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100	Keywords separated by ' - '	PACAP - Diabetes - Kidney - Oxidative stress - Apoptosis
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Molecular Mechanisms Underlying the Nephroprotective Effects of PACAP in Diabetes

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Abstract Diabetic nephropathy is the leading cause of end-stage renal failure and accounts for 30–40 % of patients entering renal transplant programmes. The nephroprotective effects of the neuropeptide pituitary adenylate cyclase-activating polypeptide (PACAP38) against diabetes have been shown previously, but the molecular mechanisms responsible for these effects remain unknown. In the present study, we showed that PACAP treatment counteracted the diabetes-induced increase in the level of the proapoptotic pp38MAPK and cleaved caspase-3 and also decreased the p60 subunit of NFκB. The examined antiapoptotic factors, including pAkt and pERK1/2, showed a slight increase in the diabetic kidneys, while PACAP treatment resulted in a notable elevation of these proteins. PCR and Western blot revealed the downregulation of fibrotic markers, like collagen IV and TGF-β1 in the kidney. PACAP treatment resulted in increased expression of the antioxidant glutathione. We conclude that the nephroprotective effect of PACAP in diabetes is, at least partly, due to its antiapoptotic, antifibrotic and antioxidative effect in addition to the previously described antiinflammatory effect.

Keywords PACAP · Diabetes · Kidney · Oxidative stress · Apoptosis

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Introduction

In the present study, we aimed at investigating the nephroprotective mechanisms of the neuropeptide pituitary adenylate cyclase-activating polypeptide (PACAP) in an experimental diabetic nephropathy. Diabetic nephropathy (DN) is a common microvascular complication of diabetes. The development as well as the progression of the disease is multifactorial due to its profound genetic background besides the well-known environmental impacts, explaining why 30–40 % of all diabetic patients suffer from this diabetic complication (Klein et al. 1984). Glucose uptake is insulin independent in neuronal, retinal, glomerular and endothelial cells, leading to excessive glucose uptake in extracellular hyperglycemia (Di Mario and Pugliese 2001). Although a few decades ago diabetic nephropathy was considered a primarily glomerular disease, nowadays increasing emphasis is put on the tubulointerstitial alterations. Indeed, the stage of tubulointerstitial injury is considered to be a reliable prognostic factor in progressive kidney diseases, including diabetic nephropathy (Nath 1998). Key factors that are involved in diabetic kidney damage are as follows: (1) oxidative stress, (2) overproduction of advanced glycation end products (AGE), (3)

apoptosis, (4) excessive production of prosclerotic growth factors and (5) inflammation due to the overproduction of proinflammatory cytokines (Gnudi 2012; Sun et al. 2013). Since the neuropeptide PACAP is known to exert antiinflammatory, antiapoptotic and antioxidant effects, it seems to be a suitable candidate to prevent the development or delay the progression of DN.

PACAP is a member of the vasoactive intestinal polypeptide (VIP)/secretin/glucagon family and exists in two biologically active forms, PACAP1-27 and PACAP1-38. PACAP38 has been shown to have more prolonged effects compared to PACAP27 in most studies (Araki and Takagi 1992; Lindén et al. 1999). PACAP acts via G-protein-coupled receptors: PAC1, specific for PACAP, and VPAC1 and VPAC2 which also bind VIP with the same affinity.

In addition to its very first known effect, namely adenylate cyclase activation in the hypophysis, PACAP exerts numerous effects in the endocrine, respiratory, gastrointestinal and urogenital systems (Miyata et al. 1989; Girard et al. 2012; Koppan et al. 2012; Moody et al. 2012; Nedvig et al. 2012; Syed et al. 2012; Wada et al. 2013). Moreover, the neuropeptide has been shown to be involved in neuroprotection and general cytoprotection. PACAP is also involved in the regulation of carbohydrate metabolism, although its exact role seems to be complex. Its ability to protect β cells and enhance insulin secretion glucose-dependently has been shown in several studies (Sakurai et al. 2011). Recently, PACAP has been proven to upregulate selenoprotein T in pancreatic β cells, leading to increased insulin secretion (Prevost et al. 2013). However, PACAP also effectively stimulates the release of adrenalin and glucagon. As a result, publications seem to be rather contradictory in the effect of PACAP treatment on blood glucose levels (Sekiguchi et al. 1994; Filipsson et al. 1998; Yada et al. 2000).

PACAP exerts renoprotective effects against several nephrotoxic agents, like hydrogen peroxide-induced oxidative stress in vitro and in vivo or ischemia/reperfusion injury (Szakaly et al. 2008; Horvath et al. 2011; Khan et al. 2012; Reglodi et al. 2012). PACAP has also been shown to attenuate kidney injuries induced by multiple myeloma, cyclosporine A, gentamicin and short-term diabetes (Arimura et al. 2006; Li et al. 2007, 2008). Recently, we have provided evidence for similar protection against 8-week diabetes-induced kidney damage (Banki et al. 2013).

