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C-peptide ameliorates renal injury in type 2 diabetic rats through protein kinase A-mediated inhibition of fibronectin synthesis

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ABSTRACT

Type 1 diabetes mellitus (T1DM) is characterized by the deficiencies of insulin and C-peptide. Mounting evidences have proved the beneficial effects of C-peptide on the renal function in T1DM. However, it is still controversial about the roles of C-peptide in T2DM nephropathy since the level of C-peptide fluctuates greatly at different stages of T2DM. In the present study, we found that the serum C-peptide concentration was much lower in GK rats with diabetic nephropathy than that in normal counterparts. A sustained supplementation of C-peptide at a physiological level could ameliorate urinary albumin, independent of blood glucose control. C-peptide treatment improved glomerulosclerosis and podocyte morphology and reduced the thickness of glomerular basement membrane as compared with saline treatment control. Moreover, it decreased fibronectin synthesis in diabetic glomeruli and in cultured rat mesangial cells accompanied by a down-regulation of RAGE and an up-regulation of PKA. Interestingly, H-89, a PKA inhibitor, could reverse the inhibition effect of C-peptide on fibronectin production in cultured mesangial cells. These findings suggest that C-peptide level is low in T2DM rats with nephropathy and a treatment with a physiological dose of C-peptide can prevent renal injury in diabetic GK rats.

1. Introduction

Diabetic nephropathy (DN) is a leading cause of end-stage renal disease worldwide. The common treatments for DN are to improve blood glucose control and normalize blood pressure. However, no satisfactory therapeutic modality is currently available. Thus it is imperative to establish effective therapeutic strategies against DN etiology.

In recent years, the impact of proinsulin C-peptide has been highlighted in the development of vascular disease. C-peptide acts in ensuring the correct folding of A and B chains to enable alignment and interchain disulfide bond formation [1]. Cleaved from insulin in secretory granules within pancreatic β cells, C-peptide is released at equimolar concentrations to insulin. Previously C-peptide has been considered to have no biological activity [2]. But recently, accumulating data have demonstrated its endocrine-like activity in both human and animal models of type 1 diabetes mellitus (T1DM). C-peptide replacement during T1DM might be beneficial in the prevention or amelioration of diabetic complications such as neuropathy, nephropathy, retinopathy, cardiovascular disease (CVD) and impaired wound healing [3,4,5,6,7,8,9]; and C-peptide could ameliorate structural and functional renal disturbances by acting independently of blood glucose control [10,11,12,13].

C-peptide has been shown to reduce renal complications of T1DM, diminish glomerular hyperfiltration [14], reduce microalbuminuria [15], decrease mesangial expansion [16] and increase endothelial nitric oxide synthase (eNOS) [17]. The mechanism of renoprotective role of C-peptide is mediated through Na+/K+-ATPase activation [18]. C-peptide also prevents glomerular hypertrophy by suppressing mesangial matrix expansion in a T1DM rat model [19].

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In T2DM, there are different opinions about the function of C-peptide. In the early stage of T2DM, the serum levels of insulin and C-peptide greatly increase over normal levels because of insulin resistance. Some studies have reported that high C-peptide level is correlated with the occurrence of macrovascular complications and cardiovascular deaths [20,21]. But a recent study of newly diagnosed T2DM has shown that C-peptide concentration is not associated with the incidence of cardiovascular events or the development of retinopathy [22]. Another larger-scale study among hospitalized T2DM patients has shown that with the progression of diabetic retinopathy, there are lower levels of fasting and 2-h postprandial C-peptide [23]. Although being in a state of insulin resistance and relative insulin insufficiency, T2DM might progress to a late-stage insulin- and C-peptide-deficiency due to pancreatic β-cell demise [24] and require exogenous insulin and C-peptide supplement. Since the natural progression of β-cell function is complex in T2DM, further studies are warranted to elucidate the action of C-peptide on DN in T2DM patients.

Here we employed Goto-Kakizaki (GK) rat as a T2DM model and detected the C-peptide level in rats developed into DN. We examined the effect of supplemented C-peptide at physiological level on renal morphology and function, and also evaluated the effects of long-term administration of homologous C-peptide at a physiological dose on inhibiting the action of AGE-RAGE system (advanced glycation end products (AGE)-receptor for AGE (RAGE) system) and on activating the protein kinase A (PKA) system.

2. Materials and methods

2.1. Animals and DN model

Eight-week-old male GK rats were purchased from Shanghai SLAC Laboratory Animal Company (Shanghai, China). All rats were maintained on a 12-h light/dark cycle and received a standard laboratory diet and water. All animal experiments were performed in accordance with the protocols and guidelines approved by the Animal Ethics Committee of China–Japan Friendship Hospital. After a 2-week high-fat diet, only rats with blood glucose levels >16.7 mM for 3 consecutive days were defined as diabetic. Then the diabetic rats received conventional chow for another 10 weeks. A DN animal model was established when the 24 h urinary albumin level surpassed 400 μg [25].

