Kallikrein protects against microalbuminuria in experimental type I diabetes

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Tissue kallikrein is the main kinin-forming enzyme in mammals, and differences in kinin levels are thought to be a contributing factor to diabetic nephropathy. Here, we determined the role of the kallikrein-kinin system in the pathogenesis of streptozotocin-induced diabetic nephropathy in wild-type and tissue kallikrein–knockout mice. All diabetic mice developed similar hyperglycemia, but the knockout mice had a significant two-fold increase in albuminuria compared to the wild-type mice before and after blood pressure elevation. Ezrin mRNA, a podocyte protein potentially implicated in albuminuria, was downregulated in the kidney of knockout mice. One month after induction of diabetes, the mRNAs of kininogen, tissue kallikrein, kinin B1, and B2 receptors were all increased up to two-fold in the kidney in both genotypes. Diabetes caused a 50% decrease in renal angiotensin-converting enzyme expression and a 20-fold increase in kidney injury molecule-1 reflecting tubular dysfunction, but there was no genotype difference. Our study found an early activation of the kallikrein-kinin system in the kidney and that this has a protective role against the development of diabetic nephropathy. The effect of tissue kallikrein deficiency on microalbuminuria in diabetic mice is similar to the effect of genetically high angiotensin-converting enzyme levels, suggesting that both observations, in part, result from a deficiency in kinins.

Kidney International (2009) **76,** 395–403; doi:10.1038/ki.2009.208; published online 10 June 2009

KEYWORDS: diabetic nephropathy; kallikrein–kinin system; microalbuminuria; renin–angiotensin system; tissue kallikrein

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Received 4 August 2008; revised 14 April 2009; accepted 21 April 2009; published online 10 June 2009

Diabetic nephropathy is one major complication of diabetes. It is a leading cause of chronic and end-stage renal failure and is epidemic worldwide. Renal involvement in type 1 diabetes has been linked to increased cardiovascular and renal morbidity and reduced life expectancy. Elevated microalbuminuria, the first clinical hallmark and initial event of diabetic nephropathy, is a risk factor for established nephropathy and subsequent renal insufficiency in diabetic patients, and is also an independent risk factor for cardiovascular disease. It is therefore of potential clinical interest to study the factors involved in the early phase of diabetic nephropathy and in the development of albuminuria.

Renal injury is causally linked to chronic hyperglycemia but additional factors modulate individual patient's risk of diabetic nephropathy. Family studies have demonstrated that genetic factors contribute to the development of diabetic renal and vascular complications.[1,2] The angiotensin I–converting enzyme (ACE) gene was one of the first genes demonstrated to contribute to the risk of diabetic nephropathy. ACE levels are genetically determined, and associated with genomic polymorphism of the ACE gene.[3–5] As the initial reports showing an association between the genetic polymorphism of ACE level and diabetic nephropathy,[6–8] several major studies have shown that genetic variations of ACE influence the renal prognosis in type 1 diabetic patients.[9–11] Moreover, a causal link has been established between genetically high ACE level and severity of kidney malfunction in diabetic mice.[12]

Angiotensin I-converting enzyme not only converts angiotensin I into angiotensin II, but it is also a major kinin-degrading enzyme. Computer simulations and experimental data suggest that a modest increase in ACE level causes a significant decrease in kinins, while having only limited effect on systemic angiotensin II production.[13,14] Kinins are potent renal vasodilators and have also antithrombotic and antifibrotic actions. These findings suggest that kinin levels may be involved in the genetic determinism of diabetic nephropathy, and that kinins provide protection against diabetes-related renal and vascular damage. Kinins exert their biological effects by stimulation of B1 and B2 receptors. In diabetes mellitus, increase in gene expression of both receptors has been observed in microvessels and kidney.[15–18]

However, conflicting data have been reported regarding the contribution of the B2 receptor[18–22] and limited information is available concerning the B1 receptor.[23] It is well established that the B1 receptor is able to take over functions of B2 receptor, making interpretation of studies based on single inhibition or deficiency difficult.[24,25]

Tissue kallikrein is the main kinin-forming enzyme in mammals. We used tissue kallikrein–deficient (TK-null) mice,[26] in which B1 and B2 receptors cannot be activated due to lack of the endogenous ligand bradykinin, to document the role of the kallikrein–kinin system (KKS) in the initiating stages of diabetic nephropathy. We induced type 1 diabetes in mice, and then compared kidney function and morphology, and gene expression of components of the KKS of TK-null mice and wild-type (WT) littermates.

