating functional

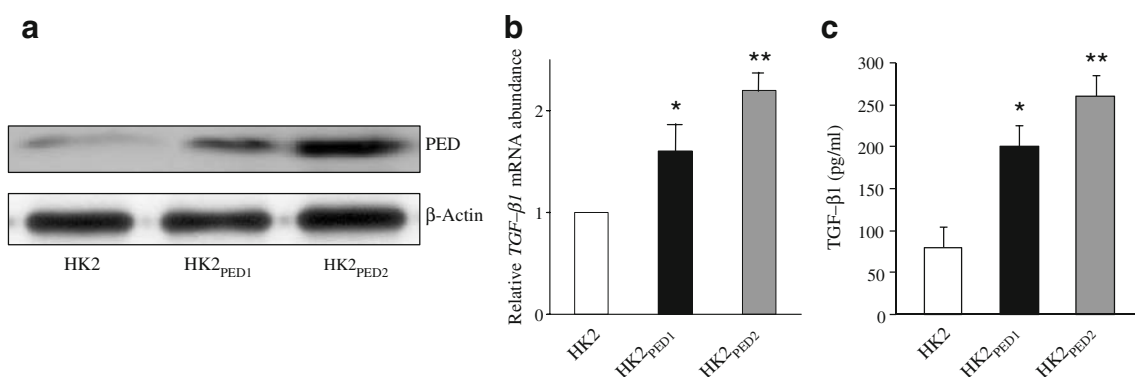


Fig. 2 Effect of *PED* overexpression on TGF-β1 in HK2 cells. HK2 cells were stably transfected with *PED* cDNA. HK_{PED1} and HK_{PED2} represent two different clones. Cell lysates were analysed by SDS-PAGE followed by blotting (a) with PED or actin antibodies. Alternatively (b), the abundance of *TGF-β1* mRNA was determined

by real-time RT-PCR analysis, using β-actin as internal standard. c TGF-β1 levels were measured in the culture medium by ELISA assay. Bars represent the means ± SD of triplicate measurements in four independent experiments. * $p<0.05$, ** $p<0.01$

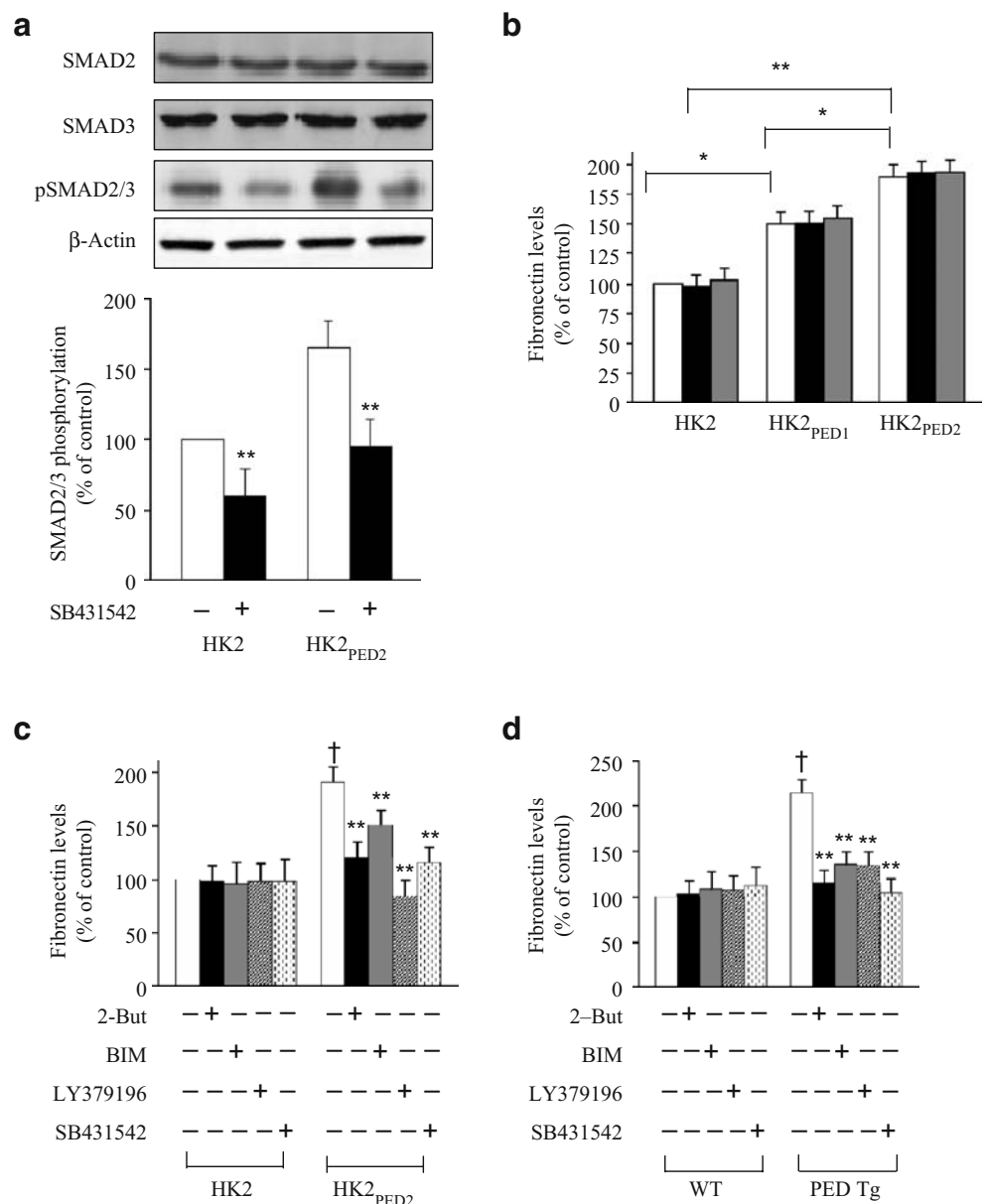
activation of the TGF- β 1 signalling pathway. Further supporting this hypothesis, treatment of HK2 cells with 0.1 μ mol/l SB431542, a TGF- β 1 receptor kinase inhibitor, reduced SMAD2 and SMAD3 phosphorylation by 40% (Fig. 3a). A 50% decrease was observed in HK_{PED2} cells. Total SMAD2 and SMAD3 levels were unchanged following exposure of both cell types to SB431542 (Fig. 3a). Results consistent with the above were obtained in HK_{PED1} cells (data not shown).