2.2. Experimental protocols

A total of 24 GK rats with DN were divided into 3 groups according to a random number table (n = 8 each). C-peptide group: an ALZET micro-amount osmotic pump (Type 2004; Alzet, USA), infused with 2 ml rat C-peptide-IIsolution (AnaSpec, Inc), was surgically implanted into the abdominal cavity. C-peptide was released at a constant rate of 50 pmol × kg^{-1} × min^{-1}. The osmotic pump was replaced with fresh solution every 4 weeks. Insulin group: 2.5 IU insulin glargine was administered via subcutaneous injection once daily. DN group: a micro-amount osmotic pump, infused with 2 ml isotonic sodium chloride, was implanted into abdominal cavity and the solution was released at the same rate as in C-peptide group. Eight specific pathogen free-grade male Wistar rats of comparable age and body weight (250–300 g) were used as controls (normal group). The treatment duration was 12 weeks in all groups.

2.3. Detection of serum C-peptide

After 2-week treatment, the animals were euthanized with 2.5% pentobarbital sodium (i.p.) and serum was isolated from venous blood. A rat C-peptide radioimmunoassay kit (R&D, Shanghai, China) was used for measuring the concentration of C-peptide.

2.4. Measurements of blood glucose and urinary albumin

Blood glucose levels were detected, via tail vein, with blood glucose meter (Johnson & Johnson, New Brunswick, NJ, USA). For measurement of urinary albumin, all rats were placed in metabolic cages for 24 h and then the urine samples were collected. A rat albumin ELISA kit (Assaypro, St. Charles, MO, USA) was used for measuring the levels of urinary albumin.

2.5. Electron microscopy

Kidney cortex section was fixed in 2% glutaraldehyde and post-fixed in 1% osmium tetroxide. Tissue sections were prepared for transmission electron microscopy. Glomerular basement membrane (GBM) and podocyte morphologies were observed using a JEOL 1010 transmission electron microscope (JEOL, Tokyo, Japan).

2.6. Histology and immunohistochemistry

Renal sections were stained with periodic acid-Schiff (PAS) according to the standard procedures. Section immunostaining was performed with primary antibody against fibronectin (Abcam, UK), RAGE (Sigma–Aldrich, Shanghai, China) and PKA (Abcam, UK). Antigen signals were detected with horseradish peroxidase-conjugated secondary antibody and visualized with DAB substrate. Then the slides were photographed under light microscope (Olympus, Tokyo, Japan).

2.7. Isolation of glomerular tissues

Rat kidneys were surgically harvested from each rat after euthanasia. Renal cortex was homogenized and passed through a 100-mesh sieve. The filtrate was applied to a 300-mesh metal sieve and the remaining glomeruli were harvested for subsequent experiments.

2.8. Culture of rat primary mesangial cells

Mesangial cells were purchased from Pricells Company (Wuhan, China) and cultured in DMEM (Gibco, USA) supplemented with 10% fetal calf serum (Gibco, USA), 0.4% bovine pituitary extract, 5 μg/ml insulin (Sigma, USA), 10 ng/ml basic fibroblast growth factor (Peprotech, U.K.), transferrin (Sigma, USA), 1.0 μg/ml epinephrine, 100 ng/ml hydrocortisone, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin.

2.9. Effects of C-peptide on fibronectin synthesis

Mesangial cells seeded in 96-well plate were cultured in serum-free medium or serum-free medium containing AGEs (400 μg/ml, Abcam, Cambridge, CB4 0FL, UK), AGEs (400 μg/ml) + insulin (1 IU/ml), AGEs (400 μg/ml) + C-peptide (5 μm), AGEs (400 μg/ml) + C-peptide (5 μm) + H-89 (10 μM) for 48 h. The supernatants were collected and the concentrations of fibronectin were detected by a rat fibronectin ELISA kit (Millipore, USA).

2.10. Real-time PCR

Total RNA was extracted with total RNA isolation kit (Qiagen, USA) and 2 μg of total RNA was reverse transcripted with reverse transcription kit (Promega, USA). Real-time PCR was performed on

an ABI 7500 sequence detection system (Applied Biosystems, Foster City, CA, USA) with a SYBR green real-time PCR Master Mix (Toyobo, Shanghai, China). Relative quantification of transcript levels was performed by $2^{-\triangle\triangle Ct}$ method. The relative amount of target mRNA was normalized to β-actin mRNA.

2.11. Western blot

Total protein was extracted with SDS Lysis Buffer (Nanjing Keygen, China). 50 μg of total protein was fractionated and transferred onto a PVDF membrane. Immunoblotting was performed with primary antibodies against actin, fibronectin, RAGE and PKA, respectively. Horseradish peroxidase-conjugated rabbit anti-mouse IgG was used as the secondary antibody. Enhanced chemiluminescence (Millipore, Shanghai, China) was used for detection.

2.12. Statistical analyses

The data were presented as mean ± standard deviation. The software SPSS17.0 was used for statistical analyses. A one-way ANOVA was used for intergroup data comparison. $P < 0.05$ and $P < 0.01$ denote statistical significance.