RESULTS

Tissue kallikrein protects against microalbuminuria

Physiological data obtained in series A and B, 1 and 2 months after the diabetes induction, respectively, are shown in Tables 1 and 2, and Figure 1. Urinary kallikrein activity was undetectable in TK-null mice. In each series, both the WT and TK-null diabetic mice developed similar hyperglycemia. Blood β-hydroxybutyrate concentrations were low (0–0.6 mM), no ketones were found in the urine, and the urinary pH was the same in all the groups (pH ≈ 6.2). Diabetes-induced changes in fluid intake, urine flow rate, and glycosuria were not altered by genotype. Genotype did not influence glomerular filtration rate, and the extent of hyperfiltration observed at 1 month, followed by return to normal values at 2 months. However, albumin excretion rate was significantly increased by diabetes ($P < 0.0001$), and it was more than twofold higher in TK-null than in WT mice 1 month after diabetes induction (genotype–diabetes interaction, $P = 0.02$) (Table 1). In the second series of diabetic mice studied longer (series B), similar genotype effect was observed (Figure 1) (genotype–diabetes interaction at 1 month and 2 months, $P = 0.03$ and 0.01, respectively) and albuminuria increased more rapidly in TK-null than in WT mice. Similar results were found when urinary albumin excretion was expressed as albumin/creatinine ratio (Table 2).

Morphological analysis

Diabetes induced similar increase in kidney weight in TK-null and WT mice (Tables 1 and 2). Light microscopy observations revealed glomerular hypertrophy ($P = 0.0012$ at 1 month (not shown) and $P = 0.0028$ at 2 months (Table 2)) without overt glomerulosclerosis in the diabetic mice and only slight and sporadic interstitial fibrosis and tubular atrophy at 2 months. Diabetes significantly increased the total glomerular surface area and the surface area of the glomerular tuft surface without genotype effect (Table 2). Mesangial morphology was not altered but diabetes induced an increase in the surface area of Bowman's capsule, which was more marked in TK-null than in WT mice. Electron microscopy showed no gross abnormalities in podocytes of the kidneys from diabetic mice except few foot process fusion/effacement at 2 months, endothelial fenestrations and glomerular basement membranes appeared similar to those of controls (not shown).

Tissue kallikrein deficiency has no effect on blood pressure

In control conditions, TK-null and WT mice had similar blood pressure measured by tail-cuff method as previously reported.[27] Blood pressure was not modified in the very early phase of the diabetes but increased later (Figure 2), with no interaction between diabetes and genotype. In other groups of freely moving mice with a telemetric implant, blood pressure was higher during active than rest period (+12 mm Hg), there was again no effect of diabetes or genotype up to 1 month after diabetes induction (not shown) (implants for telemetric measurement could not be kept functional in diabetic mice for a longer period).

Tissue-specific effect of diabetes on the expression of the kallikrein–kinin and renin–angiotensin system genes

The levels of mRNA for components of the renal KKS 1 month after inducing diabetes are shown in Figure 3. Diabetes significantly increased levels of mRNA for kininogen, TK, and B1-R and B2-R (analysis of variance, $P \leqslant 0.02$). Renal levels of ACE were significantly decreased in diabetic mice (Figure 4). The components of renin–angiotensin system were also affected by diabetes (Figure 5). The angiotensinogen mRNA levels were increased, whereas the AT1-R and AT2-R mRNA levels were significantly decreased (analysis of variance, $P \leqslant 0.01$). No genotype effect was observed for any of these parameters, except for diabetes-induced changes in the B2-R and AT2-R mRNA abundance, which were more marked in TK-null mice. TK deficiency did not alter the basal expression of KKS and renin–angiotensin system, except TK of course, renin, and AT2-R in the kidney. In the lung, the level of ACE mRNA was markedly enhanced by diabetes (Figure 4). In the liver, diabetes induced an increase in the abundance of the mRNA of kininogen and angiotensinogen (Figure 6). Gene expression of most of the components of the KKS and renin–angiotensin system, except for ACE, AT1-R, and angiotensinogen, were very low in the heart. Only the angiotensinogen mRNA level was affected by genotype ($P = 0.03$) and diabetes ($P = 0.004$) (1.00 ± 0.12, 0.73 ± 0.08, 2.53 ± 0.63, 1.25 ± 0.09, in WT CONT (control), WT DIAB (diabetic), TK-null CONT, and TK-null DIAB, respectively). Similar results for KKS and renin–angiotensin system components were obtained in mice studied at 2 months (Table 3), except for pulmonary ACE and renal TK that were not significantly different between control and diabetic mice at this time point.

