

Additive renoprotective effects of B2-kinin receptor blocker and PPAR- γ agonist in uninephrectomized *db/db* mice

Sydney CW Tang¹, Loretta YY Chan¹, Joseph CK Leung¹, Amy Shan Cheng¹, Hui Yao Lan² and Kar Neng Lai¹

We recently showed that the bradykinin B2 receptor (B2R) blocker icatibant (Icat) and the peroxisome proliferator-activated receptor- γ agonist rosiglitazone (Ros) exerted anti-inflammatory effects in renal tubular cells exposed to a diabetic milieu. This study aims to explore whether these effects can be translated to an experimental model of type 2 diabetic nephropathy (DN). *db/db* mice and their nondiabetic *db/m* littermates underwent sham operation or uninephrectomy (Unx) at 10 weeks and received vehicle (Veh), metformin (Met), Icat, Ros, or Icat plus Ros for 8 weeks before killing. Among the *db/db* group with Unx, mice that received Icat or Ros had significantly lower serum creatinine and albuminuria, which was further reduced when Icat and Ros were given in combination. These beneficial effects were not observed in the Met group that achieved similar glycemic control as Ros-treated animals. Likewise, the severity of reactive glomerular and proximal tubular hypertrophy, glomerulosclerosis, interstitial injury, cortical F4/80 and α -smooth muscle actin immunostaining, and CCL-2, ICAM-1 and TGF- β overexpression were all attenuated by Icat and Ros, and these effects were enhanced when both agents were combined. Immunohistochemical staining confirmed the proximal tubular expression of CCL-2 (inflammation) and TGF- β (fibrosis). Treatment with Icat was associated with decreased B2R, but increased, B1R expression, which was exaggerated in Unx animals. At the signaling level, Icat and Ros reduced extracellular signal-regulated kinase 1/2 and STAT1 activation, respectively. Our results suggest a deleterious role of the kallikrein-kinin system in murine-accelerated DN, which can be ameliorated by the B2R blocker Icat and enhanced by the addition of Ros. This calls for further evaluation of this novel therapeutic approach in more animal models of diabetic nephropathy.

Laboratory Investigation (2011) 91, 1351–1362; doi:10.1038/labinvest.2011.81; published online 2 May 2011

KEYWORDS: B2-kinin receptor blockade; diabetic nephropathy; inflammation; PPAR- γ agonist; renal pathology; signaling; uninephrectomy

Apart from the renin-angiotensin system, emerging literature suggests that the related kallikrein-kinin system (KKS)¹ may have an equally important pathogenetic role in diabetic nephropathy (DN) due to type 2 diabetes mellitus (T2DM). Most of the physiological actions of the KKS are attributed to the generation of bradykinin (BK), which, together with the related kinins, possess a wide range of pharmacological actions and are believed to be important mediators of inflammation.² Kinins induce their effects via two main types of receptor, designated B1 and B2, both of which belong to the superfamily of G protein-coupled receptors. BK is a selective ligand at the B2 receptor (B2R), which is present in a wide variety of tissues including the kidney. We recently

showed that high glucose (HG) induced B2R expression in cultured renal proximal tubular cells, and that BK stimulated the expression of proinflammatory and profibrotic cytokines in these cells via extracellular signal-regulated kinase (ERK)1/2 activation.³ Application of the B2R blocker, icatibant (Icat) (HOE 140), downregulated BK- and HG-induced phospho-ERK1/2 signals and the downstream secretion of IL-6, CCL-2, and TGF- β in cultured tubular cells.³

Peroxisome proliferator-activated receptors (PPARs) are a family of ligand-activated nuclear hormone receptors belonging to the steroid receptor superfamily.⁴ We have previously reported that rosiglitazone (Ros), a specific synthetic ligand of PPAR- γ , partially attenuated advanced

¹Division of Nephrology, Department of Medicine, Queen Mary Hospital, The University of Hong Kong, Hong Kong and ²Department of Medicine and Therapeutics, and Li Ka Shing Institute of Health Sciences, The Chinese University of Hong Kong, Hong Kong
Correspondence: Dr SCW Tang, Division of Nephrology, Department of Medicine, Queen Mary Hospital, The University of Hong Kong, 102 Pokfulam Road, Hong Kong. E-mail: scwtang@hku.hk

Received 25 August 2010; revised 6 December 2010; accepted 9 January 2011

glycation end-product-induced tubular inflammation through inhibition of the signal transducer and activator of transcription (JAK/STAT) signal.⁵ More recently, we demonstrated that Ros attenuated HG-induced protein kinase C, and together with Icat, conferred additive anti-inflammatory effects in cultured tubular cells exposed to HG,³ and restored nephrin depletion *in vivo*.⁶

Taken together, these *in vitro* findings suggest a potential therapeutic role of antagonizing B2R or stimulating PPAR- γ in DN, and that this combination may confer renoprotection. In this report, we explored the true implication of these *in vitro* findings in an accelerated model of murine DN that we recently used,⁶ namely the uninephrectomized *db/db* mouse.

MATERIALS AND METHODS

Antibodies and Reagents

Antibodies were rat anti-mouse F4/80 (Serotec, Oxford, UK); rabbit anti-mouse alpha-smooth muscle actin, CCL2 (formerly MCP-1), mouse monoclonal p-ERK1/2 and ERK1/2 (Abcam, Cambridge, UK); rabbit anti-mouse p-STAT1 and STAT1 (Cell Signaling Technology, Danver, MA, USA); mouse monoclonal B2R (BD Biosciences, San Jose, CA, USA); goat anti-mouse CCL2, ICAM-1 and rabbit anti-mouse B1R, TGF- β (Santa Cruz Biotechnology, Santa Cruz, CA, USA); and goat anti-mouse actin (Dako, Carpinteria, CA, USA). Isotype-matched antibodies were used as negative controls. Metformin was from Sigma (St Louis, MO, USA). The B2R receptor antagonist, Icat, and the synthetic PPAR- γ agonist, Ros were both kind gifts from Sanofi-Aventis Deutschland GmbH and GlaxoSmithKline (Compound Management Division, Stevenage, Herts, UK), respectively.

Experimental Design

All work with mice was approved by the Committee on the Use of Live Animals in Teaching and Research of The University of Hong Kong, and were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animal. Genetically diabetic male *db/db* mice and their nondiabetic *db/m* littermates on a C57.BLKS background (purchased from The Jackson Laboratory, Bar Harbor, ME, USA) underwent uninephrectomy (Unx) or sham operation at age of 10 weeks. Unx was performed in anesthetized animals through a right lateral skin incision after shaving off the overlying fur. An incision was made on the abdominal wall. The right renal pedicle was identified and ligated, followed by total removal of the right kidney. The abdominal wall and skin was then sutured. The animal was then warmed until full recovery from anesthesia. Sham-operated animals had their right renal pedicles manipulated without ligation. From week 11, *db/db* and *db/m* animals were randomly divided into the following treatment groups ($n=7$ each): for *db/m* animals; group 1, sham operation and vehicle (Veh); group 2, Unx and Veh; group 3, Unx and Met (100 mg/kg/day by oral gavage); group 4, Unx and Icat (1 mg/kg/day subcutaneously); group 5, Unx and Ros (10 mg/kg/day by oral gavage); and group 6,

Unx and Icat plus Ros. For both sham-operated or Unx *db/db* animals: group 1, Veh; group 2, Met (100 mg/kg/day by oral gavage); group 3, Icat (1 mg/kg/day subcutaneously); group 4, Ros (10 mg/kg/day by oral gavage); and group 5, Icat plus Ros. The dosage of Met was predetermined in pilot experiments to achieve a similar level of blood glucose control as Ros treatment. After 8 weeks (at 19 weeks of age), animals were killed. Twenty-four hour urine samples were collected in a metabolic cage at baseline and one day before killing. Blood samples were drawn via the tail vein at baseline and by transthoracic cardiac puncture after anesthesia before killing. After killing, the left kidney was rapidly collected and transversely bisected. The cortex from half of kidney was snap-frozen in liquid nitrogen and stored at -70°C for protein extraction. The other half was fixed with 10% buffered formalin and embedded in paraffin for histological evaluation.

Biochemical Analyses of Serum and Urine Samples

Albumin, creatinine, and glucose concentration in urine and serum samples were measured by mouse albumin ELISA quantitation kit (Bethyl Laboratories, Montgomery, TX, USA), creatinine test kit using enzymatic method (Stanbio Laboratory, Boerne, TX, USA), and glucose test kit (Stanbio), respectively.