Effect of PED overexpression on fibronectin production in HK2 cells TGF- β 1 regulates the synthesis of key extracellular matrix proteins, including fibronectin, laminin, and collagen I and IV [14, 16, 18, 19]. Thus, consistent with the augmented TGF- β 1 production, 1.5- and 1.9-fold increased

levels of fibronectin were detected, independently of glucose concentration (5.0, 11.2 and 30.0 mmol/l), in the media of HK_{PED1} and HK_{PED2}, respectively, compared with control cells (Fig. 3b). Slight, though not statistically significant, increases of laminin and both collagen I and IV were also observed (data not shown).

We have previously shown that overproduction of PED in cultured cells leads to activation of the PLD/PKC pathway [23, 27]. To study the possible involvement of PLD and PKC as potential mediators of PED-induced fibronectin production, wild-type and HK_{PED2} cells were incubated for 15 min with the PLD inhibitor 2-butanol (0.3% vol./vol.) and with the classical PKC inhibitor bisindolylmaleimide (BIM) (100 nmol/l) or the specific PKC β inhibitor LY379196 (50 nmol/l). At the concen-

Fig. 3 Effect of PED overexpression and of PLD, PKC and TGF- β 1 inhibition on fibronectin production in HK2 cells. **a** HK2 and HK2_{PED2} cells were incubated with SB431542 (0.1 μ mol/l). Total SMAD and actin levels, and SMAD2/3 phosphorylation were measured as described. A representative autoradiograph of four independent experiments is shown, with densitometric analysis of pSMAD2/3:SMAD2 ratio, normalised for actin levels, in bar graph. Values are mean \pm SD, ** p <0.01. **b** Culture media with different glucose concentrations (white bars, 5 mmol/l; black bars 11.2 mmol/l; grey bars, 30 mmol/l) from control and Ped overexpressing cells were collected and subjected to western blot with fibronectin antibodies. Bands were quantified by laser densitometry and normalised for actin of the corresponding cultured cells. Bars represent means \pm SD of three independent experiments in triplicate. * p <0.05, ** p <0.01. **c** Culture media from HK2 and HK2_{PED2} cells or **(d)** from murine tubular epithelial cells isolated from wild-type (WT) and PEDTg mice were collected, immunoblotted with fibronectin antibody. Bands were quantified by laser densitometry and normalised for actin of the corresponding cultured cells. Bars represent the mean \pm SD of duplicate determinations in five independent experiments. ** p <0.01; † p <0.01 for differences between HK2_{PED2} and HK2 cells



trations used, these compounds did not inhibit PKC α (ESM Fig. 2a) and PKC β (ESM Fig. 2b) activity in parental HK2 cells. HK_{PED2} cells displayed higher levels of PLD (data not shown), PKC α (ESM Fig. 2a) and PKC β (ESM Fig. 2b) activity. In these cells, PKC α activity was unchanged by LY379196 and reduced by 30% and 35% following exposure to 2-butanol and BIM, respectively. PKC β activity was decreased by 30%, 32% and 60%

