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ARTICLE

C-reactive protein promotes diabetic kidney disease in a mouse model of type 1 diabetes

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Abstract
Aims/hypothesis Although C-reactive protein (CRP) has been implicated as a risk factor in diabetes, its pathogenic importance in diabetic kidney disease (DKD) remains unclear. The present study investigated the potential role of CRP in DKD.
Methods Diabetes was induced by streptozotocin in human *CRP* transgenic and wild-type mice for assessment of kidney injury at 24 weeks by real-time PCR, immunohistochemistry and western blot analysis. In vitro, the pathogenic effect of CRP was investigated using human kidney tubular epithelial cells cultured with high glucose and/or CRP.
Results We found that *CRP* transgenic mice developed much more severe diabetic kidney injury than wild-type mice, as indicated by a significant increase in urinary albumin excretion and kidney injury molecule-1 abundance, enhanced infiltration of macrophages and T cells, and upregulation of pro-inflammatory cytokines (IL-1β, TNFα) and extracellular matrix (collagen I, III and IV). Enhanced renal inflammation and fibrosis in *CRP* transgenic mice was associated with upregulation of CRP receptor, CD32a, and over-activation of the TGF-β/SMAD and nuclear factor κB signalling pathways. In vitro, CRP significantly upregulated pro-inflammatory cytokines (IL-1β, TNFα, monocyte chemoattractant protein-1 [MCP-1]) and pro-fibrotic growth factors (TGF-β1, connective tissue growth factor [CTGF]) via CD32a/64. CRP was induced by high glucose, which synergistically promoted high glucose-mediated renal inflammation and fibrosis.
Conclusions/interpretation CRP is not only a biomarker, but also a mediator in DKD. Enhanced activation of TGF-β/SMAD and nuclear factor κB signalling pathways may be the mechanisms by which CRP promotes renal inflammation and fibrosis under diabetic conditions.

Keywords CRP · Diabetic nephropathy · Fibrosis · Inflammation · NF-κB · TGF-β/SMAD

Abbreviations

CRP	C-reactive protein
CTGF	Connective tissue growth factor
DKD	Diabetic kidney disease
HK-2	Human kidney epithelial cells
KIM-1	Kidney injury molecule-1
MCP-1	Monocyte chemoattractant protein-1
NF-κB	Nuclear factor κB
TEC	Tubular epithelial cell line
Tg	Transgenic
UAE	Urinary albumin excretion
Wt	Wild-type

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Introduction

Diabetic kidney disease (DKD) is the major cause of end-stage renal disease and is associated with chronic low-grade inflammation and activation of the innate immune system [1–3]. In patients with diabetes, low grade inflammation is evidenced by increased plasma levels of several biomarkers of inflammation, including C-reactive protein (CRP) [1, 2, 4, 5]. Emerging evidence suggests that CRP is associated with increased risk of cardiovascular disease and diabetes [6, 7]. In patients with type 1 or type 2 diabetes, elevated CRP levels are associated with an increase in microalbuminuria [5, 8–11]. Increasing evidence shows that inflammatory and metabolic factors associated with diabetes, such as high glucose, adipokines, modified lipoproteins and NEFA are related to blood levels of CRP and may trigger its production [11, 12]. Numerous clinical studies have demonstrated that CRP predicts the treatment outcomes in cardiovascular disease in diabetic and non-diabetic populations [6, 7, 13–15]. Indeed, treatment of diabetes with either glucose-lowering drugs such as insulin and peroxisome proliferator-activated receptor gamma, or with non-diabetes drugs including aspirin and statins has led to substantial reductions of CRP and other cardiovascular risk markers in several clinical trial studies [6, 7, 13–15]. Positive outcomes in these trials further strengthen the value and acceptance of CRP as a predictive biomarker of cardiovascular disease risk in patients with diabetes mellitus, even though the underlying reasons for the association of CRP with these diseases remains unknown.

In some studies, human CRP has been shown to promote atherosclerosis in ApoE-deficient mice and contributes to thrombosis [16, 17]. Most recently, we also demonstrated that human CRP exacerbates hypertensive cardiac remodelling under high angiotensin II conditions [18]. However, the pathogenic role of CRP in the development of diabetic complications remains unknown. Is CRP just a biomarker or is it involved in the pathogenesis of DKD? If CRP is indeed causal, then what is the mechanism of its action in diabetic renal inflammation and fibrosis? These fundamental questions were investigated in the present study, using a mouse model of type 1 DKD induced in human *CRP* transgenic (Tg) mice and in vitro using human kidney epithelial cells (HK-2).

Methods

Mouse model of streptozotocin-induced diabetes Type 1 diabetes was induced in genetically identical littermate human *CRP* Tg and wild-type (Wt) mice (C57BL/6 background, male, aged 12–14 weeks, 28–35 g) by i.p. injection of 50 mg/kg streptozotocin daily for five consecutive days, as recommended by the Animal Models of Diabetic Complications Consortium [19]. The generation and characterisation of human *CRP* Tg mice have been described previously [20]. Diabetes was induced in groups of eight mice. In addition, groups of six normal *CRP* Tg and Wt mice that had been administered sodium citrate buffer (instead of streptozotocin) were used as normal age-matched controls. Mice were killed under anaesthesia with ketamine/xyline delivered i.p. at 24 weeks and the renal cortexes were collected by carefully removing the renal pelvis and medullar tissues. Cortexes were used for real-time PCR and western blot analysis, or fixed in methyl Carnoy's for histological and immunohistochemical analysis. The experimental protocol was approved by the Animal Experimentation Ethics Committee at The Chinese University of Hong Kong.

Measurement of blood glucose, blood pressure and urinary albumin excretion Fasting (6 h) blood glucose was determined weekly for the first 2 weeks then every 4 weeks thereafter, using a blood glucose meter (Optium Xceed Systems, Doncaster, VIC, Australia). Blood pressure was performed by mouse-tail plethysmography using a blood pressure analysis system (BP2000; Visitech Systems, Apex, NC, USA) in conscious mice. Before disease induction (week 0) urine was collected for 16 h by placing mice in metabolism cages and urinary albumin excretion (UAE) assay was performed. The process was repeated after disease induction (weeks 2, 4, 8, 16, 20 and 24). Urinary albumin was measured by competitive ELISA according to the manufacturer's instructions (Exocell, Philadelphia, PA, USA), with results expressed as total urinary microalbumin.