Renal Histopathology

Four-micrometer thick paraffin-embedded sections were deparaffinized with xylene and rehydrated through a descending ethanol gradient. Histology was examined following Periodic acid-Schiff (PAS) staining, and graded as we recently described.⁷ Twenty high-power fields ($\times 400$) of renal cortex were randomly selected for assessing tubular (atrophy, casts, and vacuolization) and interstitial changes (fibrosis and inflammation), and graded from 0 to 5 (tubulointerstitial area in the cortex was graded as: 0, normal; 1, area of interstitial inflammation and fibrosis, tubular atrophy and vacuolization involving $<10\%$; 2, lesion area between 10 and 20%; 3, lesion area between 20 and 30%; 4, lesion area between 30 and 40%; and 5, lesions involving $>40\%$ of the field). Fifty randomly selected glomeruli were assessed for glomerular damage (well-developed exudative, mesangial proliferation and glomeruli hypertrophy), and graded as: 0, normal; 1, slight glomerular damage of the mesangial matrix and/or hyalinosis with focal adhesion involving $<10\%$ of the glomerulus; 2, sclerosis of 10–20%; 3, sclerosis of 20–30%; and 4, sclerosis of 30–40%; and 5, sclerosis $>40\%$ of the glomerulus. All scoring was performed in a blinded manner.

For morphometric analysis of glomerular and tubular hypertrophy, glomerular tuft area and proximal tubular area were measured in paraffin-embedded, PAS-stained sections with computer image analysis. The value was expressed as μm^2 from the mean of 50 glomeruli or 100 proximal tubules measured for each animal.

Table 1 Physical and biochemical parameters of *db/m* and *db/db* animals at baseline and after 8 weeks of treatment

	<i>db/m</i> mice						<i>db/db</i> mice									
	<i>Sham</i>		<i>Unx</i>				<i>Sham</i>		<i>Unx</i>							
	<i>Veh</i>	<i>Veh</i>	<i>Met</i>	<i>Icat</i>	<i>Ros</i>	<i>Icat+Ros</i>	<i>Veh</i>	<i>Met</i>	<i>Icat</i>	<i>Ros</i>	<i>Icat+Ros</i>	<i>Veh</i>	<i>Met</i>	<i>Icat</i>	<i>Ros</i>	<i>Icat+Ros</i>
<i>Body Weight* (g)</i>																
Baseline	25.6 ± 0.8	25.9 ± 0.8	25.5 ± 0.8	25.2 ± 1.0	25.8 ± 0.8	25.2 ± 1.2	41.7 ± 1.1	42.2 ± 0.8	41.6 ± 0.9	42.1 ± 0.9	41.7 ± 1.0	41.6 ± 1.4	42.8 ± 0.7	41.3 ± 1.2	42.0 ± 1.1	41.4 ± 1.3
8 weeks	28.5 ± 0.7	28.2 ± 0.8	28.8 ± 0.8	28.7 ± 0.7	28.6 ± 1.0	28.5 ± 1.0	50.7 ± 1.2	47.1 ± 0.9 [†]	50.6 ± 0.9	52.4 ± 1.1	52.2 ± 0.9	50.9 ± 0.9	46.8 ± 1.1 [†]	50.8 ± 0.9	52.7 ± 1.1	52.3 ± 1.3
<i>Blood glucose* (mg/dl)</i>																
Baseline	120 ± 6	124 ± 5	123 ± 6	123 ± 4	120 ± 6	122 ± 5	427 ± 11	427 ± 8	424 ± 8	425 ± 12	427 ± 7	424 ± 12	421 ± 9	427 ± 12	423 ± 11	425 ± 11
8 weeks	124 ± 4	124 ± 4	122 ± 7	123 ± 5	126 ± 4	126 ± 5	632 ± 13	248 ± 14 [†]	628 ± 10	235 ± 8 [†]	230 ± 8 [†]	633 ± 10	246 ± 13 [†]	636 ± 10	237 ± 12 [†]	230 ± 11 [†]
<i>Serum creatinine* (mg/dl)</i>																
Baseline	0.14 ± 0.03	0.15 ± 0.02	0.15 ± 0.03	0.15 ± 0.02	0.15 ± 0.02	0.14 ± 0.03	0.17 ± 0.02	0.17 ± 0.02	0.18 ± 0.02	0.17 ± 0.02	0.17 ± 0.02	0.18 ± 0.02	0.19 ± 0.02	0.17 ± 0.02	0.18 ± 0.02	0.17 ± 0.02
8 weeks	0.16 ± 0.03	0.19 ± 0.02	0.17 ± 0.02	0.17 ± 0.02	0.17 ± 0.02	0.17 ± 0.02	0.25 ± 0.03	0.25 ± 0.02	0.25 ± 0.01	0.24 ± 0.02	0.23 ± 0.02	0.35 ± 0.04 [‡]	0.36 ± 0.04 [‡]	0.32 ± 0.04 ^{‡§}	0.29 ± 0.03 ^{‡§}	0.23 ± 0.02 ^{§#}
<i>UACR* (μg/mg)</i>																
Baseline	65 ± 10	66 ± 8	67 ± 7	65 ± 3	65 ± 6	66 ± 5	1468 ± 145	1453 ± 126	1450 ± 64	1451 ± 89	1452 ± 137	1434 ± 149	1373 ± 86	1442 ± 167	1461 ± 149	1450 ± 145
8 weeks	73 ± 7	133 ± 5	132 ± 8	133 ± 9	94 ± 5	97 ± 5	1716 ± 147	1709 ± 158	1696 ± 115	1678 ± 88	1661 ± 90	2178 ± 154 [‡]	2000 ± 76 [‡]	2016 ± 129 ^{‡§}	1839 ± 133 [§]	1586 ± 113 ^{§#}

Abbreviations: Icat, icanibant; Met, metformin; Ros, rosiglitazone; Sham, sham operation; UACR, urine albumin-to-creatinine ratio; Unx, uninephrectomy; Veh, vehicle.

* $P < 0.05$ for comparisons between the corresponding *db/db* and *db/m* groups; [†] $P < 0.05$ vs all other *db/db* groups at 8 weeks; [‡] $P < 0.05$ vs sham-operated *db/db* animals; [§] $P < 0.05$ vs Unx-Veh *db/db* animals;

[#] $P < 0.05$ vs Unx-Ros or Unx-Icat *db/db* animals.

Immunohistochemical Analyses

Immunoperoxidase staining for interstitial F4/80+ macrophages, α -smooth muscle actin, CCL2, and TGF- β was performed on formalin-fixed paraffin sections (4 μ m). Briefly, sections were incubated with 0.5% H₂O₂. Nonspecific binding was blocked with blocking buffer (5% normal goat serum and 3% BSA in PBS) for 30 min. The sections were incubated with primary antibodies (1:50 with F4/80, 1:40 with α -SMA, 1:100 with CCL2, and 1:30 with TGF- β) overnight. The bound rat anti-mouse F4/80 and rabbit anti-mouse α -SMA antibodies were detected with peroxidase anti-rat and anti-rabbit antibodies, respectively, and visualized in brown using DAB substrate from the Dako Envision Plus System. Isotype-matched antibodies corresponding to the primary antibodies (Santa Cruz Biotechnology) were used as negative

controls. Immunostained F4/80-expressing macrophages and α -SMA were assessed in an observer-blinded manner as we recently described.^{6,7} Interstitial F4/80+ macrophages were counted for 25 high power ($\times 400$) interstitial/tubular fields and expressed as number of positive cells/mm². Interstitial α -SMA was assessed from the percentage area of immunostaining within the interstitium by computer image analysis.

Immunoblotting Analyses

Total protein extracts were prepared from the cortex using solubilization buffer containing 10 mM Tris, 150 mM NaCl, 5 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, 1% Triton X-100 and complete protease inhibitors. The extracts (5 μ g) were electrophoresed through 15% SDS-PAGE gel.