The protective effects of PACAP are mediated through its antiinflammatory, antiapoptotic and antioxidative effects. The antiinflammatory effects involve the inhibition of proinflammatory cytokine (i.e. tumor necrosis factor α (TNFα) and interleukin-6 (IL-6) production and NFκB activation through PAC1- and VPAC1 receptor-mediated signaling (Arimura et al. 2006). We have already proven the importance of the antiinflammatory effect in long-term diabetic nephropathy, resulting in marked downregulation of several cytokines, like cytokine-induced neutrophil chemokine (CINC-1), tissue inhibitor of metalloproteinase 1 (TIMP-1), lipopolysaccharide-induced CXC chemokine (LIX) and monokine induced by gamma interferon (MIG) (Banki et al. 2013). However, the further mechanisms leading to the significant nephroprotective effect in diabetic nephropathy remain unknown.

Materials and Methods

Animals

Adult male Wistar rats (n=33) weighing 250–300 g were housed under light/dark cycles of 12:12 h and received normal rat chow and drinking water ad libitum. Rats were randomly divided into four groups: (1) untreated control (n=10); (2) PACAP-treated control (n=6); (3) untreated diabetic (n=7); and (4) PACAP-treated diabetic (n=10). Control animals received saline i.v., while diabetic groups were administered 65 mg/kg streptozotocin (Sigma, Hungary) i.v. PACAP-treated animals were given 20 μg PACAP1-38 i.p. every second day, starting simultaneously with the streptozotocin injection.

Animals were considered diabetic in cases where they showed elevated blood glucose levels (>11 mmol/l) measured by a blood glucose monitor (Accu-Check Active, Roche, Hungary). After 8 weeks of survival, animals were sacrificed with an overdose of anaesthetics and the kidneys were removed. For RT-PCR and Western blot, kidney samples were taken from the cortex of the kidney close to the superior pole. Experimental procedures were carried out in accordance with approved protocols (University of Pécs; BA02/2000-15024/2011).

RT-PCR Analysis

Tissue samples were dissolved in Trizol (Applied Biosystems, Foster City, CA, USA) for 30 min and mechanically homogenized. After the addition of 20 % RNase-free chloroform, samples were centrifuged at 4 °C at 10,000×g for 15 min. Samples were incubated in 500 μL of RNase-free isopropanol at −20 °C for 1 h and then total RNA was harvested in RNase-free water and stored at −20 °C. The assay mixture for reverse transcriptase reaction contained 2 μg RNA, 0.112 μM oligo(dT), 0.5 mM dNTP, 200 U of High Capacity RT (Applied Bio-Systems) in 1× RT buffer. Primers used in the RT-PCR analysis of collagen IV all isotype were as follows: Col4a1: 5′-TCG GCT ATT CCT TCG TGA TG-3′ and 5′-GGA TGG CGT GGG CTT CTT-3′ (GenBank ID: NM_009931.2, 52 °C, amplimer size 209 bp); and for actin—Actb: 5′-GCC AAC CGT GAA AAG ATG A-3′ and 5′-CAA GAA GGA AGG CTG GAA AA-3′ (GenBank ID:

NM_007393, amplimer size 462 bp). Amplifications were performed in a thermal cycler (Labnet MultiGene™ 96-well Gradient Thermal Cycler; Labnet International, Edison, NJ, USA) in a final volume of 25 μL (containing 1 μL forward and reverse primers [0.4 μM], 0.5 μL dNTP [200 μM] and 5 U of Promega GoTaq® DNA polymerase in 1× reaction buffer) as follows: 95 °C, 2 min, followed by 35 cycles (denaturation, 94 °C, 1 min; annealing at 52 °C for Col4a1 or 53 °C for Actb for 1 min; extension, 72 °C, 90 s) and then 72 °C, 10 min. PCR products were analysed by electrophoresis in 1.2 % agarose gel containing ethidium bromide. Actin was used as the internal control. Optical density of signals was measured by using ImageJ 1.40 g freeware and results were normalised to actin.