Histology and immunohistochemistry Changes in renal morphology were examined in methyl Carnoy's-fixed, paraffin-embedded tissue sections (4 μm) stained with periodic acid–Schiff's reagent. Immnunohistochemistry was performed on paraffin sections using a microwave-based antigen retrieval technique [21]. The antibodies used in the study were as follows: rat anti-mouse monoclonal antibody to macrophage F4/80 (Serotec, Oxford, UK); and rabbit polyclonal antibodies to (1) T cell CD3 (SP7) and kidney injury molecule-1 (KIM-1) (both from Abcam, Cambridge, UK), (2) collagens I, III and IV (Southern Biotech, Birmingham, AL, USA), and (3) IL-1β, TNFα, TGF-β, connective tissue growth factor (CTGF), phospho-SMAD2/3, SMAD2/3 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), a phospho-nuclear factor κB (NFκB)/p65 (Ser276) subunit (Cell Signaling Technology, Beverly, MA, USA) and CRP receptor CD32a (R&D Systems, Minneapolis, MN, USA). After incubation with appropriate secondary antibodies, sections were developed with diaminobenzidine to produce a brown colour and counterstained with haematoxylin.

Abundance of IL-1β, TNFα, TGF-β, CTGF and CD32a in the entire cortex (a cross-section of the kidney) was determined

using a quantitative image analysis system (AxioVision 4; Carl Zeiss, Göttingen, Germany). Briefly, the examined cortex was outlined and positive staining identified. Then the percentage of positively stained area within the outlined area of interest was determined. The number of F4/80+, $CD3^+$, phospho-SMAD2/3+ and phospho-NFκB/p65+ cells was counted in 20 consecutive glomeruli and expressed as cells/glomerular cross-section (gcs), whereas positive cells in the tubulointerstitium were counted under high-power fields (×40) by means of a 0.0625 mm^2 graticule fitted in the eyepiece of the microscope and expressed as cells per square millimetre. Quantitative analysis of immunostaining was carried out on coded slides as previously described [22, 23].

Cell culture The tubular epithelial cell line (TEC), HK-2, was purchased from American Type Culture Collection (ATCC) and cultured in DMEM-F12 (Hyclone, Logan, UT, USA) supplemented with 10% (vol./vol.) FBS (Invitrogen Life Technologies, Gaithersburg, MD, USA). TECs were deprived of FBS for 24 h and then stimulated with D-glucose at normal (5.5 mmol/l) or high (35 mmol/l) levels in the presence or absence of recombinant human CRP (10 μg/ml; R&D Systems,) for up to 24 h or in the presence of 0, 1, 5, 10 μg/ml CRP for 6 h (the predetermined peak reaction time). To determine whether the effects of CRP and high glucose were additive, cells were cultured in the presence of either CRP (10 μg/ml), high glucose (35 mmol/l) or both in combination. To block CRP activity, antibodies (5 μg/ml) specific for the CRP receptors CD32a and CD64 (R&D Systems) were added to culture 1 h before addition of CRP (10 μg/ml). D-Mannitol (35 mmol/l) was used as an osmotic control throughout the experiments and at least three independent experiments were performed.

Real-time PCR Kidney and cultured cell total RNA was extracted using a kit (RNeasy; Qiagen, Valencia, CA, USA) according to the manufacturer's instructions and cDNA was synthesised. Real-time PCR was performed with a real-time PCR detector (Opticon 2; Bio-Rad, Hercules, CA, USA) using IQ SYBR green supermix reagent (Bio-Rad) as described previously [22]. The primers for mouse mRNA of collagen I, collagen III, *Tgfb1*, *Ctgf*, *Il1b*, *Tnfα* (also known as *Tnf*) and *Gapdh* have been described elsewhere [22, 23], while other primers were as shown in ESM Table 1. The housekeeping gene *Gapdh* was used as an internal standard. The ratio of specific mRNA: *Gapdh* mRNA was calculated and is expressed as mean±SEM.

Measurement of CRP and cytokines Human CRP in culture supernatant fraction was measured using a kit (Quantikine human CRP ELISA; R&D Systems). Protein level of cytokines (TNFα and TGF-β_1) in the culture supernatant fractions was also determined by ELISA. Monoclonal antibodies specific for TNFα and TGF-β_1 and standards (TNFα and TGF-β_1) were purchased from R&D Systems.

Western blot analysis Proteins were extracted from renal tissue with RIPA lysis buffer and analysed by western blot as previously described [22, 23]. Briefly, after blocking non-specific binding with 5% (wt/vol.) BSA, membranes were incubated overnight for 24 h at 4°C with primary antibodies against phosphorylated NF-κB/p65, phosphorylated SMAD2/3 or CRP receptor CD32a. After being washed, the membranes were then incubated with IRDye 800 conjugated secondary antibodies (Rockland Immunochemicals, Gilbertsville, PA, USA). The signals were detected with an Odyssey Infrared Imaging System (Li-COR Biosciences, Lincoln, NE, USA) and quantified with Image J (National Institutes of Health, Bethesda, MD, USA; http://rsbweb.nih.gov/ij/download.html, accessed 21 June 2011). The ratio for the protein examined was normalised against GAPDH and expressed as the mean±SEM.

Statistical analyses The data obtained are expressed as mean±SEM. Statistical analyses were performed using one-way ANOVA, followed by two-tailed Newman–Keuls post test (Prism 3.0; GraphPad Software, San Diego, CA, USA).

Results

Diabetic renal injury is exacerbated in CRP Tg mice Elevated plasma human CRP (6,387±1,413 ng/ml) was detected by ELISA at baseline in *CRP* Tg mice. After injection of multiple low doses of streptozotocin for five consecutive days, hyperglycaemia (13.9–19.4 mmol/l) developed in *CRP* Tg and Wt mice, whose blood glucose levels were not significantly different under fasting conditions over the entire 24-week study period (Fig. 1a). Also, no significant difference in blood pressure was found between the genotypes (128±2.7 mmHg in Wt vs 134±2.5 mmHg in *CRP* Tg). Surprisingly, however, *CRP* Tg mice developed more severe diabetic kidney injury than Wt mice, as evidenced by a significant increase in UAE (Fig. 1b) and marked upregulation of *Kim-1* mRNA expression and protein (Fig. 1c–e). Histologically, diabetic Wt mice (at week 24 after streptozotocin injection) had bigger glomeruli, with hypercellularity and increased mesangial matrix, thickening of the glomerular basement membrane and tubulointerstitial extracellular matrix accumulation, than age- and genotype-matched saline-treated controls; these changes were further enhanced in *CRP* Tg mice (Fig. 1f, g).