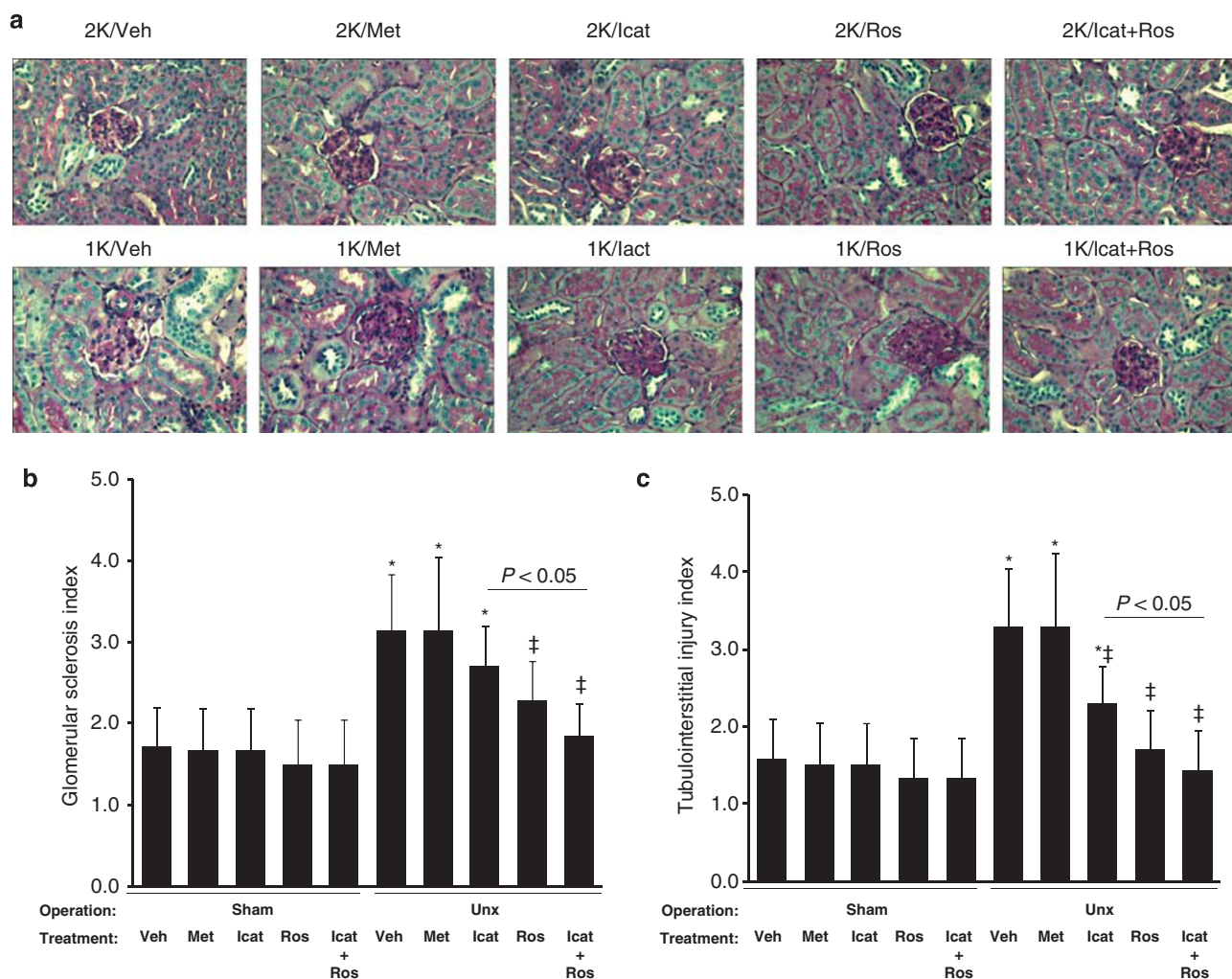


Figure 1 Morphological changes. (a) Representative renal cortical sections in *db/db* mice after sham operation (2K) or uninephrectomy (Unx) (1K) and treatment with vehicle (Veh), metformin (Met), icatibant (Icat), rosiglitazone (Ros), or Icat plus Ros for 8 weeks. Periodic acid-Schiff (PAS), objective: $\times 400$. (b) Glomerulosclerosis ($n = 7$). Fifty glomeruli were assessed in an observer-blinded manner for glomerular lesions (see text for definitions). * $P < 0.05$ vs the corresponding group of sham-operated mice; † $P < 0.05$ vs Unx-Veh or Unx-Met animals. (c) Tubulointerstitial injury ($n = 7$). Twenty high-power fields ($\times 400$) of renal cortex were randomly selected for assessing tubular (atrophy, casts, and vacuolization) and interstitial changes (fibrosis and inflammation), and graded from 0 to 5 (see text for definitions) in an observer-blinded manner. * $P < 0.05$ vs the corresponding group of sham-operated mice; † $P < 0.05$ vs uninephrectomy (Unx)-Veh or Unx-Met animals.

The proteins were transferred to PVDF membranes and probed with rabbit anti-mouse B1R (1:500 diluted), monoclonal anti-mouse B2R (1:1000 diluted), goat anti-mouse CCL2 (1:500 diluted), goat anti-mouse ICAM-1 (1:500 diluted), rabbit anti-mouse TGF- β (1:500 diluted), monoclonal anti-mouse p-ERK1/2 (1:1000 diluted), monoclonal anti-mouse ERK1/2 (1:1000 diluted), rabbit anti-mouse p-STAT1 (1:200 diluted), rabbit anti-mouse STAT1 (1:200 diluted), or goat anti-mouse actin (1:1000 diluted) in PBS-Tween for 16 h. The membrane was washed and incubated for 2 h at RT with peroxidase-labeled anti-goat, anti-mouse, or anti-rabbit immunoglobulins, and the antigen-antibody reaction was detected with ECL plus chemiluminescence (Amersham Pharmacia Biotech, Arlington, IL, USA). The immunoblots were measured by densitometry and expressed as ratio of average arbitrary-integrated values (units) after normalization with actin values.

Statistical Analysis

Animal data are presented as mean \pm s.d., and statistical differences were analyzed by three-way ANOVA followed by unpaired *t*-test with Bonferroni correction for multiple comparisons. *P*-values <0.05 were considered significant.

RESULTS

Physical and Biochemical Parameters

db/db mice were uniformly heavier than the corresponding *db/m* groups at baseline or after 8 weeks. All animals grew well and were alive before killing with steady body weight gain over the 8-week period, except that Met-treated mice had lesser weight gain than those that received other pharmacological agents (Table 1).

Biochemically, *db/db* mice had significantly higher blood glucose, serum creatinine, and albuminuria at baseline compared with *db/m* mice (Table 1). Unx had no observable effect on blood glucose levels in *db/m* or *db/db* mice. Among *db/db* animals, blood glucose levels were similar among different groups at baseline and increased significantly after 8 weeks, except that mice treated with Met or Ros had blood glucose lower than baseline levels after 8 weeks of treatment, regardless of whether Unx or sham operation was performed. Notably, there was no difference in blood glucose level between Met and Ros-treated animals (Table 1).

Among *db/m* animals that received Veh, Unx has no appreciable effect on serum creatinine and led to a modest rise in urine albumin excretion after 8 weeks compared with sham-operated animals. The effects of Unx on serum creatinine and albuminuria were markedly exaggerated in *db/db* mice compared with sham-operated *db/db* mice (Table 1). Compared with Met-treated Unx animals (Unx-Met), mice treated with Icat, Ros, or their combination had significantly lower serum creatinine. This renoprotective effect was more obvious with Icat/Ros combination than either agent alone (Table 1). Likewise, treatment with Icat or Ros reduced

albuminuria compared with Veh, and the effect was more profound in the Icat/Ros group. Among *db/db* animals without Unx, there was a numerical reduction in serum creatinine and albuminuria in the Icat/Ros group vs the control groups treated with Veh or Met only at 8 weeks. However, the difference did not reach statistical significance (Table 1).