3. Results

3.1. C-peptide level in diabetic GK rats with nephropathy

C-peptide level in T2DM rats with nephropathy was first analyzed. C-peptide level was much lower in DN rats (0.13 ± 0.02 nmol/L) than in normal group (1.49 ± 0.12 nmol/L) ($P < 0.01$). C-peptide level returned to physiological level in C-peptide group (1.43 ± 0.1 nmol/L). However, no difference existed between insulin group (0.08 ± 0.01 nmol/L) and DN group (0.13 ± 0.02 nmol/L).

3.2. Effects of C-peptide on glycemia and albuminuria

The blood glucose was as high as 20 mM in DN group. Glycemia was not significantly affected by C-peptide treatment. As expected, blood glucose in insulin treated group dramatically declined , and it was statistically lower than those of vehicle and C−peptide groups (Supplemental Table 1).

The 24 h urinary albumin levels increased gradually in DN and insulin groups. However, in C-peptide group, the 24 h urinary albumin level did not increase further but slightly decreased. After a 6-week treatment, significant differences existed in 24 h urinary albumin levels between C-peptide and DN groups ($P < 0.01$) (Table 1).

3.3. Effects of C-peptide on morphology of glomeruli and GBM

Histological examination of kidney revealed greater glomerulosclerosis in vehicle-treated diabetic rats compared to normal rats. In addition, there was an accumulation of extracellular matrix in mesangium. C-peptide but not insulin treatment improved glomerular sclerosis (Fig. 1A).

Normal ultrastructures of GBM and podocyte were observed in normal rats; while increased GBM thickness and severe effacement of foot processes appeared in DN group rats, accompanied with dramatic albuminuria. C-peptide treatment could prevent the thickening of GBM and the development of foot process effacement. However, insulin treatment almost had no effect on reducing the GBM thickness and foot process effacement (Fig. 1B).

3.4. Effects of C-peptide on the expression of fibronectin

For understanding the protection of C-peptide on GBM, fibronectin, one of the major extracellular matrix proteins, was measured in glomeruli and cultured mesangial cells. In DN glomeruli, fibronectin was markedly up-regulated in vehicle-treated rats compared with normal group. C-peptide but not insulin prevented the induction of fibronectin synthesis (Fig. 2A). The result was confirmed at both mRNA level by real-time PCR(Fig. 2B) and at protein level by Western blot (Fig. 2C).

As mesangial cell is the major cell type for extracellular matrix synthesis, we then examined the effect of C-peptide on the synthesis of fibronectin in mesangial cells. Rat primary mesangial cells were cultured with AGEs to mimic diabetic condition. As shown in Fig. 2D, AGEs promoted fibronectin releasing from mesangium; but when C-peptide was administrated with AGEs together, fibronectin concentration was lower than AGEs group. However, insulin did not inhibit fibronectin production induced by AGEs.

3.5. Effects of C-peptide on the expression of RAGE

Due to the important roles of AGEs-RAGE in DN, the effect of C-peptide on RAGE expression was investigated. In glomeruli, RAGE was up-regulated in DN group compared with normal group. C-peptide treatment, but not insulin, inhibited the induction of RAGE as revealed by immunochemistry (Fig. 3A), real-time PCR(Fig. 3B) and Western blot (Fig. 3C). Similar results were found in cultured mesangial cells as revealed by real-time PCR (Fig. 3D) and Western blot (Fig. 3E).

3.6. Effects of C-peptide on the expression of PKA

In our previous study, up-regulation of RAGE was accompanied by decreased PKA level; and PKA activation protected diabetic kidney from microvasculopathy. Therefore we examined whether C-peptide could affect the expression of PKA. In glomeruli, PKA was down-regulated in DN group versus normal group. C-peptide treatment, but not insulin, up-regulated PKA as revealed by immunohistochemistry staining (Fig. 4A), real-time PCR (Fig. 4B) and Western blot (Fig. 4C). Similar results were found in cultured mesangial cells by real-time PCR (Fig. 4D) and Western blot (Fig. 4E).

Table 1
Effect of the C-peptide treatment on albuminuria in GK diabetic nephropathy rats (μg/24 h).

Groups	Time						
	0 week	2 weeks	4 weeks	6 weeks	8 weeks	10 weeks	12 weeks
Normal	168.5 ± 25.3	167.2 ± 27.7	171.1 ± 19.4	164.8 ± 10.6	154.8 ± 21.3	166.3 ± 4.8	179.2 ± 28.5
DN	536.9 ± 35.4	586.4 ± 53.2	723.1 ± 91.7	719.8 ± 82.2	923.7 ± 100.4	1158.1 ± 182.5	1881.9 ± 161.7
C-peptide	533.3 ± 29.6	528.9 ± 40.8	490.4 ± 42.2	408.7 ± 80.7*	321.3 ± 56.7*	283.4 ± 79.5*	279.1 ± 56.4*
Insulin	560.2 ± 58.6	597.48 ± 83.2	542.2 ± 69.8	786.4 ± 98.3	1011.7 ± 181	1203.5 ± 113.1	1693.7 ± 185.4

*$P < 0.01$ vs DN group and Insulin group.