Renal inflammation is exacerbated in diabetic CRP Tg mice As inflammation is a critical process in the development

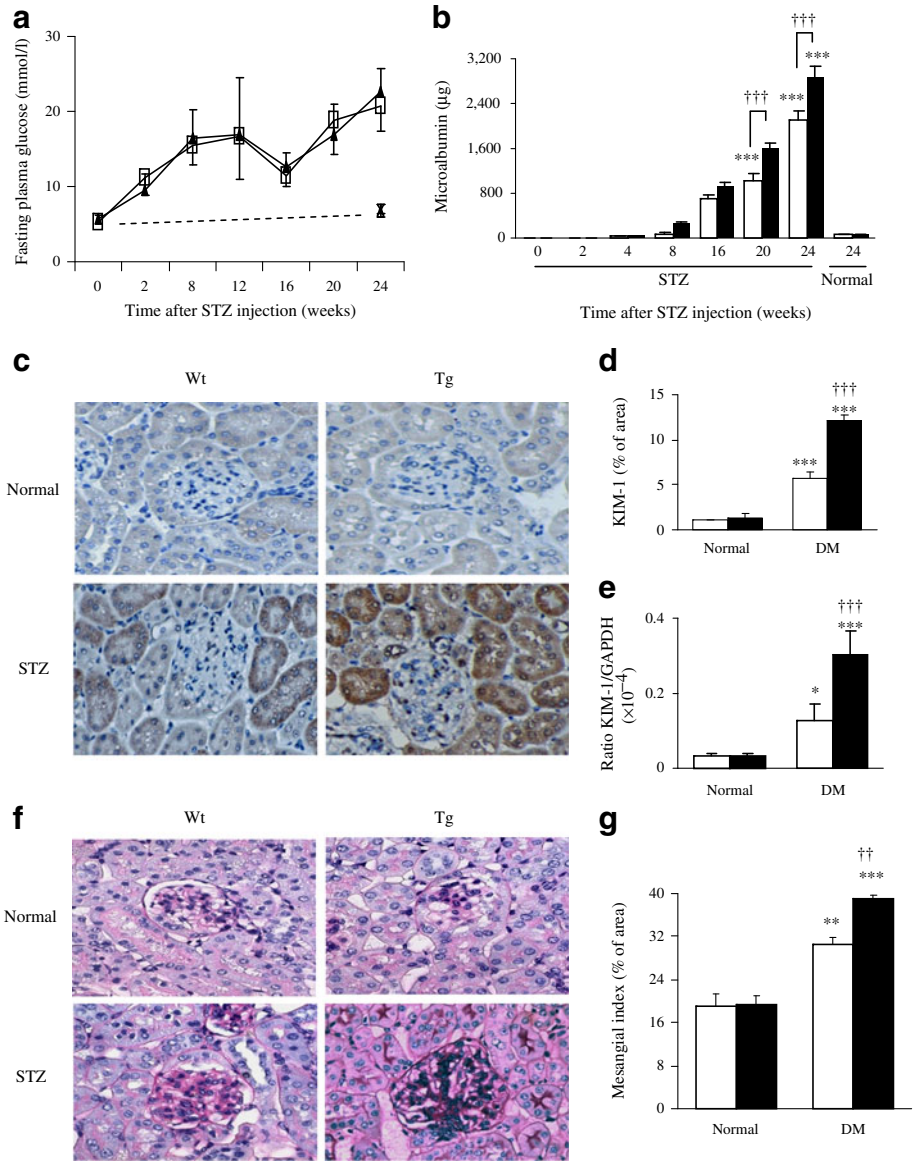

Fig. 1 Diabetic kidney injury is enhanced in *CRP* Tg mice. **a** Fasting blood glucose after streptozotocin (STZ) treatment. In diabetic Wt (white squares) and *CRP* Tg (black triangles) mice, blood glucose levels were significantly increased and maintained at equal levels over the 24-week period. White triangles, non-diabetic Wt; cross symbol, non-diabetic Tg. **b** Diabetic *CRP* Tg (black bars) mice develop more severe microalbuminuria (UAE) than diabetic Wt (white bars) mice. **c** Immunohistochemistry and (**d**) quantitative analysis of KIM-1 in mice as above (**b**). DM, diabetes mellitus. **e** Real-time PCR analysis of *Kim-1* mRNA expression showing that diabetic *CRP* Tg mice (black bars) had much greater expression of *Kim-1* by glomerular and tubular epithelial cells than did diabetic Wt mice (white bars). **f** Periodic acid–Schiff's-stained sections and (**g**) quantitative analysis of mesangial expansion showing that diabetic *CRP* Tg mice had more severe histological damage than diabetic Wt mice. Damage included glomerular hypercellularity, extracellular matrix deposition and mononuclear cells infiltrating the tubulointerstitium. Each micrograph (**c**, **f**) is representative of groups of eight or six mice, magnification ×400. Bars (**b**, **d**, **e**, **g**) represent mean±SEM from groups of eight or six mice. *$p<0.05$, **$p<0.01$, ***$p<0.001$ vs week 0 or normal; $^{\dagger\dagger}p<0.01$, $^{\dagger\dagger\dagger}p<0.001$ vs diabetic Wt mice or as indicated

of diabetic complications [1–3], we examined whether the enhanced diabetic renal injury manifest in *CRP* Tg mice is associated with increased renal inflammation. Immnunohistochemistry and real-time PCR analysis revealed that compared with age-matched normal mice, diabetic Wt mice developed significant renal inflammation, including increased renal infiltration of $CD3^+$ T cells and F4/80+ macrophages (electronic supplementary material [ESM] Fig. 1), and upregulation of pro-inflammatory cytokines including IL-1β (ESM Fig. 2a–c) and TNFα (Fig. 2a–c). All these inflammatory changes were significantly exacerbated in diabetic *CRP* Tg mice (ESM Figs 1 and 2a–c, Fig. 2a–c).