Western Blot

The specimens were put into 100 μL of ice-cold homogenization buffer containing 50 mM Tris–HCl buffer (pH 7.0), 10 μg/mL Gordox, 10 μg/mL leupeptine, 1 mM phenylmethylsulphonyl-fluoride (PMSF), 5 mM benzamidine and 10 μg/mL trypsin inhibitor as protease inhibitors. Samples were stored at −70 °C. Tissue samples were sonicated by pulsing burst for 30 s at 40 A (Cole-Parmer, IL, USA). Samples for SDS-PAGE were prepared by the addition of fivefold concentrated electrophoresis sample buffer (20 mM Tris–HCl pH 7.4, 0.01 % bromophenol blue dissolved in 10 % SDS, 100 mM β-mercaptoethanol) to kidney homogenates to set equal protein concentration of samples and boiled for 10 min. About 40 μg of protein was separated by 7.5 % SDS-PAGE gel for detection of tAkt, pAkt, pERK1/2, p38MAPK, pp38MAPK, cleaved caspase-3, TGF-β1, collagen type IV, β-actin and NFκB. Proteins were transferred electrophoretically to nitrocellulose membranes. After blocking with 5 % non-fat dry milk in phosphate-buffered saline with 0.1 % Tween 20 (PBST), membranes were washed and exposed to the following primary antibodies overnight at 4 °C: monoclonal anti-Akt, monoclonal phospho-specific anti-Akt-1 Ser473, monoclonal phospho-specific anti-ERK1/2 Thr202/Tyr204, monoclonal anti-p38 MAPK, monoclonal phospho-specific anti-p38 MAPK (1:500; Cell Signaling Technology, USA), monoclonal anti-NFκB, monoclonal anti-caspase-3 (1:500; Santa Cruz, USA), polyclonal anti-TGF-β1 antibody (1:400; Abcam, Cambridge, UK), monoclonal anti-collagen type IV antibody (1:400; Chemicon/Millipore, USA) and monoclonal anti-actin antibody (1:10,000; Sigma, Hungary) were used. After washing for 40 min in PBST, membranes were incubated with anti-mouse IgG (1:1500; Bio-Rad Laboratories, USA) or anti-rabbit IgG (1:3,000; Bio-Rad Laboratories, USA). Signals were detected by enhanced chemiluminescence (Millipore, USA) according to the instructions of the manufacturer. Actin was used as the internal control. Optical density of Western blot signals was measured by using ImageJ 1.40 g freeware and the results were normalised to actin.

Biochemical Assay of Oxidative Stress Markers

Malondialdehyde (MDA) was measured as previously described (Placer et al. 1966). Briefly, MDA was measured in kidney homogenates with the addition of TBA (saturated thiobarbituric acid in 10 % perchloric acid)-TCA (20 % trichloroacetic acid) reagent. After incubation at 100 °C for 20 min, samples were placed in ice-cold water and were centrifuged for 15 min at 4,000 rpm. MDA concentration was determined spectrophotometrically by measuring the absorbance at 532 nm against TBA+TCA reagent. From the concentration vs. extinction curve, the MDA value of the tissue sample could be calculated in micromole per gram tissue weight.

Reduced glutathione (GSH) was quantified as described by Sedlak and Lindsay (1968). After adding 10 % TCA, kidney homogenates were centrifuged for 15 min at 4,000 rpm. TRIS-buffer was added to the supernatant and samples were measured at 412 nm after adding DTNB to the mixture. Values of glutathione were expressed in micromole per gram tissue weight.

Kidney homogenates were centrifuged for 20 min at 16,000 rpm, and the supernatant was used to measure the concentration of superoxide dismutase (SOD) as described previously (Misra and Firdovich 1972). SOD inhibited the transformation of adrenaline to adrenochrome, which absorbed maximally at 480 nm. Quantification of SOD is based on the degree of inhibition. The value of SOD was given in international units per gram tissue weight.

Electron Microscopy

Kidney samples were cut into maximum 1 mm^3 pieces and were fixed in 5 % glutaraldehyde. Post-fixation was performed with 1 % osmium tetroxide. After dehydration in ascending alcohol and subsequent transfer to propylene oxide, samples were embedded in Araldite resin. Semithin sections were cut by a ultramicrotome (Leica Ultracut R) and stained by toluidine blue. Ultrathin sections were prepared from the area of interest and were contrasted by uranyl-acetic acid and lead citrate. Slides were eventually examined using JEOL 1200 EX-II electron microscope.

Statistical Analysis

Statistical analysis was performed by Microsoft Office Excel and GraphPad software. Analysis of variance (ANOVA) with Bonferroni correction was used to detect significant differences between groups. p value less than 0.05 was considered to be statistically significant.

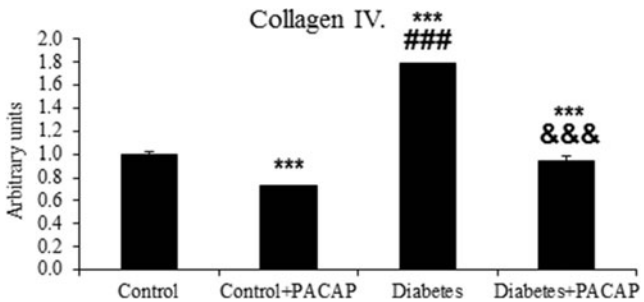
Results

RT-PCR analysis revealed marked elevation in the collagen IV mRNA, a collagen uniquely present in the basement membrane. PACAP treatment successfully counteracted this increase (Fig. 1). We then measured the protein expression of two key factors involved in the fibrotic processes of diabetic nephropathy, namely collagen IV and TGF-β1. Diabetes resulted in excessive expression of collagen IV and TGF-β1, which was attenuated by PACAP38, reaching the level of normal kidneys in the case of collagen IV (Figs. 2 and 3a, b).