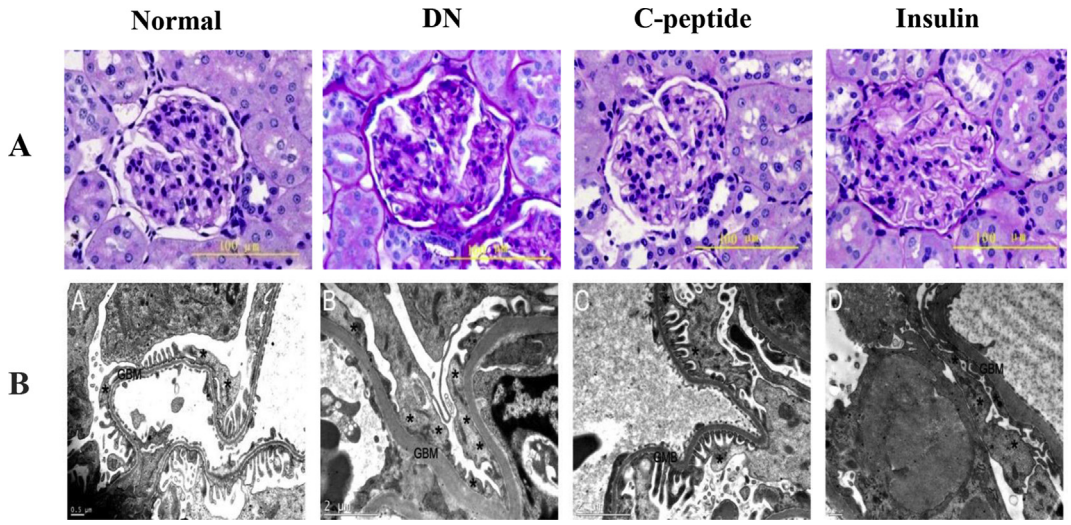

Fig. 1. Effects of C-peptide on structure of GBM. Diabetic GK rats with nephropathy were treated for 12 weeks with vehicle, C-peptide or insulin. Normal rats of a comparable age were used as normal control. 1A: Representative glomerular morphology of all groups by PAS staining. 1B: Transmission electron microscopic analyses of kidney samples. Stars indicated the areas of podocyte foot process effacement.

3.7. Effects of PKA inhibitor on fibronectin synthesis

H-89, a PKA inhibitor, was used to determine the role of PKA in the function of C-peptide on fibronectin synthesis. The amount of fibronectin increased in AGEs group (274.6 ± 22.0 μg/L) versus control group (186.2 ± 13.4 μg/L) ($P < 0.01$). C-peptide treatment could inhibit fibronectin release (212.2 ± 19.4 μg/L) induced by AGEs ($P < 0.01$), while H-89 (286.9 ± 29.3 μg/L) reversed the effect of C-peptide on fibronectin release ($P < 0.01$). These data suggested that C-peptide inhibited fibronectin synthesis mediated by PKA.

4. Discussion

C-peptide has been proven to be a bioactive peptide with beneficial effects on renal function in T1DM [12,14]. However, whether T2DM rats also respond to C-peptide treatment is unknown. The present study demonstrated the therapeutic effects of C-peptide in GK rats with DN.

As a non-obese Wistar substrain with an onset of T2DM in early life, GK rat was selected because of mild hyperglycemia and renal structural changes such as glomerular hypertrophy and thickening of glomerular basement membrane [26]. These characteristics mimicked the manifestations of human DN.

C-peptide level stays high in the early stage of T2DM due to insulin resistance and pancreatic β-cell compensation. However, DN usually occurrs during the late stage of T2DM which is featured by dysfunction of pancreatic β-cell. The deficiencies of C-peptide and insulin have been observed in progressive β-cell loss during the later stage of T2DM [9,24], and C-peptide level in rats with DN is much lower than normal. It has been reported that patients with