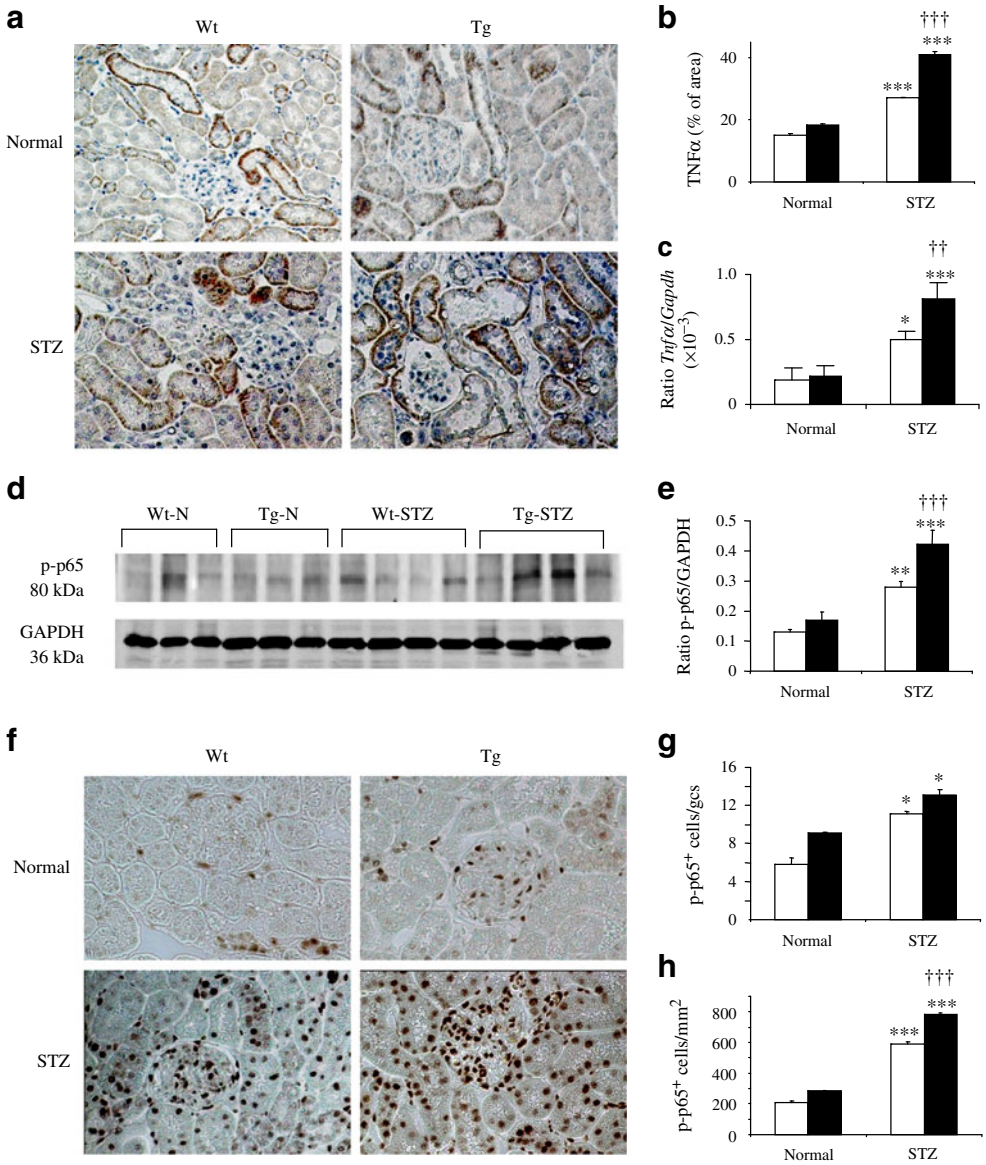

Fig. 2 Renal inflammation is enhanced in *CRP* Tg mice with diabetes. **a** Immunohistochemical staining and (**b**) quantitative analysis of TNFα in Wt (white bars) and Tg (black bars) mice. **c** Real-time PCR analysis of *Tnfα*. Results show that TNFα protein levels and mRNA expression were enhanced in *CRP* Tg mice with diabetes. **d** Western blot and (**e**) semi-quantitative analysis of phospho-NF-κB/p65 (p-p65). **f** Immunohistochemistry and semi-quantitative analysis of nuclear translocation of phospho-NF-κB/p65 (p-p65) in (**g**) glomeruli and (**h**) and tubulointerstitium. Results show that activation of NF-κB/65 was significantly increased in *CRP* Tg mice with diabetes. Magnification (**a**, **f**) ×400. Data (**b**, **c**, **e**, **g**, **h**) represent the mean±SEM for groups of eight or six animals. *$p<0.05$, **$p<0.01$, ***$p<0.001$ vs normal; $^{\dagger\dagger}p<0.01$, $^{\dagger\dagger\dagger}p<0.001$ vs diabetic Wt mice. gcs, glomerular cross-section; STZ, streptozotocin

Renal fibrosis is enhanced in diabetic CRP Tg mice We next investigated whether increased renal fibrosis contributes to enhanced diabetic kidney injury in *CRP* Tg mice. As shown in Fig. 3 and ESM Fig. 2d–f, immunohistochemistry and real-time PCR analysis demonstrated that diabetic Wt mice developed significant collagen I, III and IV matrix accumulation and mRNA upregulation. All of these fibrotic changes were significantly enhanced in diabetic *CRP* Tg mice.

Enhanced CRP signalling and activation of NF-κB and TGF-β/SMAD3 signalling are a key mechanism by which CRP promotes diabetic renal injury Next, we investigated the signalling mechanisms that might underlie CRP exacerbation of diabetic renal injury. We first examined levels of the CRP receptor, CD32a. As shown in Fig. 4, a significant upregulation of CD32a was evident in normal *CRP* Tg mice compared with normal Wt mice. Likewise under diabetic conditions, CD32a abundance was significantly increased in diabetic Wt mice, presumably by glomerular and tubular epithelial cells; this increase was further aggravated in the diabetic kidney of *CRP* Tg mice, demonstrating enhanced CRP signalling in the diabetic kidney of that group.