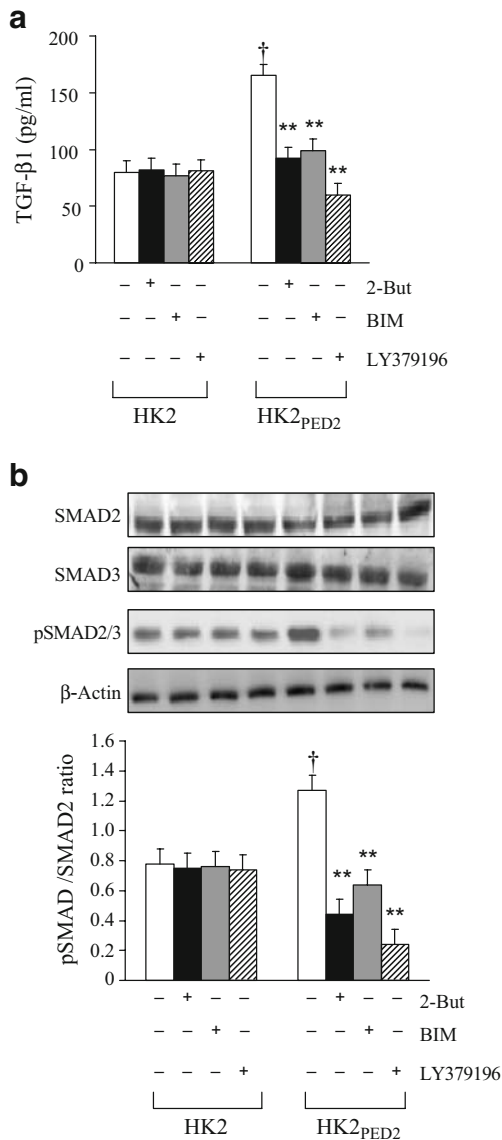


Fig. 4 Effect of PLD and PKC on TGF- β 1 signalling in HK2 cells overexpressing *PED*. HK2 and HK2_{PED2} cells were incubated with 2-butanol (But), BIM, LY379196 or SB431542 for 15 min. **a** TGF- β 1 levels were measured in the culture media by ELISA assay. Bars represent means \pm SD of triplicate measurements in four independent experiments. **b** Cells were solubilised and lysates analysed by SDS-PAGE and western blotted with total Smad2 or Smad3, p-Smad2/3 and actin antibodies. The autoradiograph shown is representative of four independent experiments. Densitometric analysis of pSMAD2/3:SMAD2 ratio is shown in bar graph, with values as mean \pm SD. * p < 0.05, ** p < 0.01; $\dagger p$ < 0.05 for differences between HK2_{PED2} and HK2

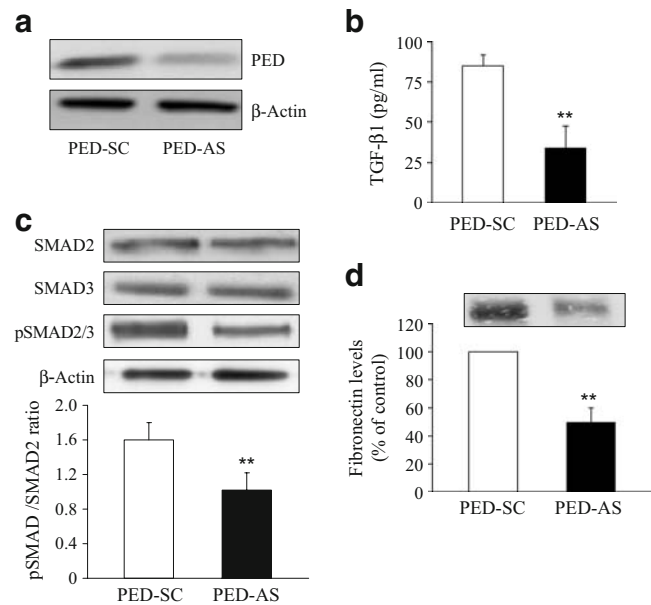


Fig. 5 Role of *PED* depletion in TGF- β 1 signalling. HK2 cells were transiently transfected with *PED* antisense (PED-AS) or scrambled (PED-SC) oligonucleotides. **a** *PED* was detected by western blot, as previously described. The autoradiograph is representative of four independent experiments. **b** TGF- β 1 was measured by ELISA. Bars represent means \pm SD of duplicate measurements in four independent experiments. **c** Total SMAD2 and SMAD3 levels, and SMAD2/3 phosphorylation were detected by western blot, with densitometric analysis (bar graph) of pSMAD2/3:SMAD2 ratio. Values are mean \pm SD. **d** Fibronectin from the culture media of the cells was analysed by western blot and quantified by bar graph, mean \pm SD of duplicate determinations in four independent experiments. ** p < 0.01

following exposure to 2-butanol, BIM and LY379196 respectively. Finally, no change of PKC α (ESM Fig. 2a) and PKC β (ESM Fig. 2b) activity was induced by treatment with SB431542, either in HK2 or in HK_{PED2} cells, indicating that TGF- β 1 was not upstream to PKC activation.

In parallel with the unmodified PKC activity, neither PLD nor PKC inhibitors reduced fibronectin production by native HK2 cells (Fig. 3c). In contrast, exposure of HK_{PED2} cells for 15 min to 0.3% 2-butanol, 100 nmol/l BIM and 50 nmol/l LY379196 decreased fibronectin by 37%, 22% and 55%, respectively. A similar effect was also observed following treatment with SB431542 for 15 min, which reduced fibronectin levels by 40% in HK_{PED2} cells, without affecting control cells (Fig. 3c). To confirm these data, we also measured fibronectin production in primary cultures from the kidneys of wild-type and PEDTg mice. As shown in Fig. 3d, exposure to 2-butanol, BIM, LY379196 and SB431542, respectively reduced the levels of fibronectin by 47%, 38%, 38% and 50% in PEDTg-derived cells only. Thus, these data indicate that PLD, PKC β and TGF- β 1 signalling are required for regulation of fibronectin release by cells overexpressing *PED*.