Morphological Changes

Representative photomicrographs demonstrating renal histopathological changes following sham operation or Unx and various treatments for 8 weeks were shown in Figure 1a. Moderately advanced glomerulosclerosis and tubulointerstitial damage occurring after Unx in *db/db* mice was

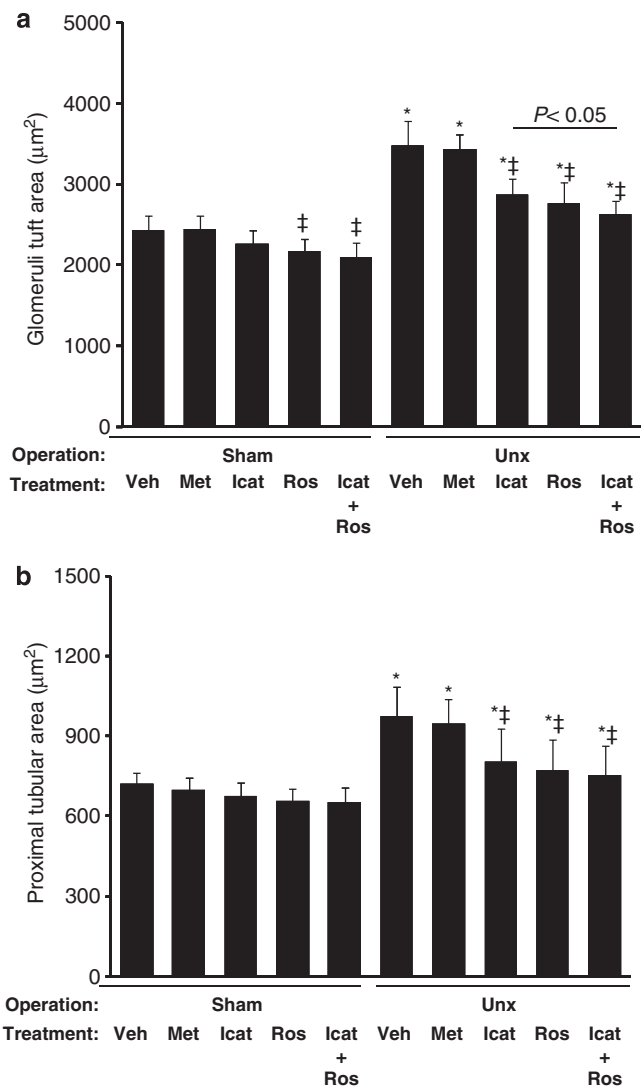


Figure 2 Morphometric measurements of glomerular tuft area (a) and proximal tubules area (b) in *db/db* mice after sham operation or uninephrectomy (Unx) and treatment with vehicle (Veh), metformin (Met), icatibant (Icat), rosiglitazone (Ros), or Icat plus Ros for 8 weeks (*n* = 7). **P* < 0.05 vs the corresponding group of sham-operated mice; ‡*P* < 0.05 vs Unx-Veh or Unx-Met animals in the same operation group.

attenuated by Icat or Ros but not with Met, and the Icat/Ros combination conferred an additive effect (Figures 1b and c). There was no appreciable treatment effect among sham-operated animals.

Glomerular Tuft and Proximal Tubule Areas

Both glomerular tuft and proximal tubule areas, assessed using computed-assisted morphometry, were increased following Unx, and the degree of which was significantly attenuated in mice treated with Icat or Ros (Figure 2). The additive effect of Icat and Ros combined was apparent in terms of glomerular hypertrophy. In addition, Ros or Icat/Ros gave rise to a small, but numerically, significant reduction in glomerular hypertrophy in *db/db* mice that did not undergo Unx (Figure 2).

Macrophage Infiltration

Macrophage infiltration into the interstitium was assessed by immunostaining for F4/80 in kidney sections. As shown in Figure 3, the increased F4/80-positive macrophages in Unx mice was attenuated by Icat or Ros treatment, and the effect was additive with Icat/Ros combination. These effects were not observed among animals that underwent sham operation without Unx.

Interstitial Fibrosis

There was diffuse α -smooth muscle actin staining in Unx mice. Treatment with Icat or Ros attenuated α -smooth muscle actin expression, and the effect was more pronounced in *db/db* mice treated with Icat/Ros combination (Figure 4). On the other hand, there was only low level of α -smooth

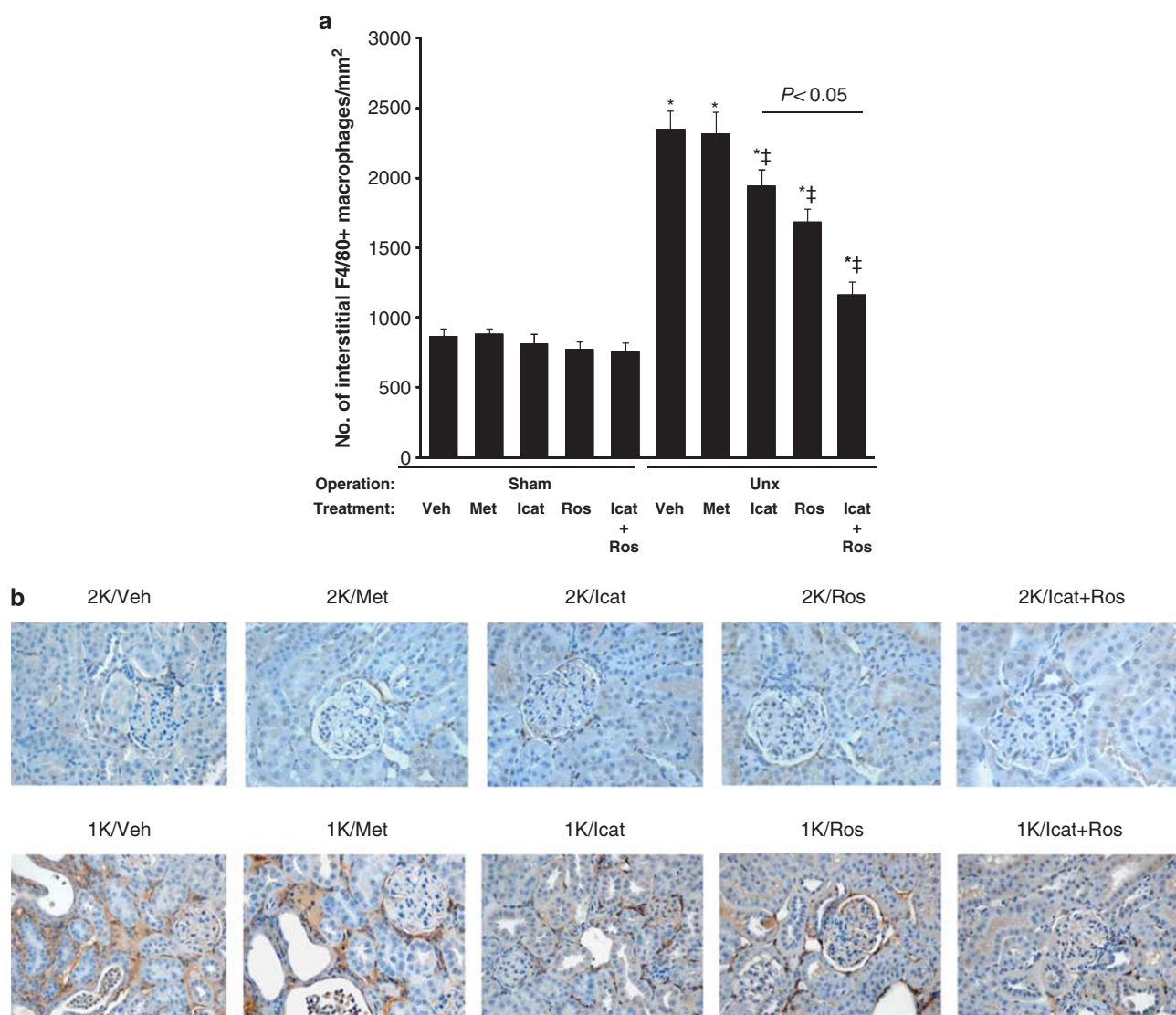


Figure 3 Macrophage infiltration. **(a)** Interstitial infiltration by F4/80-expressing cells in *db/db* mice after sham operation or uninephrectomy (Unx) and treatment with vehicle (Veh), metformin (Met), icatibant (Icat), rosiglitazone (Ros), or Icat plus Ros for 8 weeks ($n = 7$). $*P < 0.05$ vs the corresponding group of sham-operated mice; $^{\ddagger}P < 0.05$ vs Unx-Veh or Unx-Met animals. **(b)** Representative renal cortical sections of F4/80 immunostaining. 2K, sham-operated mouse with two intact kidneys; 1K, uninephrectomized mouse with one kidney. Objective: $\times 400$.