Next we investigated the levels of anti- and proapoptotic proteins by Western blot. PACAP alone caused increased expression of the phosphorylated form of Akt. Diabetic nephropathy is accompanied by excessive apoptosis, shown by the upregulation of the phosphorylated forms of the proapoptotic pp38MAPK. However, the antiapoptotic Akt and ERK1/2 were also activated. PACAP treatment in diabetic animals led to a remarkable increase in the activation of the antiapoptotic factors, like pAkt and pERK1/2, and decreased the level of pp38MAPK. PACAP treatment was effective in decreasing the elevated cleaved caspase-3 levels observed in diabetic animals. We then aimed at measuring the level of p60 NFκB, a protein known to control both cytokine production and cell survival. We found that p60 NFκB was upregulated in the diabetic samples. PACAP treatment in diabetic kidneys resulted in a remarkable decrease in the p60 subunit of NFκB (Figs. 2 and 3c–h).

Biochemical assay of the oxidative stress markers revealed a significant elevation in the kidney GSH concentration of the PACAP-treated diabetic group compared to the untreated diabetic one. No changes were observed in the kidney SOD or MDA concentration of the diabetic groups compared to that of the intact animals; however, PACAP caused a significant increase in the SOD level of the control animals (Fig. 4).

Electron microscopy revealed segmental thickening of the glomerular basement membrane (GBM) in several parts of the untreated diabetic glomeruli. The thickness of these parts of the GBM was significantly greater than the GBM in control,



Q2 **Fig. 1** mRNA expression pattern of collagen type IV in control, PACAP-treated control, diabetic and PACAP-treated diabetic kidneys. For RT-PCR reactions, actin was used as the control. ***$p<0.001$ vs. control; ###$p<0.001$ vs. control + PACAP; &&&$p<0.001$ vs. diabetes

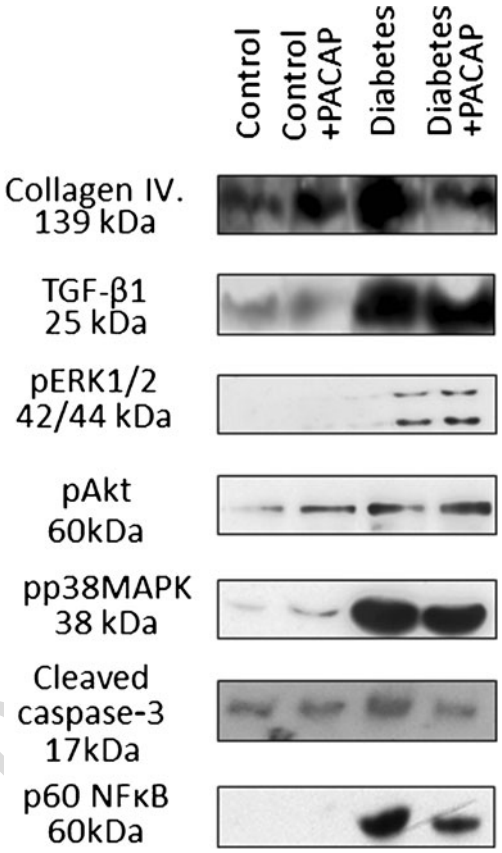


Fig. 2 Western blots of collagen type IV, TGF-β1, pERK1/2, pAkt, pp38MAPK, cleaved caspase-3 and p60 NFκB in untreated or PACAP-treated control and diabetic animal groups. For Western blot analysis, β-actin was used as a control

PACAP-treated control and PACAP-treated diabetic kidneys. However, we could not detect any changes between the non-thickened part of the GBM in diabetic animals and the thickness of the GBM in the control. Most importantly, PACAP-treated diabetic animals did not show this focal segmental thickening; there was no difference between PACAP-treated or untreated control and PACAP-treated diabetic animals. Podocytes in the PACAP-treated diabetic kidneys did not show any morphological alterations compared to the control groups, although severe podocyte injury was present in the diabetic glomeruli with marked foot process broadening and extensive flattening (Fig. 5).

Discussion

In the present study, we demonstrated that in vivo PACAP treatment exhibits protective effect through inhibiting apoptotic, fibrotic and oxidative pathways, key mediators in the development and progression of diabetic nephropathy and preventing diabetes-induced podocyte injury in 8-week diabetes. The present experiment was based on our previous finding, showing that PACAP38 effectively counteracted the