Because NF-κB and TGF-β/SMAD signalling pathways are activated in the diabetic kidney and both have been shown to play a pathogenic role in the development of DKD [24–28], we examined whether enhanced diabetic kidney injury in *CRP* Tg mice is associated with enhanced activation of these two signalling pathways. Western blot analysis demonstrated that phosphorylation of NF-κB/p65 subunit was notably increased in the diabetic kidney of Wt

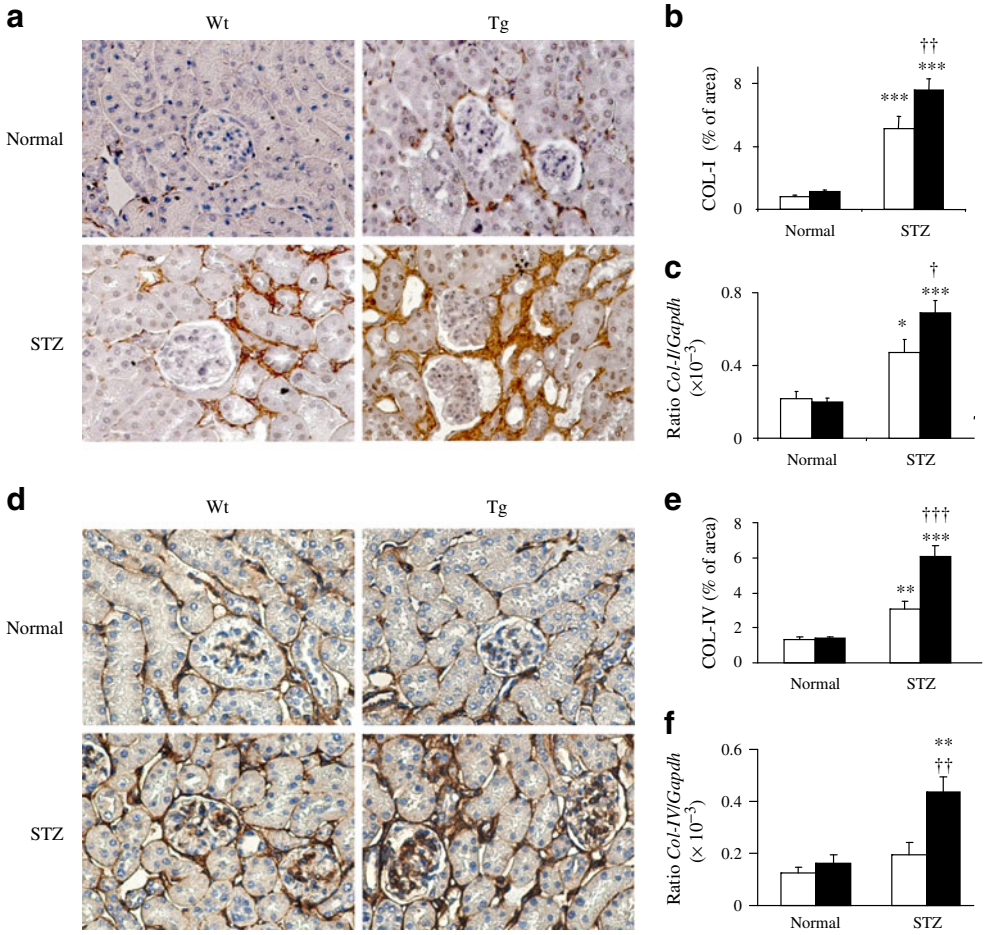

Fig. 3 Renal fibrosis is enhanced in *CRP* Tg mice with diabetes. **a** Immunohistochemical staining and (**b**) quantitative analysis of collagen I (COL-I) in Wt (white bars) and Tg (black bars) mice. **c** Real-time PCR analysis of *Col-I* (also known as *Col1a1*) mRNA expression. **d** Immunohistochemical staining and (**e**) quantitative analysis of collagen IV (COL-IV). **f** Real-time PCR analysis of *Col-IV* (also known as *Col4a4*) mRNA expression. Results show that increased collagen I and IV protein and mRNA expression in diabetic Wt mice was further enhanced in *CRP* Tg mice. Magnification (**a**, **d**) ×200. Data (**b**, **c**, **e**, **f**) represent the mean± SEM for groups of eight or six animals. *$p<0.05$, **$p<0.01$, ***$p<0.001$ vs normal; $^{\dagger}p<0.05$, $^{\dagger\dagger}p<0.01$, $^{\dagger\dagger\dagger}p<0.001$ vs diabetic Wt mice. STZ, streptozotocin

mice and even more increased in *CRP* Tg mice (Fig. 2d, e). Enhanced activation of NF-κB signalling in the diabetic kidney in *CRP* Tg mice was further evidenced by increased nuclear localisation of phosphorylated NF-κB/p65 in the glomeruli and tubulointerstitium (Fig. 2f–h).

Enhanced renal fibrosis in *CRP* Tg mice was also associated with increased TGF-β/SMAD signalling in the diabetic kidney (Fig. 5). Real-time PCR and immunohistochemistry analysis detected higher levels of renal TGF-β1 and CTGF protein and mRNA expression (Fig. 5a–f) in diabetic *CRP* Tg than in diabetic Wt mice. This was associated with enhanced SMAD signalling as demonstrated by higher levels of phosphorylated SMAD2/3 by western blot (Fig. 5g, h) and the enhancement of nuclear localisation of phosphorylated SMAD2/3 in many glomerular and tubulointerstitial cells (Fig. 5i–k).

CRP is induced by high glucose and signals through CD64 and CD32a to mediate renal inflammation and fibrosis directly and additively with high glucose in vitro To investigate the mechanism whereby CRP is elevated in patients with diabetes, we tested the hypothesis that CRP may be induced in human renal TEC (HK-2) under high glucose conditions. As shown in Fig. 6, real-time PCR and ELISA revealed that high glucose was able to induce CRP protein and mRNA expression. More importantly, although human TECs cultured with high glucose or CRP alone produced low-grade inflammatory and fibrotic responses as demonstrated by upregulation of pro-inflammatory cytokines (IL-1β, TNFα) and pro-fibrotic growth factors (TGF-β1, CTGF), the combination of CRP and high glucose had an additive effect on upregulation of pro-inflammatory cytokines IL-1β and TNFα, and of pro-fibrotic growth factors TGF-β1 and CTGF (Fig. 6c–f).