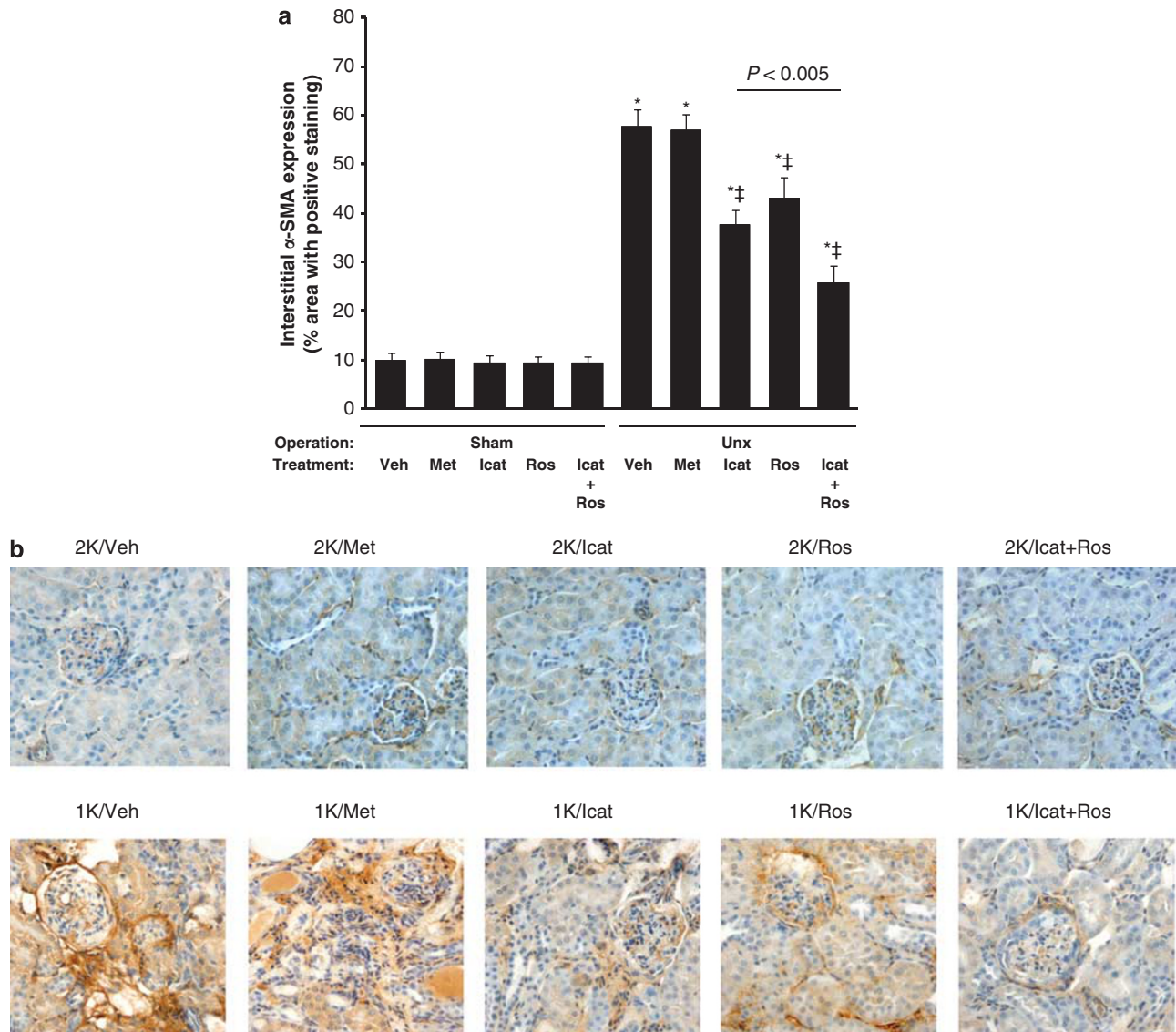


Figure 4 Interstitial fibrosis. (a) Immunostaining of α -smooth muscle actin in *db/db* mice after sham operation or uninephrectomy (Unx) and treatment with vehicle (Veh), metformin (Met), icatibant (Icat), rosiglitazone (Ros), or Icat plus Ros for 8 weeks ($n = 7$). * $P < 0.05$ vs the corresponding group of sham-operated mice; † $P < 0.05$ vs Unx-Veh or Unx-Met animals. (b) Representative renal cortical sections of α -smooth muscle actin immunostaining. 2K, sham-operated mouse with two intact kidneys; 1K, uninephrectomized mouse with one kidney. Objective: $\times 400$.

muscle actin expression in sham-operated animals and the various pharmacological treatments made no difference in its expression.

Expression of Cortical CCL2, ICAM-1, and TGF- β

CCL2, ICAM-1, and TGF- β may mediate interstitial inflammatory cell infiltration and fibrosis. Although their expression was uniformly upregulated after Unx in *db/db* animals, both Icat and Ros significantly suppressed the expression of these molecules compared with mice that received Veh only. The suppressive effect was more pronounced after treatment with Icat/Ros combination than either agent alone (Figure 5). These effects were not apparent among animals that underwent sham operation without Unx.

As we recently demonstrated upregulation of CCL2 and TGF- β in cultured proximal tubular cells to HG,³ we next performed immunohistochemical staining to localize these two cytokines in mouse kidney sections. Both CCL2 (proinflammatory) and TGF- β (profibrotic) were present in renal proximal tubules of *db/db* mice (Figure 6) and the staining intensity after various treatments mirrored those of the western blots as described above.

Expression of Cortical B1R and B2R

Expression of cortical B1R was upregulated after Unx across all treatment groups (Figure 7a). Treatment with Icat (either alone or combined with Ros) increased cortical B1R expression compared with Veh in either sham-operated or Unx