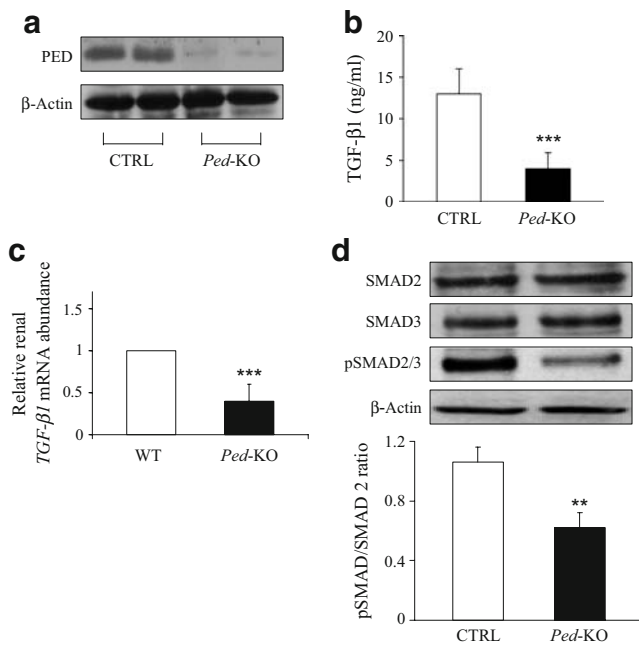


Fig. 6 Determination of serum and renal TGF- β 1 levels, and SMAD2/3 phosphorylation in *Ped*-KO. **a** Kidneys from wild-type and *Ped*-KO were dissected, solubilised and western blotted with anti-PED or actin antibodies. Blots were revealed by ECL and autoradiography (representative autoradiograph of four independent experiments). **b** Serum TGF- β 1 levels were determined in *Ped*-KO and control mice ($n=11$ per group) by ELISA assay. Bars are the mean \pm SD of duplicate determinations. *** $p<0.001$. **c** The abundance of *Ped* mRNA was determined by real-time RT-PCR analysis of total RNA isolated from kidneys of the KO and control mice, using β -actin as internal standard. Bars represent the mean \pm SD of four independent experiments in each of which reactions were performed in triplicate using the pooled total RNAs obtained from five mice per genotype. *** $p<0.001$. **d** Tissue lysates from kidneys of the KO and control mice ($n=5$ per group) were pooled and analysed by SDS-PAGE followed by blotting with total SMAD2 or SMAD3, p-SMAD2/3 and actin antibodies. The autoradiograph is representative of three independent experiments, with densitometric analysis of pSMAD2/3:SMAD2 ratio in bar graph, mean \pm SD. ** $p<0.01$

Effect of PLD and PKC inhibition on TGF- β 1 signalling in HK2 cells We then measured TGF- β 1 production and function in parental HK2 and in HK_{PED2} cells following PLD and PKC inhibition. 2-Butanol and BIM had no significant effect in the HK2 cells, but reduced TGF- β 1 levels in HK_{PED2} cells by 45% and 40%, respectively (Fig. 4a). Interestingly, the selective block of PKC β with LY379196 led to 65% reduction of TGF- β 1 levels in HK_{PED2} cells, while being ineffective in HK2 cells. The effect of PKC β inhibition was also well detectable in terms of reduction of SMAD2 phosphorylation, as well as of SMAD3 (data not shown), with an almost complete block following treatment with LY379196 of HK_{PED2} cells (Fig. 4b).

Role of TGF- β 1 signalling in PED-mediated fibronectin production Next, HK2 cells were transiently transfected

Table 2 Characterisation of wild-type and *Ped*-KO mice after streptozotocin treatment

Variables	Wild-type	<i>Ped</i> -KO
Fasting serum insulin (pmol/l)	70.6 \pm 5.2	39.6 \pm 8.6**
Fasting blood glucose (mmol/l)	4.0 \pm 0.4	4.5 \pm 0.5
Body weight (g)	30 \pm 2	28 \pm 2
Urine excretion (ml/day)	1.4 \pm 0.6	1.1 \pm 0.2
Urinary albumin excretion (μ g/day)	13.4 \pm 4	13 \pm 2
Plasma creatinine (μ mol/l)	11.5 \pm 4.4	9.7 \pm 4.4
Systolic BP (mmHg)	112 \pm 1.7	109 \pm 3.8
Diastolic BP (mmHg)	82 \pm 0.9	77 \pm 0.8
Heart rate (beats/min)	372 \pm 15	369 \pm 13.9
NEFA (nmol/l)	0.78 \pm 0.05	0.82 \pm 0.03
Triacylglycerol (mmol/l)	0.20 \pm 0.03	0.24 \pm 0.04