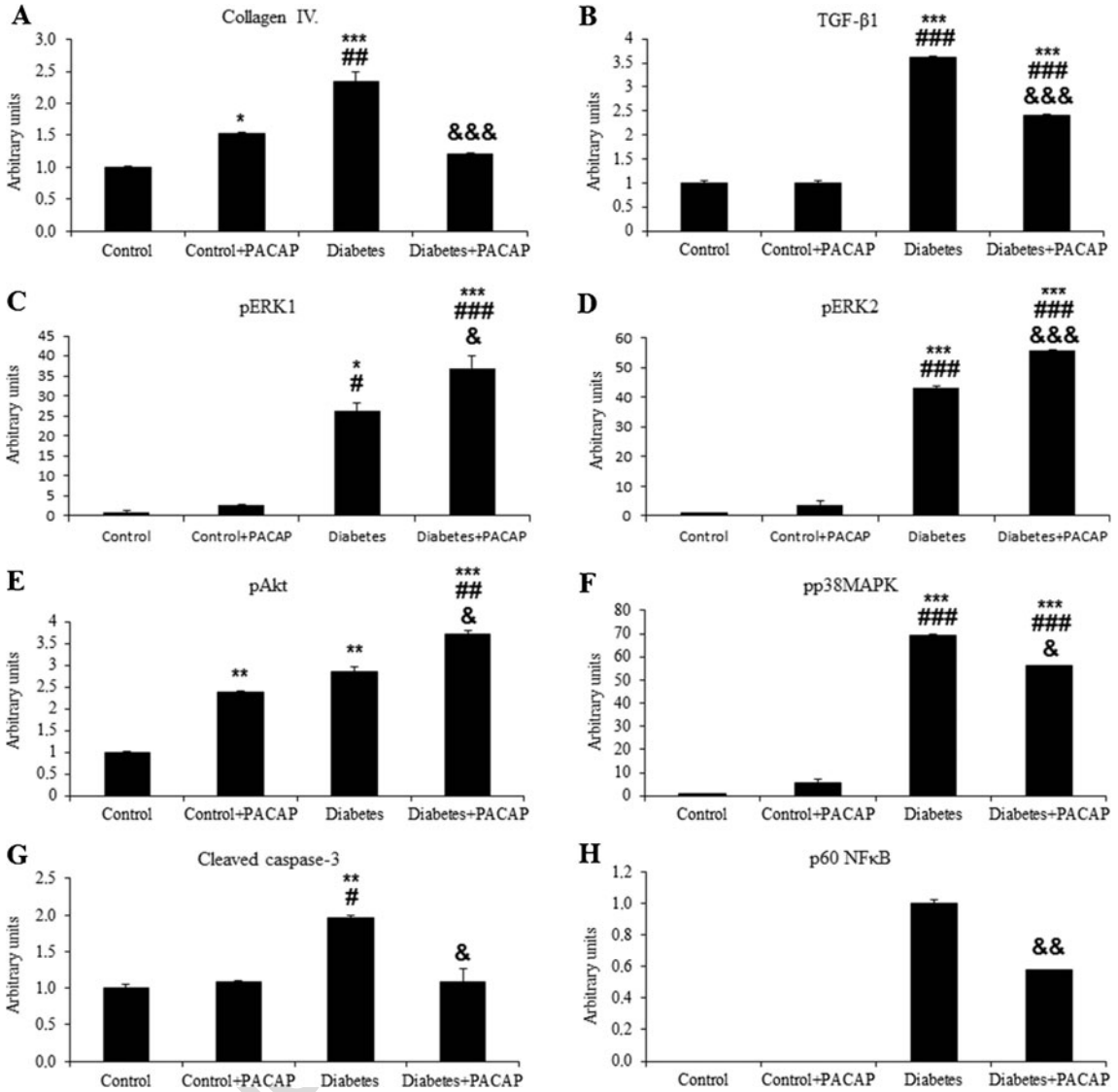


Fig. 3 Effect of 8-week PACAP treatment and diabetes on the protein expression of collagen type IV (**a**), TGF-β1 (**b**), pERK1 (**c**), pERK2 (**d**), pAkt (**e**), pp38MAPK (**f**), cleaved caspase-3 (**g**) and p60 NFκB (**h**). *$p<0.05$; **$p<0.01$; ***$p<0.001$ vs. control; $^{\#}p<0.05$; $^{\#\#\#}p<0.01$; $^{\#\#\#}p<0.001$ vs. control+PACAP; &$p<0.05$; &&$p<0.01$; &&&$p<0.001$ vs. diabetes

histological alterations of 8-week diabetic nephropathy. Decreased PAS-positive area expansion, glycogen deposits in tubular epithelial cells and significantly diminished vascular hyalinosis proved the ameliorative effect of PACAP. Moreover, we provided evidence that this effect is at least partially mediated through its antiinflammatory effect (Banki et al. 2013).