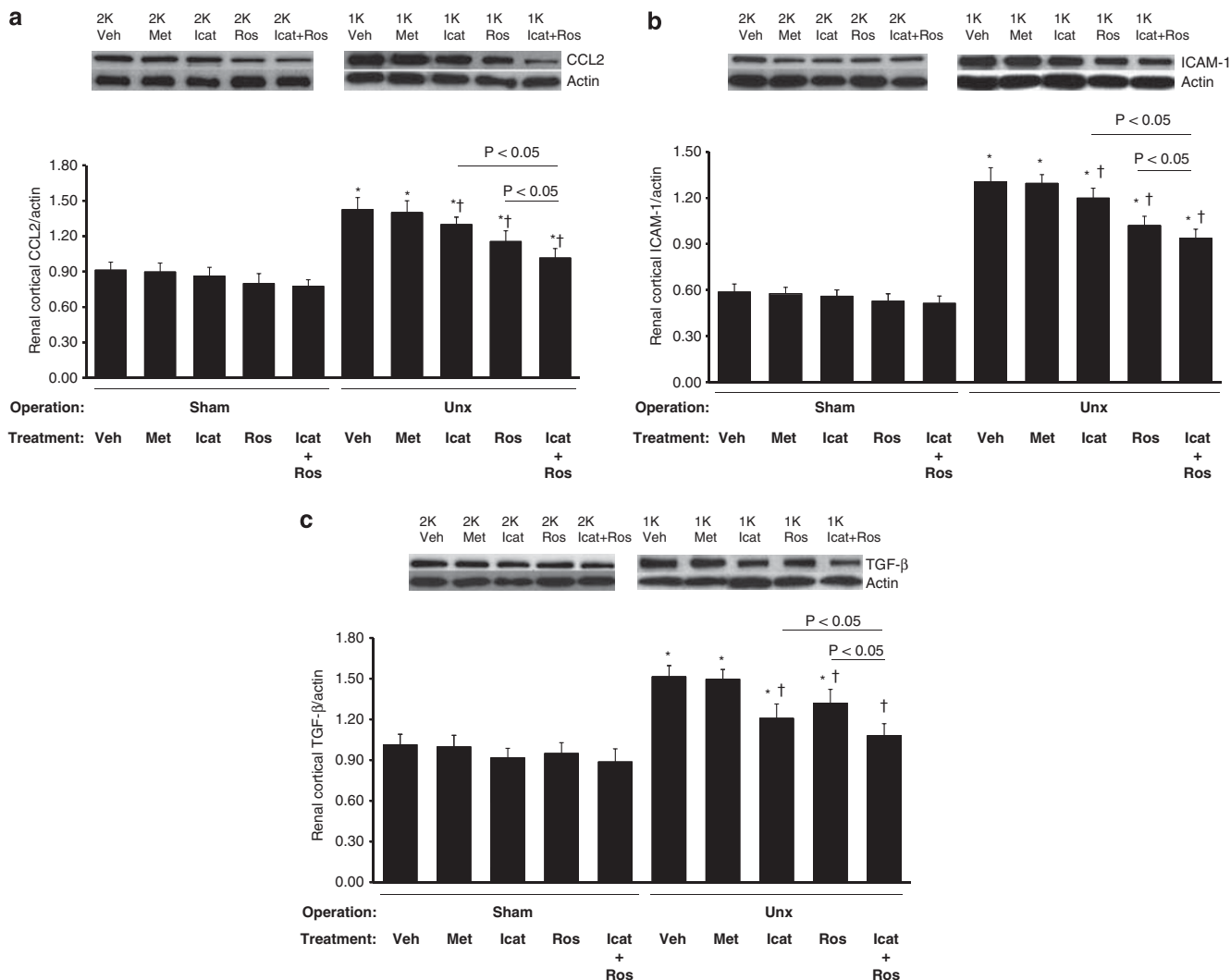


Figure 5 Renal cortical CCL2 (a), ICAM-1 (b), and TGF- β (c) expression in *db/db* mice after sham operation or uninephrectomy (Unx) and treatment with vehicle (Veh), icatibant (Icat), rosiglitazone (Ros), or Icat plus Ros for 8 weeks ($n = 7$). * $P < 0.05$ vs the corresponding group of sham-operated mice; † $P < 0.05$ vs Unx-Veh or Unx-Met animals. Panel above each chart: representative western blots. 2K, sham-operated mice with two intact kidneys; 1K, uninephrectomized mice with one kidney.

animals. Metformin or Ros alone had no effect on B1R expression in either operation group.

Expression of cortical B2R was also upregulated after Unx (Figure 7b). Treatment with Icat (either alone or combined with Ros) significantly reduced cortical B2R expression compared with Veh in either sham-operated or Unx animals. Metformin or Ros alone had no effect on B2R expression in either operation group.

ERK1/2 and STAT1 Signal Transduction

Compared with sham-operated mice, both phospho-ERK1/2 (Figure 8) and phospho-STAT1 (Figure 9) signals were upregulated in Unx-*db/db* animals. Treatment with Icat, but not Ros, Met, or Veh, significantly downregulated phospho-ERK1/2 activation in Unx animals. These effects were not

observed among Sham-*db/db* animals (Figure 8). On the other hand, phospho-STAT1 signals were significantly suppressed by Ros but not by Icat or other pharmacological treatments, and these effects were only observed among Unx, but not sham-operated, *db/db* mice (Figure 9).

DISCUSSION

This current study is an *in vivo* application of our recent *in vitro* observation of the anti-inflammatory action of Icat and Ros on renal tubular cells exposed to a diabetic milieu. When applied to an animal model of accelerated renal injury in *db/db* mice that resembles human T2DM, the *in vitro* results were reproduced *in vivo* in that serum creatinine and the degree of albuminuria were both lowered by Icat or Ros, and that these parameters were further improved when the two

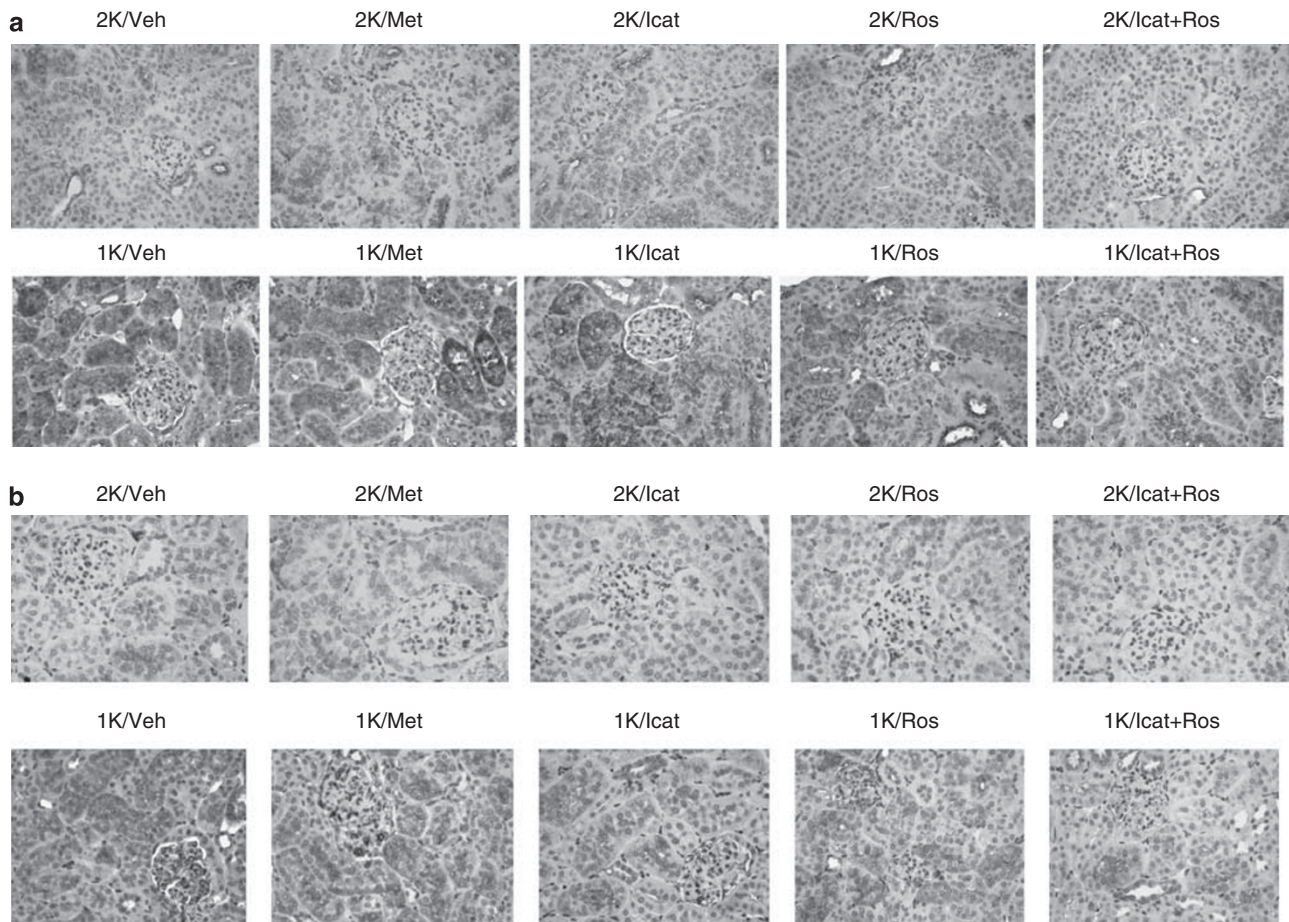


Figure 6 Immunohistochemical staining for CCL2 (a) and TGF- β (b) in *db/db* mice after sham operation or uninephrectomy (Unx) and treatment with vehicle (Veh), icatibant (Icat), rosiglitazone (Ros), or Icat plus Ros for 8 weeks. 2K, sham-operated mice with two intact kidneys; 1K, uninephrectomized mice with one kidney.

agents were combined, showing an additive effect. Of note is the fact that these actions were independent of glycemic control, as Met-treated animals, titrated to achieve a similar level of blood glucose, did not show these benefits. Furthermore, we used a control group of nondiabetic *db/m* animals that received Unx and the same pharmacological treatment as *db/db* animals. Unx *per se* in *db/m* littermates only brought about mild biochemical changes. On the other hand, the combined effect of Unx and diabetes in the *db/db* strain was one of robust biochemical derangement. This underscores that the renal disease in the Unx-*db/db* model is predominantly due to the effect of diabetes and not due to the effects of reduced nephron mass. Furthermore, *db/db* animals without Unx displayed only modest biochemical and histological renal disease in which the therapeutic effects of the studied pharmacological agents became difficult to interpret. This underscores the value of applying Unx to accelerate nephropathy in studying the therapeutic efficacy of any potential renoprotective regimen in this strain of murine diabetic model within a reasonable time frame (8 weeks in

the present study). One effect of Unx is compensatory hypertrophy of the remnant kidney. Here, we showed that glomerular and proximal tubule hypertrophy, secondary to Unx, was also attenuated by Icat or Ros treatment.