We then examined the mechanism by which CRP induces renal inflammation and fibrosis in vitro. It was apparent that CRP alone was able to upregulate pro-inflammatory cytokine (IL-1β, TNFα) and pro-fibrotic growth factor (TGF-β1, CTGF) production by renal tubular epithelial cells (HK-2) in a time- and dosage-dependent manner, with production being increased at 3 h and peaking from 6 to 12 h (ESM Fig. 3). Monocyte chemoattractant protein-1 (MCP-1) abundance was also significantly increased in a dose-dependent manner after CRP stimulation, as shown in ESM Fig. 3e. Blockade of binding of human CRP to its receptors with neutralising antibodies to

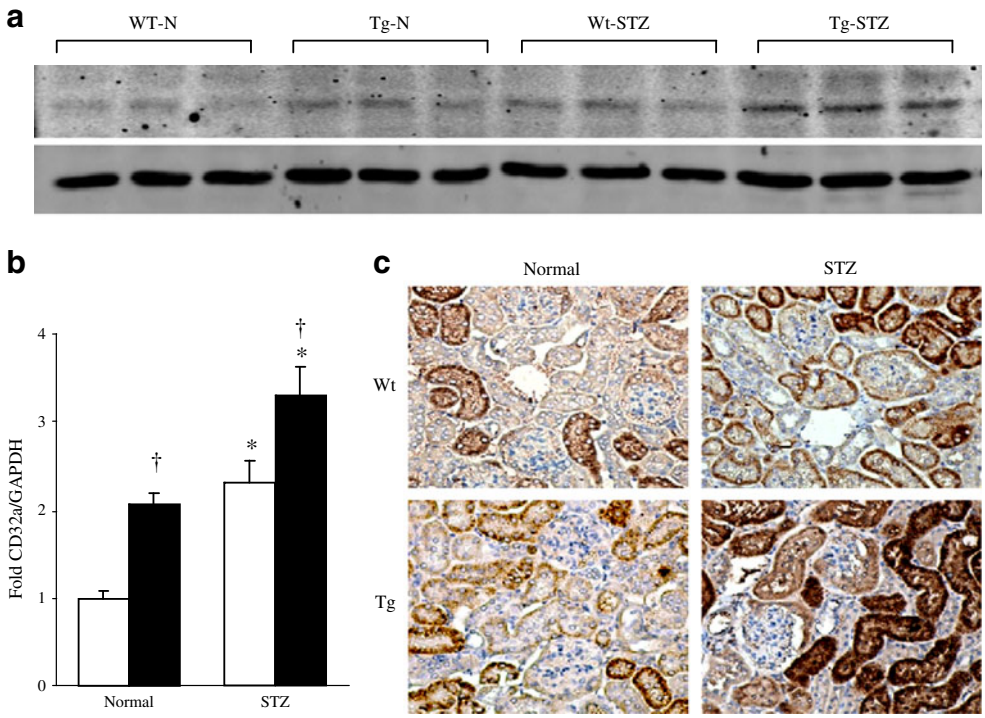

Fig. 4 CD32a abundance is enhanced in diabetic kidneys of *CRP* Tg mice. **a** Western blot and (**b**) semi-quantitative analysis of CD32a in Wt (white bars) and Tg (black bars) mice. **c** Immunohistochemical analysis of CD32a; magnification ×400. Results reveal that CD32a protein was significantly increased in the diabetic kidney of Wt mice and this was further enhanced in diabetic *CRP* Tg mice. Interestingly, compared with normal Wt mice, CD32a was readily upregulated in normal *CRP* Tg mice. Note that CD32a was largely expressed by glomerular and tubular epithelial cells. Each lane (**a**) or image (**c**) is representative of a group of eight mice; data (**b**) are expressed as mean±SEM for a group of eight mice. *$p<0.05$ vs normal; †$p<0.05$ vs Wt mice. STZ, streptozotocin

either CD64 or CD32a was able to partially abolish CRP-stimulated expression of pro-inflammatory cytokines (*Il1b*, *Tnf*α) and growth factors (*Tgfb1*, *Ctgf*) in human TECs (ESM Fig. 4). Interestingly, ELISA analysis revealed that addition of neutralising antibodies to CD64 or CD32a was also capable of inhibiting high glucose-induced TNFα and TGF-β1 production (ESM Fig. 5), suggesting a pathogenic importance for CRP signalling in the pathogenesis of renal inflammation and fibrosis under diabetic conditions.

Discussion

The present study provides direct biological evidence for the pathogenic importance of human CRP in DKD. We found that diabetic kidney injury was worsened in *CRP* Tg mice compared with Wt, as demonstrated by a significant increase in UAE and *Kim-1* expression. We also found that enhanced diabetic renal injury in *CRP* Tg mice was associated with a pronounced upregulation of renal inflammation and fibrosis, which is mediated through activation of TGF-β/SMAD and NF-κB signalling pathways. Together, these findings suggest that CRP may not only be a biomarker, but also a mediator in the development of DKD.

There are several mechanisms through which CRP may promote DKD. First, enhanced renal inflammation may be a mechanism by which CRP promotes diabetic kidney injury. It has been shown that the NF-κB signalling pathway is activated in human diabetic nephropathy and CRP is capable of inducing production of pro-inflammatory cytokines such as IL-1β and TNFα in cultured monocytes or endothelial cells via an NF-κB-dependent mechanism [26, 29–32]. In the present study, we also demonstrated that renal inflammation, including IL-1β and TNFα production, and $CD3^+$ T cell and F4/80+ macrophage infiltration, was significantly enhanced in the diabetic kidney of *CRP* Tg mice compared with diabetic Wt mice. In vitro, CRP alone induced production of pro-inflammatory cytokines such as IL-1β and TNFα. We also found that CRP stimulates MCP-1 production by HK-2 cells. These results are consistent with other studies in human endothelial cells and monocytes [33, 34]. More importantly, CRP itself was induced by high glucose, which, in turn, promoted high glucose-mediated renal inflammation. This finding suggests that CRP may function as an inflammatory mediator or cofactor of high glucose to promote diabetic renal inflammation. Importantly, inhibition of high glucose-induced TNFα production by blockade of CRP signalling (with neutralising antibodies to CD32a and CD64) provided additional evidence of the pathogenic activity of CRP in renal inflammation under diabetic conditions. Enhanced activation of the NF-κB signalling pathway in the diabetic kidney of *CRP* Tg mice, coupled with the ability of CD64- and CD32a-neutralising antibodies to block CRP-induced IL-1β and TNFα production, suggests that CRP probably acts via the CD64/CD32a–NF-κB pathway to exacerbate renal inflammation. This is consistent with previous reports [26, 31, 32].