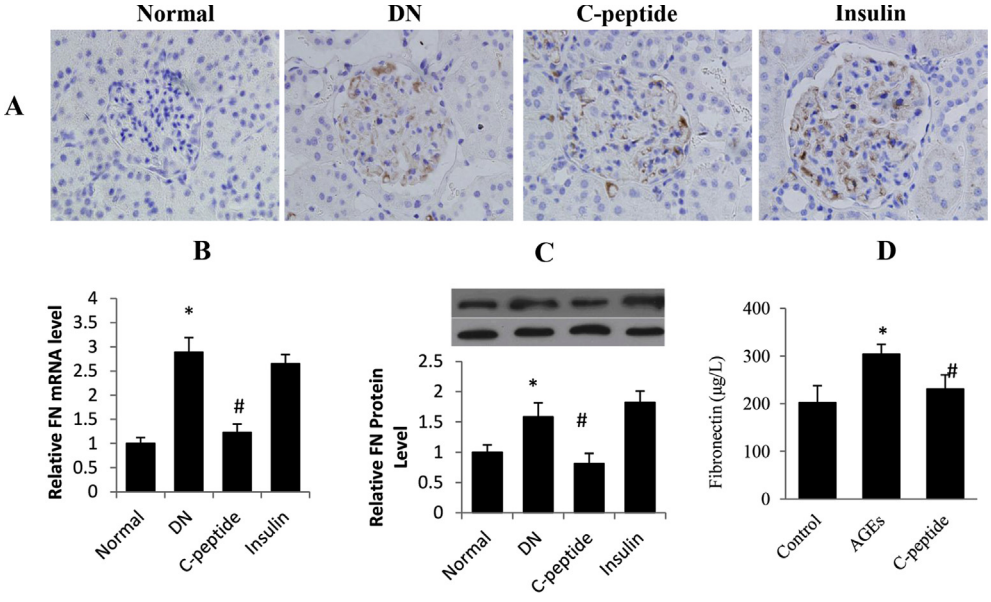

Fig. 2. Effects of C-peptide on fibronectin production in GBM and cultured mesangial cells. The kidneys of rats in all groups were harvested after a 12-week treatment. Immunohistochemical staining (A), real-time PCR (B) and Western blot (C) were used for detecting fibronectin. In addition, the effects of C-peptide on the release of fibronectin by primary cultured rat mesangial cells were detected by ELISA (D). In Figs. B and C * $P < 0.01$ vs. normal group, and #$P < 0.01$ vs. DN group. In Fig. D *$P < 0.01$ vs. control group and #$P < 0.01$ vs. AGEs group.

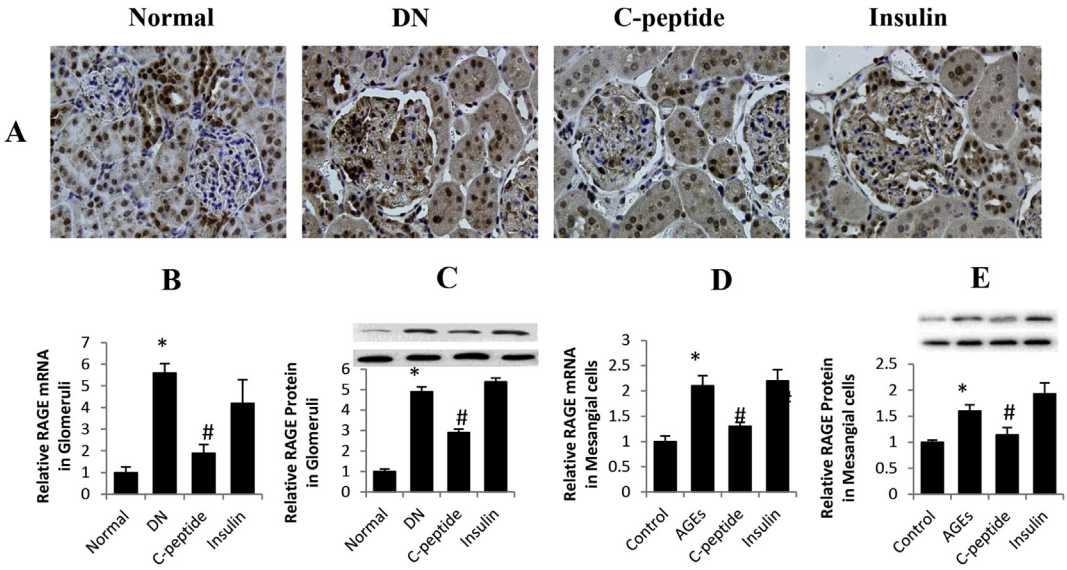

Fig. 3. Effects of C-peptide on the expression of RAGE. After a 12-week treatment of vehicle, C-peptide or insulin, kidneys were immunohistochemically stained for RAGE (3A). The expression of RAGE in glomeruli was evaluated by real-time PCR (3B) and Western blot (3C) $^{*}P < 0.01$ vs. normal group. In addition, the expression of RAGE in cultured mesangial cells was also evaluated by real-time PCR (3D) or Western blot (3E) $^{*}P < 0.01$ vs. control group and $\#P < 0.01$ vs. AGEs group.

progressive diabetic retinopathy has lower levels of fasting and 2 h postprandial C-peptide [23]. These observations suggest that diabetic microvascular diseases might be associated with low C-peptide level.

Supplementation with C-peptide may be an alternative treatment for T1DM and the late stage T2DM with low C-peptide level. Dose selection of C-peptide might be based on the relative amounts of C-peptide and its putative receptor. The receptor for C-peptide is not yet elucidated, but binding studies have demonstrated specific binding sites in various cell types. For example, Rigler R et al. demonstrated that there are 1000–1500 C-peptide binding sites per cell on renal tubular cells, and co-addition of excess intact C-peptide is able to displace binding [27]. This study provides evidence for the existence of a specific GPCR (G-protein-coupled receptor) for C-peptide, which can be fully saturated within the physiological C-peptide concentration range. Thus, in the presence of normal pancreatic β-cell function, these receptors should be fully occupied by prevailing C-peptide concentrations, and therefore no further response may be expected with exogenous administration. This feature may explain the absence of response when C-peptide is administered to healthy subjects [28] and indicate that the therapeutic dose of C-peptide is preferred at physiological level.