Mice were analysed as described in the [Methods](#)

Data are the means \pm SD of determinations in seven wild-type and seven *Ped*-KO mice

** $p<0.01$

with SMAD2- or SMAD3- specific phosphorothioate anti-sense oligonucleotides (SMAD2 AS and SMAD3 AS) to block production of the two proteins. SMAD2 AS and SMAD3 AS caused a > 70% reduction of SMAD2 and -3 compared with scrambled oligonucleotides, both in wild-type and in HK_{PED2} cells (ESM Fig. 3a). Specific depletion of the SMAD2 and SMAD3 isoforms was followed by a 30% and 35% fibronectin reduction in HK2 cells and by 60% and 65% reductions in HK_{PED2} clones, compared with the respective cell clone treated with scrambled control oligonucleotides (ESM Fig. 3b). Similar results were obtained in HK_{PED1} cells (data not shown). Thus, overpro-

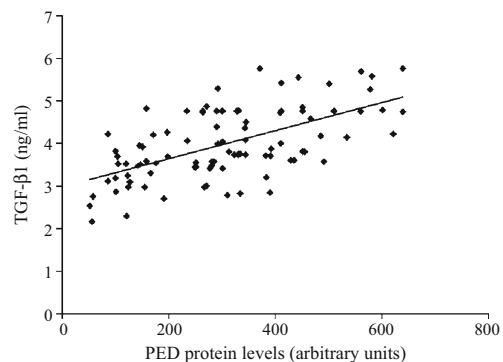


Fig. 7 Correlation between PED levels from PBLs and plasma TGF- β 1 levels in type 2 diabetic patients. PED protein levels were analysed by western blot in PBLs from 95 type 2 diabetic patients and bands were quantified by laser densitometry as described. Plasma TGF- β 1 levels were measured by ELISA. Linear regression analysis revealed a positive correlation between PED and TGF- β 1 levels ($r=0.593$, $p<0.001$)

duction of PED upregulated fibronectin production through a TGF- β 1/SMAD2/3-dependent pathway.

PED depletion decreases TGF- β 1 signalling and fibronectin production To assess whether endogenous PED controls TGF- β 1 and fibronectin production, HK2 cells were transiently transfected with a *PED*-specific phosphorothioate antisense oligonucleotide (PED-AS). PED-AS treatment induced a 65% PED depletion compared with a scrambled oligonucleotide (PED-SC) (Fig. 5a).

TGF- β 1 levels were about 60% lower in PED-AS cells than in the controls (Fig. 5b) and, in parallel, SMAD2, as well as SMAD3 (data not shown) phosphorylation was decreased by about 50% in the presence of the antisense (Fig. 5c). However, total SMAD protein levels were unchanged. Consistently, fibronectin secretion was reduced by about 50% in cells treated with PED-AS compared with those treated with PED-SC (Fig. 5d). Consistent results were obtained in *Ped*-KO [32] (Fig. 6a). In spite of normal glucose levels (Table 2), *Ped* gene ablation coincided with a 65% and 60% decrease of TGF- β 1 plasma levels and renal mRNA content, respectively, compared with control mice (Fig. 6b, c). SMAD2 phosphorylation was also reduced by 40% in kidney extracts of *Ped*-KO (Fig. 6d). SMAD3 phosphorylation also displayed a similar decrease (data not shown).

Correlation between individual levels of PED and plasma TGF β in humans We had analysed plasma TGF- β 1 levels and PED protein abundance in PBLs in 95 type 2 diabetic patients matched for age, BMI, waist circumference, systolic and diastolic BP, and fasting HDL-cholesterol [24]. As shown in Fig. 7, there was a positive correlation between the individual levels of PED and plasma TGF- β 1.

Discussion

The molecular mechanisms responsible for the onset and/or progression of renal complications of diabetes mellitus are still poorly understood. While hyperglycaemia plays a crucial role [2–5], other environmental conditions as well as specific genetic backgrounds may strongly contribute to the clinical appearance of the disorder, either as protective or as predisposing factors [39]. A further complicating element for understanding the molecular determinants is the lack of animal models fully representative of human diabetic nephropathy [40]. We have previously generated a transgenic mouse ubiquitously expressing high levels of *PED*, a gene whose expression is elevated in a large proportion of individuals with type 2 diabetes and in their first-degree relatives [21, 24]. In mice, PED overproduction impairs glucose tolerance by affecting

insulin action and secretion [25, 41], and the animals develop overt diabetes following administration of high-fat diets [25].

Here we show that PEDTg mice also displayed mesangial expansion, and mildly elevated plasma creatinine levels and urinary albumin excretion between 6 and 9 months of age, in the absence of frank hyperglycaemia. Indeed, blood glucose, systolic and diastolic blood pressure, heart rate and HbA_{1c} did not significantly change in wild-type and PEDTg mice, when early signs of renal disorder were found. The renal functional changes were paralleled by elevation of TGF β 1 levels, a cytokine which has been commonly found elevated in patients with diabetic nephropathy [11–15]. However, none of these abnormalities was detectable in younger littermates, suggesting that while PED overproduction represents an early defect, it may also play a role in the slow progression toward renal damage.