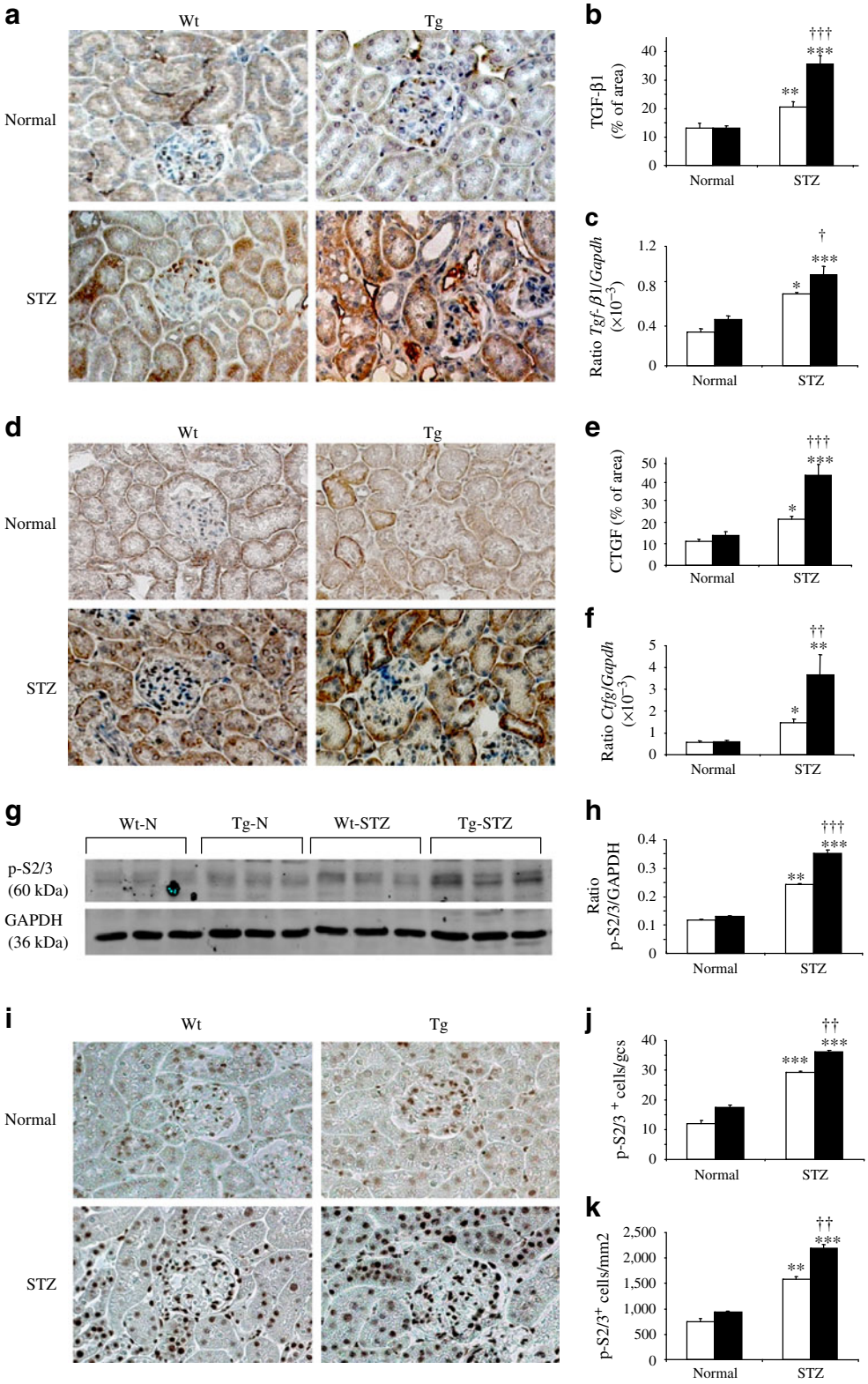

Fig. 5 Mechanisms of enhanced renal fibrosis in *CRP* Tg mice with diabetes. **a** Immunohistochemical staining and (**b**) quantitative analysis of TGF-β1 in Wt (white bars) and Tg (black bars) mice. **c** Real-time PCR analysis of *Tgfb1* mRNA expression. **d** Immunohistochemical staining and (**e**) quantitative analysis of CTGF. **f** Real-time PCR analysis of *Ctgf* mRNA expression. Results show that increased TGF-β1 and CTGF protein levels and mRNA expression in diabetic Wt mice was further enhanced in *CRP* Tg mice. **g** Western blot analysis and (**h**) semi-quantitative data of phospho-SMAD2/3. **i** Immunohistochemistry and semi-quantitative data of phospho-SMAD2/3 (p-S2/3) nuclear translocation in (**j**) glomeruli and(**k**) tubulointerstitium. Results show that increased SMAD2/3 phosphorylation and nuclear translocation in diabetic Wt mice were further enhanced in diabetic kidneys of *CRP* Tg mice. Magnification (**a**, **d**, **i**) ×400; data (**b**, **c**, **e**, **f**, **h**, **j**, **k**) represent the mean±SEM for groups of eight or six animals. *$p<0.05$, **$p<0.01$, ***$p<0.001$ versus normal; $^{\dagger}p<0.05$, $^{\dagger\dagger}p<0.01$, $^{\dagger\dagger\dagger}p<0.001$ versus diabetic Wt mice. gcs, glomerular cross-section; STZ, streptozotocin

Enhancement of renal fibrosis in DKD might be achieved via increased TGF-β/SMAD signalling. Renal fibrosis, characterised by increased accumulation of extracellular matrix within glomeruli and tubulointerstitium, is a hallmark of the final common pathway leading to loss of renal function associated with DKD. Activation of TGF-β/SMAD signalling has been shown in experimental models and in human diabetic kidneys [24, 25, 27, 28], and is