Albuminuria is a sensitive marker of DN. DN rats were treated with physiological supplementation of C-peptide for 12 weeks. The most profound effect of C-peptide therapy was the amelioration of albuminuria in diabetic rats without a significant change of blood

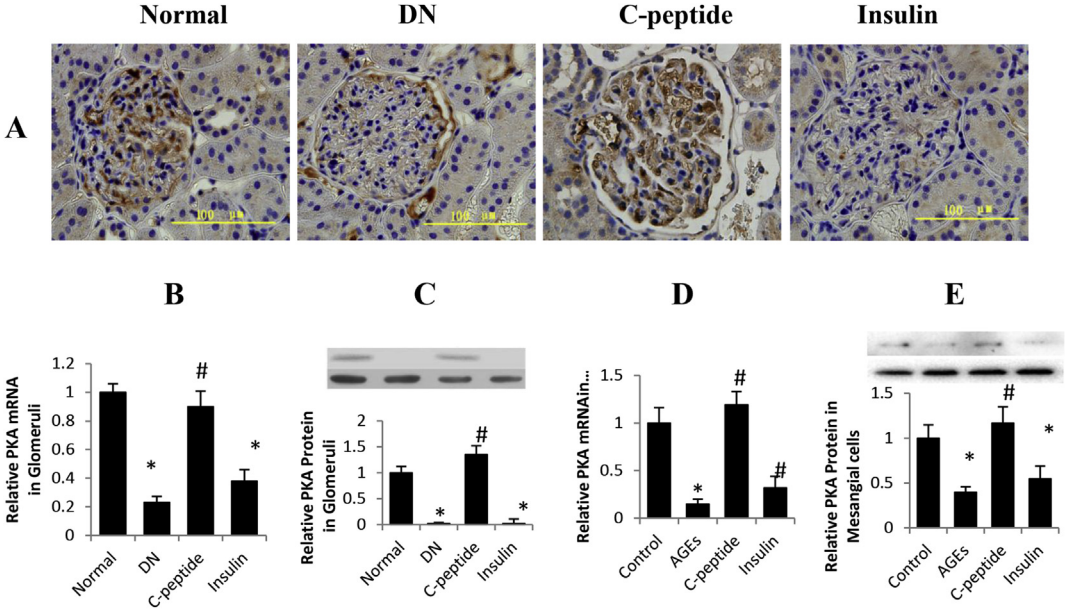

Fig. 4. Effects of C-peptide on the expression of PKA. Kidneys tissue was immunohistochemically stained for PKA (4A). The expression of PKA in glomeruli was evaluated by real-time PCR (4B) or Western blot (4C) $^{*}P < 0.01$ vs. normal group, and $\#P < 0.01$ vs. DN group. In addition, the expression of PKA in cultured mesangial cells was also evaluated by real-time PCR (4D) or Western blot (4E) $^{*}P < 0.01$ vs. control group, and $\#P < 0.01$ vs. AGEs group.

glucose. It was consistent with previous studies [29]. The development of albuminuria is associated with an injury of glomerular filtration barrier. Analyzing the histological results, we suggested that the mechanism of reducing albuminuria by C-peptide included reducing glomerulosclerosis, normalizing the structure of glomerular filtration barrier and preventing GBM thickening and podocyte effacement. C-peptide has been reported to prevent mesangial matrix expansion in T1DM [16]. Fibronectin, a major composition of GBM, increased during DN. C-peptide suppressed fibronectin induction in rat glomeruli and cultured mesangial cells. C-peptide can also reduce urinary albumin by normalizing renal hyperfiltration. Insulin treatment in patients with newly diagnosed T1DM often failed to normalize renal hyperfiltration, but C-peptide replacement treatment or patients with T2DM who had maintained endogenous insulin and C-peptide secretion generally did not develop glomerular hyperfiltration [28].

C-peptide has been shown to initiate multiple signaling cascades to exert cellular functions. Physiological concentrations of C-peptide activates extracellular signal regulated kinase, phosphatidylinositol 3-kinase, protein kinase C, elevates intracellular calcium, and stimulates transcription factors NF-κB and peroxisome proliferator-activated receptor- γ [5]. C-peptide prevents or ameliorates reactive oxygen species (ROS) production through inhibiting protein kinase C (PKC)-dependent NADPHoxidase 2 (NOX2) activity, which ultimately attenuates cytosolic and mitochondrial ROS cycling [4]. This effect may reduce AGE formation under hyperglycaemic conditions. On the other side, in the present study, the direct effect of C-peptide on the AGE pathway was investigated. Studies have revealed AGEs as a major environmental factor for diabetic renal injury and RAGE as a major genetic factor [30]. Here C-peptide treatment could depress the expression of RAGE in renal glomeruli and cultured mesangial cells. A blockade of RAGE inhibited the development of DN and enhanced wound repair in murine models [31].