As we mentioned earlier, the effect of exogenous PACAP on glucose homeostasis is not fully elucidated. However, the pivotal role of endogenous PACAP on regulating blood sugar level seems to be more evident. Chronic administration of the antagonist PACAP6-27 was found to deteriorate insulin sensitivity and glucose tolerance in mice (Green et al. 2006). Newborn PACAP knockout mice showed decreased glucose and intrahepatic glucagon levels, but significantly higher insulin levels compared to the control PACAP$^{+/+}$ mice (Gray et al. 2001). Although PACAP protects pancreatic β cells against streptozotocin-induced apoptosis, under our experimental circumstances, we could not find significant changes between the blood sugar levels of the PACAP-treated and untreated diabetic rats after 8 weeks of survival, meaning that this factor cannot be responsible for the ameliorative effect in diabetic nephropathy (Onoue et al. 2008; Banki et al. 2013).

In the present study, we found that diabetes resulted in upregulated NFκB levels compared to the controls, while PACAP treatment effectively diminished the renal NFκB expression. Several previous studies revealed that PACAP, which is structurally similar to the related VIP peptide, prevents NFκB translocation to the nucleus via inhibition of IκB phosphorylation both in vitro and in vivo (Leceta et al. 2000; Delgado and

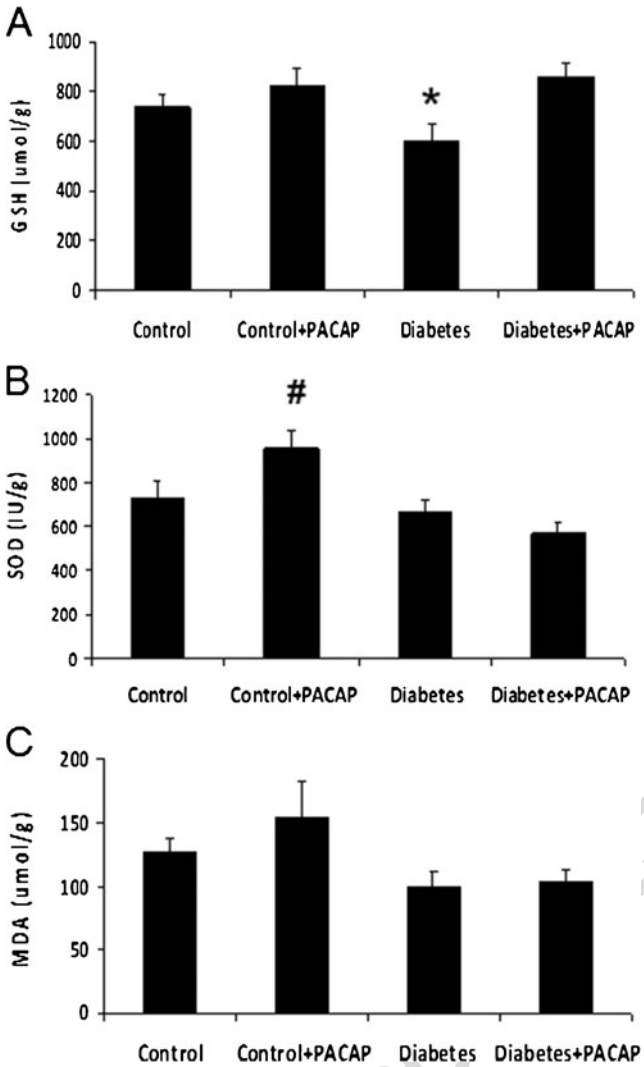


Fig. 4 Changes in renal expression of glutathione (GSH; **a**), superoxide dismutase (SOD; **b**) and malondialdehyde (MDA; **c**). Values are given as mean micromoles per gram, international units per gram and micromoles per gram ± SEM, respectively. *$p<0.05$ vs. diabetes + PACAP; $^{\#}p<0.05$ vs. control

Ganea 2001; Delgado et al. 2002; Ganea and Delgado 2003). Antioxidants and inhibitors of the renin-angiotensin-aldosterone system, which exert their nephroprotective effect partially through controlling the action of NFκB, are extensively used in the treatment of diabetic nephropathy (Liu et al. 2009). In diabetic nephropathy, NFκB can be activated by mesangial cells, hyperglycemia-induced ROS production, renin-angiotensin-aldosterone system (RAAS) activation, TGF-β1, TNFα and interleukins (Iwamoto et al. 2005; Gnudi 2012). NFκB plays an important role in several renal diseases, such as nephritis, proteinura and tubulointerstitial disorders, and it is the key transcriptional regulator of diabetic kidney disease (Sakurai et al. 1996; Iwamoto et al. 2005). It activates the transcription of a variety of factors involved in cell proliferation and inflammation contributing to the progression of diabetic nephropathy (Navarro-González et al. 2011). Mesangial cell proliferation, tubular cell damage and excessive secretion of proinflammatory cytokines, chemokines and adhesion molecules occur as a result of NFκB activation (Mezzano et al. 2004; Chen et al. 2008).