Indeed, participation of the KKS in DN has become increasingly apparent. We recently observed expression of kallikrein in the proximal tubules of human diabetic kidney tissue,³ whereas Campell *et al*⁸ reported increased plasma levels of tissue kallikrein in type 2 diabetic subjects. However, there are conflicting *in vitro* and animal data, as to whether KKS overactivation is good (protective) or bad (destructive) in DN. Data supporting a renoprotective role of KKS include the following: *in vitro*, BK has been shown to reduce mesangial cell (MC) proliferation under diabetic milieu.⁹ Fibronectin secretion induced by macrophage-conditioned medium was reduced by perindoprilat or BK treatment.¹⁰ *In vivo*, Akita diabetic mice with absent B2R had more severe albuminuria and glomerulosclerosis,¹¹ and senescence-associated phenotypes¹² than wild type (WT) animals. In streptozotocin (STZ)-induced rats, Icat significantly

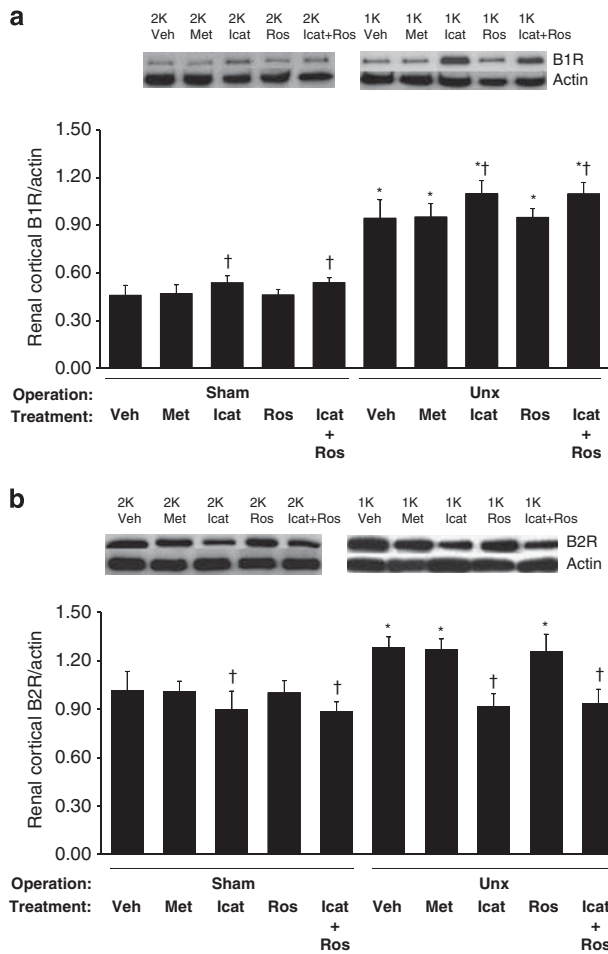


Figure 7 Renal cortical expression of the bradykinin receptors B1R (a) and B2R (b) in *db/db* mice after sham operation or uninephrectomy (Unx) and treatment with vehicle (Veh), icatibant (Icat), rosiglitazone (Ros), or Icat plus Ros for 8 weeks ($n = 7$). * $P < 0.05$ vs the corresponding group of sham-operated mice; [†] $P < 0.05$ vs animals that underwent the same operation and treated with vehicle, metformin (Met) or Ros. Upper panels: representative western blots. 2K, sham-operated mouse with two intact kidneys; 1K, uninephrectomized mouse with one kidney.

attenuated the anti-proteinuric effect of ramipril.¹³ More recently, Bodin *et al*¹⁴ showed that KLK-knock out STZ-induced mice had more severe albuminuria than WT. In an indirect manner, Buleon *et al*¹⁵ showed that B2R blockade reduced the renoprotective effect of ramipril in *db/db* mice.

In contrast, data supporting a deleterious role of KKS include: *in vitro*, BK induced proliferation of quiescent renal cells¹⁶ and upregulated CTGF, TGF- β , and collagen I mRNA via ERK1/2 activation in MC.¹⁷ *In vivo*, renal B2R and TGF- β mRNA expression was significantly increased in STZ-induced animals.¹⁷ Targeted deletion of B2R protected against the development of DN in which B2R $-/-$ mice injected with STZ displayed less albuminuria and glomerular and tubular injuries¹⁸ than WT animals. There was a compensatory rise in renal B1R expression in B2R $-/-$ mice. In STZ-induced

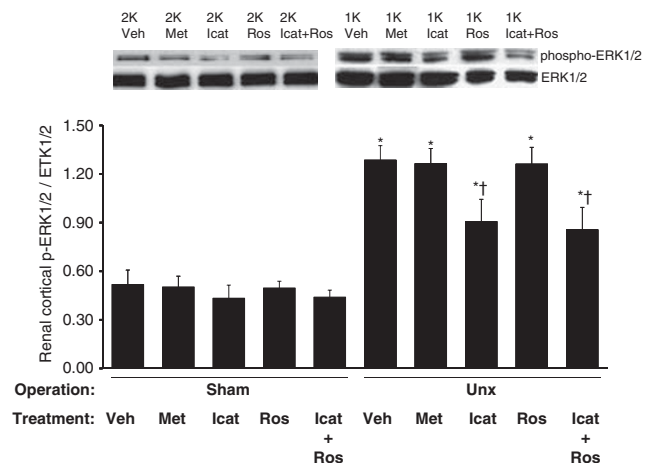


Figure 8 Renal cortical phospho-ERK1/2 signals after receiving sham operation or uninephrectomy (Unx) and treatment with vehicle (Veh), icatibant (Icat), rosiglitazone (Ros), or Icat plus Ros for 8 weeks ($n = 7$). * $P < 0.05$ vs the corresponding group of sham-operated mice; [†] $P < 0.05$ vs Unx-Veh or Unx-Met animals. Upper panels: representative western blots. 2K, sham-operated mouse with two intact kidneys; 1K, uninephrectomized mouse with one kidney.

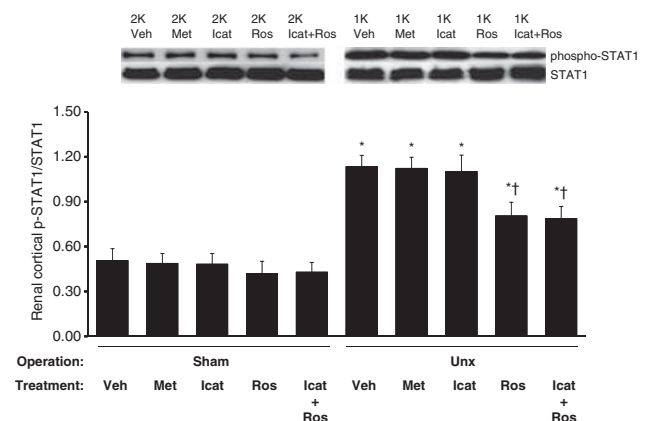


Figure 9 Renal cortical phospho-STAT1 signals after receiving sham operation or uninephrectomy (Unx) and treatment with vehicle (Veh), icatibant (Icat), rosiglitazone (Ros), or Icat plus Ros for 8 weeks ($n = 7$). * $P < 0.05$ vs the corresponding group of sham-operated mice; [†] $P < 0.05$ vs Unx-Veh or Unx-Met animals. Upper panels: representative western blots. 2K, sham-operated mouse with two intact kidneys; 1K, uninephrectomized mouse with one kidney.

diabetic hyperfiltrating rats, acute treatment with KLK inhibitor, aprotinin, nearly normalized GFR and renal blood flow,¹⁹ and normalized renal function,²⁰ whereas blocking B1R or B2R markedly reduced proteinuria.²¹

The discrepancies in these results may be related to the different cell types used and different genetic backgrounds of the studied animals. But more importantly, these animal models, directly exploring the effects of the KKS in DN, are mostly restricted to T1DM, such as via STZ induction or in Akita mice, though there is indirect data of the influence of

B2R blockade on ACE inhibition in T2DM. Studies directly exploring the effects of the KKS are lacking in a model of T2DM, which accounts for over 90% of the diabetic burden in the general population. Our results now provide a more definitive answer to the true effect of blocking B2R in an accelerated T2DN model. Although Icat evidently reduced B2R expression, B1R was upregulated in response to B2R blockade. This compensatory phenomenon is also observed by other researchers in mouse models.^{18,22,23} The exact role of the B1R in the diabetic kidney warrants further investigation.