Our previous study showed that an up-regulation of RAGE was accompanied by decreased PKA; and PKA activation might protect the kidney of diabetic animals [25]. Recent studies have indicated that PKA activation can reduce the generation of ROS in endothelial cells [32]. Here we found that C-peptide could up-regulate PKA level and PKA inhibitor could abolish the effect of C-peptide on fibronectin synthesis. These results indicate an important role of PKA in the action of C-peptide. The down-regulation of PKA by AGEs could be reversed by blocking the mesangial cells with anti-RAGE, which indicated the up-regulation of PKA by C-peptide may partly result from down-regulation of RAGE (Supplemantal Fig 1). In addition, PKA signaling was reported to crosstalk with ERK signaling pathways, which mediated the renoprotection of C-peptide [33].

Therefore C-peptide treatment down-regulates RAGE and activates the PKA system in diabetic kidney. As a result, fibronectin content in GBM was reduced. In short, C-peptide treatment could effectively prevent the development of nephropathy in T2DM.

Conflict of interest

The authors declare no competing financial interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.02.022.

Transparency document

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References

[1] D.F. Steiner, On the role of the proinsulin C-peptide, Diabetes 27 (Suppl. 1) (1978) 145–148.
[2] B.J. Hoogwerf, J.P. Bantle, H.E. Gaenslen, B.Z. Greenberg, B.J. Senske, R. Francis, F.C. Goetz, Infusion of synthetic human C-peptide does not affect plasma glucose, serum insulin, or plasma glucagon in healthy subjects, Metabolism 35 (1986) 122–125.
[3] M.P. Bhatt, Y.C. Lim, J. Hwang, S. Na, Y.M. Kim, K.S. Ha, C-peptide prevents hyperglycemia-induced endothelial apoptosis through inhibition of reactive oxygen species-mediated transglutaminase 2 activation, Diabetes 62 (2013) 243–253.
[4] M.P. Bhatt, Y.C. Lim, Y.M. Kim, K.S. Ha, C-peptide activates AMPKalpha and prevents ROS-mediated mitochondrial fission and endothelial apoptosis in diabetes, Diabetes 62 (2013) 3851–3862.
[5] C.E. Hills, N.J. Brunskill, P.E. Squires, C-peptide as a therapeutic tool in diabetic nephropathy, Am. J. Nephrol. 31 (2010) 389–397.
[6] H. Kamiya, W. Zhang, K. Ekberg, J. Wahren, A.A. Sima, C-Peptide reverses nociceptive neuropathy in type 1 diabetes, Diabetes 55 (2006) 3581–3587.
[7] Y.C. Lim, M.P. Bhatt, M.H. Kwon, D. Park, S. Lee, J. Choe, J. Hwang, Y.M. Kim, K.S. Ha, Prevention of VEGF-mediated microvascular permeability by C-peptide in diabetic mice, Cardiovasc Res. 101 (2014) 155–164.
[8] Y.C. Lim, M.P. Bhatt, M.H. Kwon, D. Park, S. Na, Y.M. Kim, K.S. Ha, Proinsulin C-Peptide prevents impaired wound healing by activating angiogenesis in diabetes, J. Invest. Dermatol. 135 (2015) 269–278.
[9] J. Wahren, A. Kallas, A.A. Sima, The clinical potential of C-peptide replacement in type 1 diabetes, Diabetes 61 (2012) 761–772.
[10] K. Ekberg, T. Brismar, B.L. Johansson, B. Jonsson, P. Lindstrom, J. Wahren, Amelioration of sensory nerve dysfunction by C-Peptide in patients with type 1 diabetes, Diabetes 52 (2003) 536–541.
[11] T. Forst, D.D. De La Tour, T. Kunt, A. Pfutzner, K. Goitom, T. Pohlmann, S. Schneider, B.L. Johansson, J. Wahren, M. Lobig, M. Engelbach, J. Beyer, P. Vague, Effects of proinsulin C-peptide on nitric oxide, microvascular blood flow and erythrocyte Na+,K+-ATPase activity in diabetes mellitus type I, Clin. Sci. (Lond.) 98 (2000) 283–290.
[12] B.L. Johansson, K. Borg, E. Fernqvist-Forbes, A. Kernell, T. Odergren, J. Wahren, Beneficial effects of C-peptide on incipient nephropathy and neuropathy in patients with Type 1 diabetes mellitus, Diabet. Med. 17 (2000) 181–189.
[13] A.A. Sima, W. Zhang, K. Sugimoto, D. Henry, Z. Li, J. Wahren, G. Grunberger, C-peptide prevents and improves chronic Type I diabetic polyneuropathy in the BB/Wor rat, Diabetologia 44 (2001) 889–897.
[14] B. Samnegard, S.H. Jacobson, G. Jaremko, B.L. Johansson, M. Sjoquist, Effects of C-peptide on glomerular and renal size and renal function in diabetic rats, Kidney Int. 60 (2001) 1258–1265.
[15] Y. Maezawa, K. Yokote, K. Sonezaki, M. Fujimoto, K. Kobayashi, H. Kawamura, T. Tokuyama, M. Takemoto, S. Ueda, T. Kuwaki, S. Mori, J. Wahren, Y. Saito, Influence of C-peptide on early glomerular changes in diabetic mice, Diabetes Metab. Res. Rev. 22 (2006) 313–322.
[16] B. Samnegard, S.H. Jacobson, G. Jaremko, B.L. Johansson, K. Ekberg, B. Isaksson, L. Eriksson, J. Wahren, M. Sjoquist, C-peptide prevents glomerular hypertrophy and mesangial matrix expansion in diabetic rats, Nephrol. Dial. Transpl. 20 (2005) 532–538.
[17] T. Wallerath, T. Kunt, T. Forst, E.I. Closs, R. Lehmann, T. Flohr, M. Gabriel, D. Schafer, A. Gopfert, A. Pfutzner, J. Beyer, U. Forstermann, Stimulation of endothelial nitric oxide synthase by proinsulin C-peptide, Nitric Oxide 9 (2003) 95–102.
[18] L. Nordquist, K. Shimada, T. Ishii, D.T. Furuya, A. Kamikawa, K. Kimura, Proinsulin C-peptide prevents type-1 diabetes-induced decrease of renal Na+-K+-ATPase alpha1-subunit in rats, Diabetes Metab. Res. Rev. 26 (2010) 193–199.
[19] W. Sun, X. Gao, X. Zhao, D. Cui, Q. Xia, Beneficial effects of C-peptide on renal morphology in diabetic rats, Acta Biochim. Biophys. Sin. 42 (2010) 893–899. Shanghai.
[20] C. Lebherz, N. Marx, C-Peptide and its career from innocent bystander to active player in diabetic atherogenesis, Curr. Atheroscler. Rep. 15 (2013) 339.
[21] D. Walcher, N. Marx, Advanced glycation end products and C-peptide-modulators in diabetic vasculopathy and atherogenesis, Semin. Immunopathol. 31 (2009) 103–111.
[22] M. Pikkemaat, O. Melander, S. Molstad, G. Garberg, K.B. Bostrom, C-peptide concentration, mortality and vascular complications in people with Type 2 diabetes. The skaraborg diabetes register, Diabet. Med. 32 (2015) 85–89.
[23] X. Cai, X. Han, S. Zhang, Y. Luo, Y. Chen, L. Ji, Age at diagnosis and C-peptide level are associated with diabetic retinopathy in Chinese, PLoS One 9 (2014) e91174.
[24] M. Massi-Benedetti, M. Orsini-Federici, Treatment of type 2 diabetes with combined therapy: what are the pros and cons? Diabetes Care 31 (Suppl. 2) (2008) S131–S135.