Activation of NFκB under diabetic conditions is assumed to mediate ROS-induced apoptotic changes (Aoki et al. 2011). Extracellular signal-regulated kinase (ERK) 1/2, p38 mitogen activated protein kinase (MAPK) and Jun kinase-mediated pathways are stimulated upon NFκB activation. In this study, we showed that PACAP increased the expression of antiapoptotic factors, like pAkt and pERK1/2, while down-regulated the proapoptotic pp38MAPK. The markedly increased expression of the antiapoptotic proteins in untreated diabetic animals may result from the induction of the protective compensatory mechanisms. The antiapoptotic effect of PACAP was associated with decreased cleaved caspase-3. Caspase-3, as an effector caspase, plays a critical role in receptor-mediated, mitochondria-dependent and endoplasmic reticulum stress-induced apoptotic mechanisms, which are involved in hyperglycemia-induced podocyte loss (Susztak et al. 2006; Tunçdemir and Oztürk 2011; Gui et al. 2012). Apoptosis is a rare event in the normal kidney; however, it is present in human diabetic kidney biopsies, similar to other kidney disorders. Not only proximal and distal tubular cells but also endothelial and mesangial cells are affected by apoptosis in diabetes (Woo 1995; Kumar et al. 2004).

Similar to the pathomechanism of the tubulointerstitial injury in myeloma kidney, tubular epithelial cells start to produce excessive amount of proinflammatory cytokines in diabetic nephropathy as well. TGF-β1 is a prosclerotic cytokine produced by mesangial and proximal tubular cells (Gilbert et al. 1998). It is the key mediator of hyperglycemia-induced changes in the kidney, accelerating the production of extracellular matrix in excess through epithelial-to-mesenchymal cell transformation (di Paolo et al. 1996; Hills Paul and Squires 2010). The accumulation of fibronectin, collagen IV and laminin is caused by simultaneous overproduction and decreased breakdown of proteins in mesangial matrix, glomerular and tubular basal membrane and interstitium, resulting in a severely damaged renal morphology and function (Mauer et al. 1984; Steffes et al. 1989). Similar to our results in rat kidney, PACAP was found to significantly lower the TGF-β1 production in stimulated macrophages (Sun et al. 2000). Li et al. reported that PACAP provided a protective effect against early diabetic nephropathy. They found that this effect is mediated via inhibiting TGF-β1 and TNFα pathways, resulting in remarkably attenuated histological changes in the PACAP-treated animals. Similar to Li et al., in the present study, we showed decreased TGF-β1 and collagen IV levels in the PACAP-treated diabetic compared to the untreated diabetic kidneys (Li et al. 2008). Therefore, these findings provide explanation for the suppressed extracellular matrix expansion observed in the histological sections of PACAP-treated animals. Antioxidants and AGE inhibitors,