Apart from biochemical improvement, there was also histological improvement, and reduced macrophage infiltration and interstitial fibrosis. This is not surprising considering the downregulation of the key molecules responsible for these changes, namely CCL-2, ICAM-1, and TGF- β , which is in keeping with our *in vitro* observation in proximal tubular cells.³ Here, we also confirmed that CCL-2 and TGF- β were localized to the proximal tubules of *db/db* mice. Moreover, the dose of Icat used here was based on previous studies showing efficacious blockade of the action of BK in a mouse model of angioedema²⁴ and in human subjects for prophylaxis against angioedema attacks.²⁵

Although Ros has been shown clinically to confer renoprotection in T2DM subjects,^{26,27} its mechanism of action is as yet not fully understood. We recently showed that in proximal tubular cells, Ros partially ameliorated AGE-induced CXCL8 and ICAM-1 overexpression,⁵ and HG-induced IL-6, CCL-2, VEGF, and TGF- β overexpression.³ In the current animal, such *in vitro* benefits were confirmed, independent of the glucose-lowering effect of Ros. Furthermore, we have also shown that Ros can restore nephrin depletion in the same model of accelerated murine DN.⁶

In addition to the independent efficacies of Icat and Ros, we also showed that the two agents provided additive renoprotective actions leading to both functional and histological improvements. This is not surprising considering the fact that Icat and Ros function through different signal transduction pathways. Our previous *in vitro* data showed that Icat intercepted the ERK1/2 pathway,³ whereas Ros attenuated STAT1⁵ and PKC³ overactivation, with paralleled attenuation of HG-induced IL-6, CCL-2, VEGF, and TGF- β overexpression. Here, we confirmed *in vivo* that ERK1/2 and STAT1 signals were attenuated by Icat and Ros, respectively.

In conclusion, our results suggest a deleterious role of the KKS in murine accelerated type 2 DN, which can be ameliorated by the B2R blocker Icat through downregulation of ERK1/2-driven CCL-2, ICAM-1, and TGF- β superinduction in the kidney. Such effects may be enhanced by the addition of Ros that intercepts STAT1 signaling. Our findings call for further evaluation of antagonizing the KKS as a potential therapeutic approach in more animal models of DMN.

ACKNOWLEDGEMENTS

This study is supported by a General Research Fund of the Research Grants Council (Grant number: HKU 7764/07M) of Hong Kong. The bradykinin B2 receptor antagonist, icatibant (HOE 140), and the synthetic PPAR- γ agonist, rosiglitazone were both kind gifts from Sanofi-Aventis Deutschland GmbH and GlaxoSmithKline (Compound Management Division, Stevenage, Herts, UK), respectively.

DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

1. Kakoki M, Smithies O. The kallikrein-kinin system in health and in diseases of the kidney. *Kidney Int* 2009;75:1019–1030.
2. Marceau F, Regoli D. Bradykinin receptor ligands: therapeutic perspectives. *Nat Rev Drug Discov* 2004;3:845–852.
3. Tang SC, Chan LY, Leung JC, *et al*. Bradykinin and high glucose promote renal tubular inflammation. *Nephrol Dial Transplant* 2010;25:698–710.
4. Evans RM. The steroid and thyroid hormone receptor superfamily. *Science* 1988;240:889–895.
5. Tang SC, Leung JC, Chan LY, *et al*. Activation of tubular epithelial cells in diabetic nephropathy and the role of the peroxisome proliferator-activated receptor- γ agonist. *J Am Soc Nephrol* 2006;17:1633–1643.
6. Tang SC, Leung JC, Chan LY, *et al*. Renoprotection by rosiglitazone in accelerated type 2 diabetic nephropathy: role of STAT1 inhibition and nephrin restoration. *Am J Nephrol* 2010;32:145–155.
7. Tang SC, Leung JC, Chan LY, *et al*. Angiotensin converting enzyme inhibitor but not angiotensin receptor blockade or statin ameliorates murine adriamycin nephropathy. *Kidney Int* 2008;73:288–299.
8. Campbell DJ, Kladis A, Zhang Y, *et al*. Increased tissue kallikrein levels in type 2 diabetes. *Diabetologia* 2010;53:779–785.
9. Alric C, Pecher C, Cellier E, *et al*. Inhibition of IGF-I-induced Erk 1 and 2 activation and mitogenesis in mesangial cells by bradykinin. *Kidney Int* 2002;62:412–421.
10. Pawluczyk IZ, Patel SR, Harris KP. The role of bradykinin in the antifibrotic actions of perindoprilat on human mesangial cells. *Kidney Int* 2004;65:1240–1251.
11. Kakoki M, Takahashi N, Jennette JC, *et al*. Diabetic nephropathy is markedly enhanced in mice lacking the bradykinin B2 receptor. *Proc Natl Acad Sci USA* 2004;101:13302–13305.
12. Kakoki M, Kizer CM, Yi X, *et al*. Senescence-associated phenotypes in Akita diabetic mice are enhanced by absence of bradykinin B2 receptors. *J Clin Invest* 2006;116:1302–1309.
13. Tschöpe C, Seidl U, Reinecke A, *et al*. Kinins are involved in the antiproteinuric effect of angiotensin-converting enzyme inhibition in experimental diabetic nephropathy. *Int Immunopharmacol* 2003;3:335–344.
14. Bodin S, Chollet C, Goncalves-Mendes N, *et al*. Kallikrein protects against microalbuminuria in experimental type I diabetes. *Kidney Int* 2009;76:395–403.
15. Buleon M, Allard J, Jaafar A, *et al*. Pharmacological blockade of B2-kinin receptor reduces renal protective effect of angiotensin-converting enzyme inhibition in *db/db* mice model. *Am J Physiol Renal Physiol* 2008;294:F1249–F1256.
16. Goldstein RH, Wall M. Activation of protein formation and cell division by bradykinin and des-Arg9-bradykinin. *J Biol Chem* 1984;259:9263–9268.
17. Tan Y, Wang B, Keum JS, *et al*. Mechanisms through which bradykinin promotes glomerular injury in diabetes. *Am J Physiol Renal Physiol* 2005;288:F483–F492.
18. Tan Y, Keum JS, Wang B, *et al*. Targeted deletion of B2-kinin receptors protects against the development of diabetic nephropathy. *Am J Physiol Renal Physiol* 2007;293:F1026–F1035.
19. Harvey JN, Jaffa AA, Margolius HS, *et al*. Renal kallikrein and hemodynamic abnormalities of diabetic kidney. *Diabetes* 1990;39:299–304.
20. Zuccollo A, Frontera M, Cueva F, *et al*. Effects of aprotinin on the kallikrein-kinin system in type I diabetes (insulinitis). *Immunopharmacology* 1997;37:251–256.

21. Zuccollo A, Navarro M, Catanzaro O. Effects of B1 and B2 kinin receptor antagonists in diabetic mice. *Can J Physiol Pharmacol* 1996;74: 586–589.
22. Duka I, Kintsurashvili E, Gavras I, *et al*. Vasoactive potential of the b(1) bradykinin receptor in normotension and hypertension. *Circ Res* 2001;88:275–281.
23. Griol-Charhbili V, Messadi-Laribi E, Bascands JL, *et al*. Role of tissue kallikrein in the cardioprotective effects of ischemic and pharmacological preconditioning in myocardial ischemia. *FASEB J* 2005;19:1172–1174.
24. Han ED, MacFarlane RC, Mulligan AN, *et al*. Increased vascular permeability in C1 inhibitor-deficient mice mediated by the bradykinin type 2 receptor. *J Clin Invest* 2002;109:1057–1063.
25. Deeks ED. Icatibant. *Drugs* 2010;70:73–81.
26. Miyazaki Y, Cersosimo E, Triplitt C, *et al*. Rosiglitazone decreases albuminuria in type 2 diabetic patients. *Kidney Int* 2007;72: 1367–1373.
27. Trivedi H, Lu N, Andresen BT, *et al*. Slower decline of renal function after initiation of rosiglitazone in diabetics – a pilot study. *Clin Nephrol* 2009;72:181–185.