Since glucose tolerance of these animals is significantly reduced, and plasma triacylglycerol and NEFA are increased [25], we cannot exclude the possibility that the renal abnormalities observed in PEDTg mice could be linked to the metabolic phenotype. Consistent with this hypothesis, individuals with impaired glucose tolerance display a higher risk of developing nephropathy [42], and microalbuminuria often precedes the onset of diabetes [43]. Nevertheless, studies in HK2 human proximal tubular cells and in primary cultures from the kidneys have shown that selective overexpression of *PED* in kidney cells may, at least in part, be directly responsible for renal derangement. In this regard, forced expression of *PED* increased the production of extracellular matrix (ECM) proteins, whose accumulation is a hallmark of mesangial expansion [17]. In addition, these changes occurred independently of glucose concentration in the extracellular media (Fig. 3b) and of insulin treatment (data not shown), indicating that elevation of PED levels is sufficient to impair ECM production.

The potential mechanisms leading to hyperproduction of ECM proteins are likely to involve hyperactivation of PKC β and the TGF- β 1 signalling pathways. PED binds PLD, increasing its intracellular stability and leading to the accumulation of diacylglycerol [23, 27]. This, in turn, determines abnormal activation of conventional PKC isoforms, including PKC α and PKC β . Increased PKC β activity may account, at least in part, for the renal phenotype observed in PEDTg mice. In HK2 cells, inhibition of PKC β largely reversed the effect of *PED* overexpression. In particular, the increase in *TGF- β 1* mRNA levels and signalling caused by PED in HK2 cells was almost completely blocked by the PKC β inhibitor. Interestingly, both PKC β hyperactivity and elevated TGF- β 1 levels are commonly found in diabetic nephropathy, both in vitro and in vivo [44–46]. In several experimental

and clinical studies, inhibition of PKC (LY333531) has been shown to delay or halt the progression of diabetic complications [47]. Part of the beneficial effect of PKC inhibition is due to the consequent downregulation of TGF- β 1 [48]. Consistently, higher circulating levels of TGF- β 1 have been detected in PEDTg mice and are paralleled by increased *Tgf- β 1* mRNA content in kidney extracts. However, the question of whether other cell types contribute to the increase of TGF- β 1 content in the bloodstream is currently not resolved and under investigation in our laboratory.

Inhibition of PLD and PKC β , as well as of TGF- β 1 receptor activity, or genetic silencing of *SMAD2* and *SMAD3* decreased production of fibronectin. Thus, *PED* overexpression may lead to increased PKC β activity, which enhances expression of *TGF- β 1*, which in turn upregulates fibronectin production. Further support of this hypothesis was seen in the fact that inhibition of *PED* by antisense oligonucleotides in HK2 cells was paralleled by reduced PKC β activity and TGF- β 1 expression and signalling, as well as by decreased fibronectin production. Similarly to *PED*-depleted cells, kidney and total TGF- β 1 levels and PKC β activity were reduced in animals bearing the complete ablation of *Ped* gene, indicating a cause–effect relationship.

It remains unknown whether *PED* overproduction is involved in diabetic nephropathy in humans. However, in a cohort of patients with type 2 diabetes [24], we found a positive correlation between levels of *PED* measured in PBLs and plasma TGF β 1 levels. It could therefore be inferred that high expression levels of *PED* deranges renal function in a subset of diabetic individuals, thereby facilitating the onset and/or progression of diabetic nephropathy.

The mechanism leading to the increase of *PED* cellular levels are only partially known and may be due to altered transcriptional control [49] or to post-translational modifications [50]. The contribution of genetic variations and epigenetic modifications is currently under investigation in our laboratory.

Thus, high expression levels of *PED* determine an increase of PKC β activity and of TGF- β 1 expression in cellular and animal models, as well as in humans. They therefore may represent an early abnormality, contributing to the derangement of ECM deposition and to progression toward nephropathy.

Acknowledgements This study was supported by the European Community's FP6 EUGENE2 (LSHM-CT-2004-512013) and PRE-POBEDIA (201681) projects, the European Foundation for the Study of Diabetes and grants from the Associazione Italiana per la Ricerca sul Cancro (AIRC) and the Ministero dell'Università e della Ricerca Scientifica (PRIN and FIRB). The financial support of Telethon–Italy is also gratefully acknowledged.

Duality of interest The authors declare that there is no duality of interest associated with this manuscript.