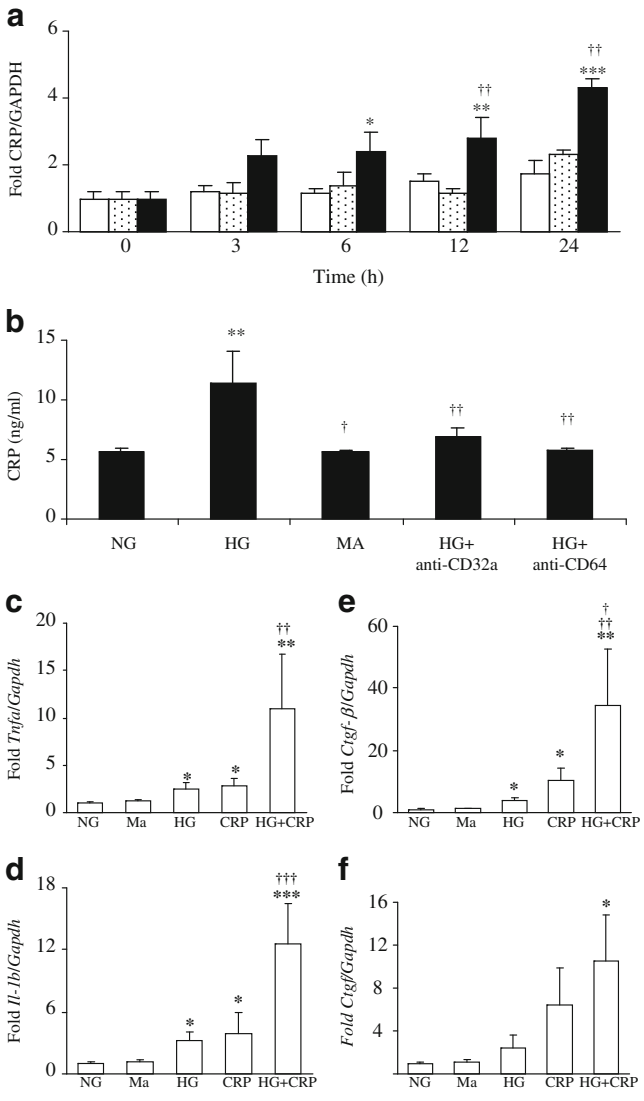

Fig. 6 CRP is induced by high glucose, but promotes high glucose-stimulated pro-inflammatory and pro-fibrotic responses in human TECs (HK-2). **a** High glucose (35 mmol/l) (HG, black bars), but not mannitol (35 mmol/l) (MA, dotted bars), induces *CRP* mRNA expression in HK-2 TECs in a time-dependent manner. NG, normal glucose. **b** ELISA revealed that addition of HG (35 mmol/l), but not mannitol (35 mmol/l), induced CRP protein production at 24 h after stimulation, a process that was blocked by addition of neutralising antibodies to CD32a and CD64. **c** Addition of HG (35 mmol/l) or CRP (10 μg/ml) alone, but not mannitol (35 mmol/l), was able to induce *Tnfα*, *Il1b* (**d**), *Tgfb1* (**e**) and *Ctgf* (**f**) mRNA expression, which was additively increased when combined together. Data represent the mean±SEM for at least three independent experiments. *$p<0.05$, **$p<0.01$, ***$p<0.001$ versus normal; $^{\dagger}p<0.05$, $^{\dagger\dagger}p<0.01$, $^{\dagger\dagger\dagger}p<0.001$ versus HG or CRP alone

responsible for extracellular matrix production in vitro under high glucose and AGEs conditions [25, 35]. Thus, blockade of TGF-β with neutralising anti-TGF-β antibodies and targeting of the TGF-β/SMAD signalling pathway by deletion of SMAD3 were able to inhibit diabetic kidney injury [27, 28, 36, 37]. A novel and significant finding in the present study was that CRP alone was able to induce pro-fibrotic growth factor production, including that of TGF-β1 and CTGF, which was further enhanced in the presence of high glucose, indicating that CRP may act as a pro-fibrotic mediator that induces and promotes renal fibrosis under diabetic conditions. This may account for enhanced TGF-β/SMAD signalling in the diabetic kidney of *CRP* Tg mice. The ability of neutralising antibodies to CD32a or CD64 to block CRP- or high glucose-induced renal TGF-β as well as CTGF production revealed that CRP may bind CD32a or CD64, thus mediating renal fibrosis through the TGF-β signalling pathway under high glucose conditions.

Enhanced insulin resistance may be another plausible mechanism by which CRP worsens diabetic kidney injury. In patients with type 2 diabetes, for example, insulin resistance is associated with CRP [38]. This relationship holds in human *CRP* Tg mice, in which overexpression of human *CRP* promotes the development of insulin resistance under high-fat diet conditions [39]. Similar results have also been obtained by other studies, showing that increased CRP causes insulin resistance by impairing skeletal muscle glucose uptake, which is mediated by Fc γ receptor IIB [40]. Thus, it is likely that increased CRP and metabolic alterations may synergistically mediate diabetic kidney injury in *CRP* Tg mice, although this is not yet proven and further studies are warranted.

Finally, we found no evidence that hypertension developed in human *CRP* Tg mice under normal or diabetic conditions. This is consistent with previous reports that in human *CRP* Tg mice atherosclerosis and ischaemia-induced cardiac remodelling can be worsened without alteration of blood pressure [17, 41]. This is further supported by our recent finding that human *CRP* Tg mice had normal blood pressure, but promoted angiotensin II-induced cardiac remodelling under hypertensive conditions similarly to that seen in littermate non-Tg mice [18]. In contrast, mice overexpressing rabbit *CRP* and rats after adeno-associated virus-mediated human *CRP* gene transfer did develop hypertension [42, 43]. The mechanisms leading to discrepancy in the development of hypertension in animals that overexpress *CRP* remain largely unclear. The question of whether the genetic variation in response to CRP between the species contributes to the development of hypertension can only be answered by further investigations.

In summary, CRP may exacerbate the kidney complications of diabetes by promoting renal inflammation and fibrosis, a process achieved via the CD32a- and CD64-mediated activation of NF-κB and TGF-β/SMAD signalling pathways. These results suggest that targeted lowering of CRP might be of therapeutic benefit in DKD.

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Contribution statement F.L. and H.Y.C. conceived experiments, analysed data and drafted the article. X.R.H and A.J.S. generated *CRP* Tg mice, conceived experiments and revised the manuscript. A.C.K. C., L Z. and P.F. analysed data and revised the manuscript. H.Y.L. designed, supervised and wrote the article. All authors approved the final version of the manuscript.

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

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