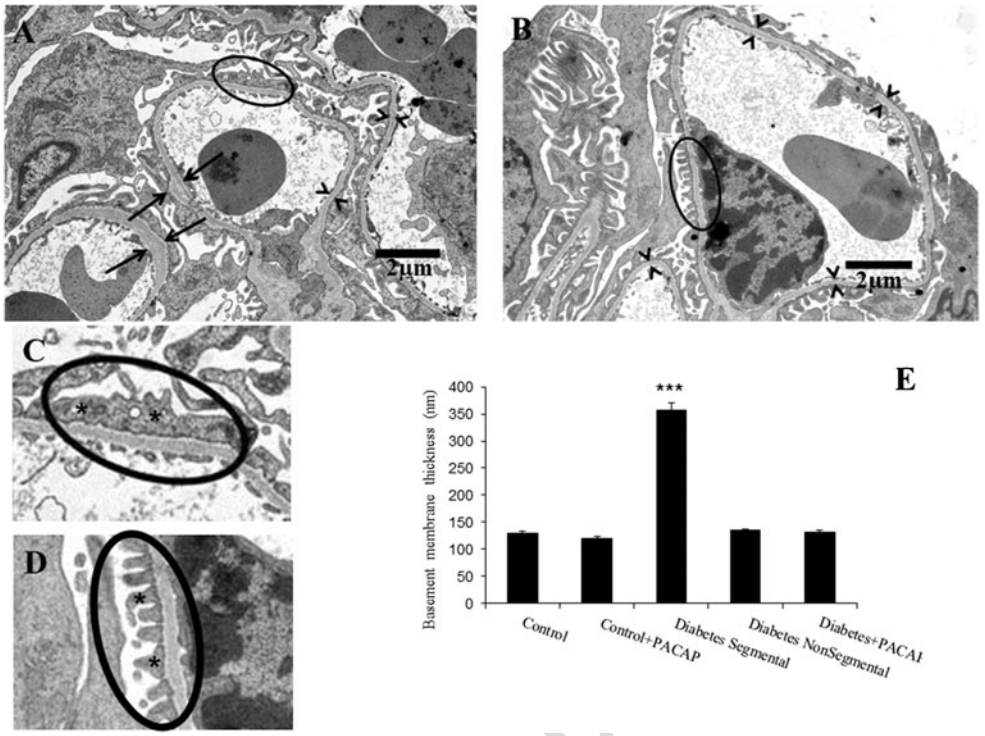


Fig. 5 Representative electron microscopic images of diabetic (**a**) and PACAP-treated diabetic kidneys (**b**). Focal segmental thickening of the glomerular basement membrane (GBM) (*paired arrows*) was observed in the diabetic kidney, while other parts of the GBM remained unchanged (*paired arrowheads*). Fusion of the foot processes of the podocytes (*encircled*) was also remarkable. PACAP treatment effectively counteracted the diabetes-induced GBM thickening and podocyte injury (**b**). *Scale bar*= 2 μm. *Inserts* show *enlarged encircled parts* of pictures **a** and **b** to visualize the severe injury of the podocyte foot processes (fused foot processes marked by *asterisks*, **c**), while PACAP treatment resulted in intact podocyte foot processes (foot processes marked by *asterisks*, **d**). Thickness of GBM (**e**). Basement membrane thickness measurements were performed in the entire basement membrane in control, control + PACAP and in diabetes + PACAP-treated groups. Data are given separately for segmental thickenings in diabetic animals (*bar*: diabetes segmental) and for areas showing no segmental thickening (*bar*: diabetes nonsegmental). Data show mean ± SEM. ***$p<0.001$ vs. all other groups

which are already commonly used in clinical practice, also effectively reduce TGF-β1, fibronectin and collagen IV levels (Ha et al. 1999; Kelly et al. 2001). The results of anti-TGF-β1 treatments are promising; therefore, any candidate, which downregulates the TGF-β1 pathway, could be effective in the treatment of diabetic nephropathy (Sharma et al. 1996; Ziyadeh et al. 2000).

The importance of oxidative stress in the development of diabetes and diabetic complications is well known (Usuki et al. 2011). The diabetogenic effect of streptozotocin in pancreatic β cells also involves the overproduction of nitric oxide (NO) and superoxide (O_2^-) (Nukatsuka et al. 1988; Kaneto et al. 1995). The mitochondrial free radicals were shown to stimulate the polyol-PKC, hexosamine and AGE pathways, contributing to the progression of DN. The vital role of glutathione is based on its ability to neutralize electrophils produced by metabolic processes or external stimuli in order to prevent the organism against their harmful effects. Conditions associated with high levels of free radicals, like diabetes, probably inactivate the mitochondrial GSH carriers, resulting in depressed antioxidant mechanisms. Non-enzymatic glycation of renal mitochondrial proteins was shown to be at least partially responsible for these changes (Lash 2006). Moreover, increased activity of NADPH oxidase results in severely lowered NADPH levels, leading to impaired glutathione recovery. Therefore, not only the increased oxidative stress but also the inactivated defence mechanisms contribute to the oxidative damage of the renal cells, leading to more severe consequences of the oxidative agents (Stanton 2011; Gnudi 2012). Changes in the concentration and redox status of GSH lead to mitochondrial DNA damage and induction of apoptotic pathways (Marchetti et al. 1997; Davis et al. 2001). We found that PACAP is capable of normalising the decreased GSH levels in the diabetic kidney, suggesting an antioxidative effect of PACAP. This finding is similar to our previous studies, where PACAP was found to exert antioxidative effects in oxidative stress-induced renal and hepatic cell damage, intestinal cold preservation and warm ischemic injury (Ferencz et al. 2010a, 2010b; Horvath et al. 2011). Usually diabetes is also associated with decreased superoxide dismutase (SOD) and elevated malondialdehyde (MDA) levels; however, under our experimental circumstances, we could not detect diabetes-induced changes in these factors.

Based on these data, PACAP seems to be a promising candidate in treating diabetic nephropathy. Similar to other

studies showing that PACAP is a strong cytoprotective agent, we have also provided evidence that PACAP is highly effective in diabetic nephropathy. This efficacy is most probably due to PACAP acting at several levels and directly and/or indirectly (via antiinflammatory, antiapoptotic and antioxidant mechanisms) affecting more cell types in the kidney. However, several aspects have to be examined before the clinical application of PACAP. In rats, no side effects were observed after systemic administration of PACAP. Indeed, even a human study proved that systemic infusion of the peptide causes no changes in the physiological parameters apart from transient flushing (Li et al. 2007). The other drawback of systemic PACAP administration is the poor bioavailability, since the half life of the peptide in the circulation is only 5–10 min due to its rapid degradation by dipeptidyl peptidase IV (DPPIV) (Banks et al. 1993; Bourgault et al. 2008). However, nowadays, several studies aim at finding an easy and reliable way of PACAP treatment (Onoue et al. 2011).

In summary, our present study demonstrated the molecular mechanisms involved in the protective effect of PACAP. Besides the contribution of the previously proven antiinflammatory effect, antiapoptotic, antioxidative and antifibrotic mechanisms are responsible for the protective effect of PACAP in 8-week diabetic nephropathy in rats.

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