References

1. Wolf G (2004) New insights into the pathophysiology of diabetic nephropathy: from haemodynamics to molecular pathology. *Eur J Clin Invest* 34:785–796
2. The Diabetes Control and Complications Trial Research Group (1993) The effect of intensive treatment of diabetes on the development and progression of long-term complications in insulin-dependent diabetes mellitus. *The Diabetes Control and Complications Trial Research Group*. *N Engl J Med* 329:977–986
3. UK Prospective Diabetes Study (UKPDS) Group (1998) Intensive blood-glucose control with sulphonylureas or insulin compared with conventional treatment and risk of complications in patients with type 2 diabetes (UKPDS 33). *UK Prospective Diabetes Study (UKPDS) Group*. *Lancet* 352:837–853
4. Members of the ADVANCE collaborative group (2008) Intensive blood glucose control and vascular outcomes in patients with type 2 diabetes. *N Engl J Med* 358:2560–2572
5. Bilous R (2008) Microvascular disease: what does the UKPDS tell us about diabetic nephropathy? *Diabet Med* 25(Suppl 2):25–29
6. Fogarty DG, Hanna LS, Wantman M, Warram JH, Krolewski AS, Rich SS (2000) Segregation analysis of urinary albumin excretion in families with type 2 diabetes. *Diabetes* 49:1057–1063
7. Imperatore G, Knowler WC, Pettitt DJ, Kobes S, Bennett PH, Hanson RL (2000) Segregation analysis of diabetic nephropathy in Pima Indians. *Diabetes* 49:1049–1056
8. Boright AP, Paterson AD, Mirea L et al (2005) Genetic variation at the ACE gene is associated with persistent microalbuminuria and severe nephropathy in type 1 diabetes: the DCCT/EDIC Genetics Study. *Diabetes* 54:1238–1244
9. Flyvbjerg A (2000) Putative pathophysiological role of growth factors and cytokines in experimental diabetic kidney disease. *Diabetologia* 43:1205–1223
10. Wolf G (2003) Growth factors and the development of diabetic nephropathy. *Curr Diab Rep* 3:485–490
11. Azar ST, Salti I, Zantout MS, Major S (2000) Alteration in plasma transforming growth factor β in normoalbuminuric type 1 and type 2 diabetic patients. *J Clin Endocrinol Metab* 85:4680–4682
12. Pfeiffer A, Middleberg-Bisping K, Drewes C, Schatz H (1996) Elevated plasma levels of transforming growth factor β -1 in NIDDM. *Diabetes Care* 18:1113–1117
13. Ziyadeh FN (2004) Mediators of diabetic renal disease the case for TGF- β as the major mediator. *J Am Soc Nephrol* 15(Suppl 1):S55–S57
14. Wang W, Koka V, Lan HY (2005) Transforming growth factor- β and Smad signalling in kidney diseases. *Nephrology* 10:48–56
15. Sharma K, Ziyadeh FN (1995) Hyperglycemia and diabetic kidney disease. The case for transforming growth factor- β as a key mediator. *Diabetes* 44:1139–1146
16. Ha H, Yu MR, Lee HB (2001) High glucose-induced PKC activation mediates TGF- β 1 and fibronectin synthesis by peritoneal mesothelial cells. *Kidney Int* 59:463–470
17. Bloomgarden ZT (2005) Diabetic nephropathy. *Diabetes Care* 28:745–751
18. Cheng J, Grande JP (2002) Transforming growth factor- β signal transduction and progressive renal disease. *Exp Biol Med* 227:943–956
19. Ten Dijke P, Hill CS (2004) New insights into TGF- β -Smad signalling. *Trends Biochem Sci* 29:265–273
20. Araujo H, Danziger N, Cordier J, Glowinski J, Chneiweiss H (1993) Characterization of PEA-15 a major substrate for protein kinase C in astrocytes. *J Biol Chem* 268:5911–5920