[25] H. Wang, Y.W. Jiang, W.J. Zhang, S.Q. Xu, H.L. Liu, W.Y. Yang, J.N. Lou, Differential activations of PKC/PKA related to microvasculopathy in diabetic GK rats, Am. J. Physiol. Endocrinol. Metab. 302 (2012) E173–E182.
[26] A.O. Phillips, K. Baboolal, S. Riley, H. Grone, U. Janssen, R. Steadman, J. Williams, J. Floege, Association of prolonged hyperglycemia with glomerular hypertrophy and renal basement membrane thickening in the Goto Kakizaki model of non-insulin-dependent diabetes mellitus, Am. J. Kidney Dis. 37 (2001) 400–410.
[27] R. Rigler, A. Pramanik, P. Jonasson, G. Kratz, O.T. Jansson, P. Nygren, S. Stahl, K. Ekberg, B. Johansson, S. Uhlen, M. Uhlen, H. Jornvall, J. Wahren, Specific binding of proinsulin C-peptide to human cell membranes, Proc. Natl. Acad. Sci. U S A 96 (1999) 13318–13323.
[28] C.E. Hills, N.J. Brunskill, Cellular and physiological effects of C-peptide, Clin. Sci. (Lond.) 116 (2009) 565–574.
[29] L. Rebsomen, A. Khammar, D. Raccah, M. Tsimaratos, C-Peptide effects on renal physiology and diabetes, Exp. Diabetes Res. 2008 (2008) 281536.
[30] S. Sakurai, H. Yonekura, Y. Yamamoto, T. Watanabe, N. Tanaka, H. Li, A.K. Rahman, K.M. Myint, C.H. Kim, H. Yamamoto, The AGE-RAGE system and diabetic nephropathy, J. Am. Soc. Nephrol. 14 (2003) S259–S263.
[31] M. Brownlee, Biochemistry and molecular cell biology of diabetic complications, Nature 414 (2001) 813–820.
[32] H. Oeseburg, R.A. de Boer, H. Buikema, P. van der Harst, W.H. van Gilst, H.H. Sillje, Glucagon-like peptide 1 prevents reactive oxygen species-induced endothelial cell senescence through the activation of protein kinase A, Arterioscler. Thromb. Vasc. Biol. 30 (2010) 1407–1414.
[33] G.C. Liu, G.Y. Oudit, F. Fang, J. Zhou, J.W. Scholey, Angiotensin-(1-7)-induced activation of ERK1/2 is cAMP/protein kinase A-dependent in glomerular mesangial cells, Am. J. Physiol. Ren. Physiol. 302 (2012) F784–F790.