21. Condorelli G, Vigliotta G, Iavarone C et al (1998) PED/PEA-15 gene controls glucose transport and is overexpressed in type 2 diabetes mellitus. *EMBO J* 7:3858–3866
22. Condorelli G, Trencia A, Vigliotta G et al (2002) Multiple members of the mitogen-activated protein kinase family are necessary for PED/PEA-15 anti-apoptotic function. *J Biol Chem* 277:11013–11018
23. Condorelli G, Vigliotta G, Trencia A et al (2001) Protein kinase C (PKC)- α activation inhibits PKC- ζ and mediates the action of PED/PEA-15 on glucose transport in the L6 skeletal muscle cells. *Diabetes* 50:1244–1252
24. Valentino R, Lupoli G, Raciti GA et al (2006) The PEA15 gene is overexpressed and related to insulin resistance in healthy first-degree relatives of patients with type 2 diabetes. *Diabetologia* 49:3058–3066
25. Vigliotta G, Miele C, Santopietro S et al (2004) Overexpression of the ped/pea-15 gene causes diabetes by impairing glucose-stimulated insulin secretion in addition to insulin action. *Mol Cell Biol* 24:5005–5015
26. Zhang Y, Redina O, Altshuller YM et al (2000) Regulation of expression of phospholipase D1 and D2 by PEA-15, a novel protein that interacts with them. *J Biol Chem* 275:35224–35232
27. Viparelli F, Cassese A, Doti N et al (2008) Targeting of PED/PEA-15 molecular interaction with phospholipase D1 enhances insulin sensitivity in skeletal muscle cells. *J Biol Chem* 283:21769–21778
28. Caruso M, Miele C, Oriente F et al (1999) In L6 skeletal muscle cells, glucose induces cytosolic translocation of protein kinase C α and transactivates the insulin receptor kinase. *J Biol Chem* 274:28637–28644
29. Li Y, Soos TJ, Li X et al (2004) Protein kinase C θ inhibits insulin signaling by phosphorylating IRS1 at Ser(1101). *J Biol Chem* 279:45304–45307
30. Ishizuka T, Kajita K, Natsume Y et al (2004) Protein kinase C (PKC) β modulates serine phosphorylation of insulin receptor substrate-1 (IRS-1)—effect of overexpression of PKC β on insulin signal transduction. *Endocr Res* 30:287–299
31. Nakajima K, Yamauchi K, Shigematsu S et al (2000) Selective attenuation of metabolic branch of insulin receptor down-signaling by high glucose in a hepatoma cell line HepG2 cells. *J Biol Chem* 275:20880–20886
32. Kitsberg D, Formstecher E, Fauquet M et al (1999) Knock-out of the neural death effector domain protein PEA-15 demonstrates that its expression protects astrocytes from TNF α -induced apoptosis. *J Neurosci* 19:8244–8251
33. Ciccarelli M, Santulli G, Campanile A et al (2008) Endothelial α 1-adrenoreceptors regulate neovascularization. *Br J Pharmacol* 153:936–946
34. Kasahara M, Mukoyama M, Sugawara A et al (2000) Ameliorated glomerular injury in mice overexpressing brain natriuretic peptide with renal ablation. *J Am Soc Nephrol* 11:1691–1701
35. Ziyadeh FN, Hoffman BB, Han DC et al (2000) Long-term prevention of renal insufficiency, excess matrix gene expression, and glomerular mesangial matrix expansion by treatment with monoclonal antitransforming growth factor- β antibody in db/db diabetic mice. *Proc Natl Acad Sci U S A* 97:8015–8020
36. Terryn S, Jouret F, Vandennebe F et al (2007) A primary culture of mouse proximal tubular cells, established on collagen-coated membranes. *Am J Physiol Renal Physiol* 293:F476–F485
37. Hao C, Beguinot F, Condorelli G et al (2001) Induction and intracellular regulation of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) mediated apoptosis in human malignant glioma cells. *Cancer Res* 61:1162–1170
38. Miele C, Caruso M, Calleja V et al (1999) Differential role of insulin receptor substrate (IRS)-1 and IRS-2 in L6 skeletal muscle cells expressing the Arg1152 \rightarrow Gln insulin receptor. *J Biol Chem* 274:3094–3102
39. Granier C, Makni K, Molina L, Jardin-Watelet B, Ayadi H, Jarraya F (2008) Gene and protein markers of diabetic nephropathy. *Nephrol Dial Transplant* 23:792–799
40. Breyer MD, Böttinger E, Brosius FC 3rd et al (2004) AMDCC. Mouse models of diabetic nephropathy. *J Am Soc Nephrol* 16:27–45
41. Miele C, Raciti GA, Cassese A et al (2007) PED/PEA-15 regulates glucose-induced insulin secretion by restraining potassium channel expression in pancreatic beta-cells. *Diabetes* 56:622–633
42. Singleton JR, Smith AG, Russell JW, Feldman EL (2003) Microvascular complications of impaired glucose tolerance. *Diabetes* 52:2867–2873
43. Mykkanen L, Haffner SM, Kuusisto J, Pyorala K, Laakso M (1994) Microalbuminuria precedes the development of NIDDM. *Diabetes* 43:552–557
44. Koya D, Jirousek MR, Lin YW, Ishii H, Kuboki K, King GL (1997) Characterization of protein kinase C β isoform activation on the gene expression of transforming growth factor- β , extracellular matrix components, and prostanoids in the glomeruli of diabetic rats. *J Clin Invest* 100:115–126
45. Meier M, Park JK, Overheu D et al (2007) Deletion of protein kinase C- β isoform in vivo reduces renal hypertrophy but not albuminuria in the streptozotocin-induced diabetic mouse model. *Diabetes* 56:346–354
46. Kelly DJ, Zhang Y, Hepper C et al (2003) Protein kinase C β inhibition attenuates the progression of experimental diabetic nephropathy in the presence of continued hypertension. *Diabetes* 52:512–518
47. Das Evcimen N, King GL (2007) The role of protein kinase C activation and the vascular complications of diabetes. *Pharmacol Res* 55:498–510
48. Koya D, Haneda M, Nakagawa H et al (2000) Amelioration of accelerated diabetic mesangial expansion by treatment with a PKC β inhibitor in diabetic db/db mice, a rodent model for type 2 diabetes. *FASEB J* 14:439–447
49. Ungaro P, Teperino R, Mirra P et al (2008) Molecular cloning and characterization of the human PED/PEA-15 gene promoter reveal antagonistic regulation by hepatocyte nuclear factor 4 α and chicken ovalbumin upstream promoter transcription factor II. *J Biol Chem* 283:30970–30979
50. Perfetti A, Oriente F, Iovino S et al (2007) Phorbol esters induce intracellular accumulation of the anti-apoptotic protein PED/PEA-15 by preventing ubiquitinylation and proteasomal degradation. *J Biol Chem* 282:8648–8657