Expression of two genes expressed in podocytes and potentially involved in albuminuria was studied. Expression of the nephrin gene was not affected by genotype or diabetes. The level of ezrin mRNA was lower in TK-null mice (genotype effect, $P < 0.05$), and was increased by diabetes at 1 month ($P < 0.01$) (WT CONT: 1.00 ± 0.10, WT DIAB:

Table 1 | Physiological parameters and kidney weight 1 month after induction of diabetes (series A)

	Wild-type		TK-null		ANOVA (*P*-value)	
	CONT	DIAB	CONT	DIAB	Genotype	Diabetes
Body weight (g)	21.9 ± 0.3	19.3 ± 0.9***	21.1 ± 0.3	18.9 ± 0.5***	0.30	<0.0001
Water intake (ml/24 h)	5.44 ± 0.21	27.50 ± 1.95***	4.53 ± 0.32	26.59 ± 1.32***	0.39	<0.0001
Food intake (g/24 h)	3.17 ± 0.14	5.54 ± 0.17***	2.97 ± 0.16	5.33 ± 0.14***	0.25	<0.0001
Urine flow rate (ml/24 h)	0.98 ± 0.07	21.76 ± 2.36***	0.87 ± 0.08	23.02 ± 1.11***	0.55	<0.0001
Urine osmolality (mosm/kg H_2O)	3096 ± 146	996 ± 34***	3501 ± 202	972 ± 49***	0.24	<0.0001
Urine excretion						
Glucose (μmol/24 h)	2.82 ± 0.22	10942 ± 933***	5.96 ± 2.32	10221 ± 594***	0.44	<0.0001
Sodium (μmol/24 h)	129 ± 10	521 ± 48***	118 ± 12	600 ± 31***	0.20	<0.0001
Potassium (μmol/24 h)	253 ± 26	1096 ± 34***	221 ± 31	874 ± 29***	0.0005	<0.0001
Urea (μmol/24 h)	1933 ± 128	4955 ± 332***	1700 ± 124	4673 ± 271***	0.26	<0.0001
Kallikrein activity (pKat/24 h)	844 ± 92	932 ± 128	11 ± 4	5 ± 2	0.49	<0.0001
Albumin (μg/24 h)	4.0 ± 1.0	21.7 ± 2.3***	5.7 ± 0.8	54.6 ± 9.2***,##	<0.0001	0.0123
Hematocrit, %	45.1 ± 1.1	44.5 ± 1.9	44.6 ± 1.0	44.9 ± 0.8	0.99	0.93
Blood glucose (mg per 100 ml)	125 ± 6	459 ± 39***	110 ± 4	459 ± 26***	0.72	<0.0001
Plasma sodium (mmol/l)	143 ± 1	141 ± 0	145 ± 1	145 ± 1	0.009	0.33
Plasma potassium (mmol/l)	5.1 ± 0.2	5.2 ± 0.1	4.9 ± 0.1	4.6 ± 0.2	0.08	0.76
Plasma creatinine (mmol/l)	11.1 ± 1.1	13.7 ± 1.8	10.9 ± 0.5	17.1 ± 2.3*	0.36	0.02
Creatinine clearance (ml/24 h)	252 ± 30	341 ± 30*	209 ± 18	291 ± 37*	0.18	0.019
Kidney weight						
Absolute (mg)	255 ± 11	287 ± 14***	240 ± 10	298 ± 10***	0.82	0.0005
Relative (mg/10 g body weight)	115 ± 5	150 ± 7***	107 ± 3	157 ± 6***	0.96	<0.0001

ANOVA, analysis of variance; CONT, control groups; DIAB, diabetic groups; TK-null, tissue kallikrein–deficient mice.
Results are means ± s.e.m., *n*=10–12/group.
Fisher *post hoc* test: DIAB vs CONT, *$P<0.05$, ***$P<0.0001$; TK-null vs wild type: ##$P<0.01$.

Table 2 | Physiological parameters and renal morphometric data 2 months after induction of diabetes (series B)

	Wild-type		TK-null		ANOVA (*P*-value)	
	CONT	DIAB	CONT	DIAB	Genotype	Diabetes
Body weight (g)	22.6 ± 0.3	17.1 ± 0.6***	22.8 ± 0.7	18.2 ± 0.7***	0.27	<0.0001
Water intake (ml/24 h)	7.70 ± 0.37	23.23 ± 2.63***	5.95 ± 1.20	21.68 ± 1.74***	0.35	<0.0001
Food intake (g/24 h)	2.69 ± 0.25	4.74 ± 0.39***	2.84 ± 0.25	4.53 ± 0.38***	0.93	<0.0001
Urine flow rate (ml/24 h)	0.72 ± 0.11	17.54 ± 1.21***	0.72 ± 0.11	15.54 ± 1.76***	0.32	<0.0001
Urine osmolality (mosm.kg/H_2O)	2544 ± 202	990 ± 20***	2494 ± 172	1008 ± 55***	0.91	<0.0001
Urine excretion						<0.0001
Glucose (μmol/24 h)	2.07 ± 0.45	11578 ± 922***	2.50 ± 0.47	9259 ± 0.26		<0.0001
Urea (μmol/24 h)	933 ± 113	2841 ± 178***	909 ± 89	2659 ± 238***	0.53	$P<0.0001$
Kallikrein activity (pKat/24 h)	307 ± 93	289 ± 64	3 ± 2##	0 ± 0##	$P<0.0001$	0.85
Albumin (μg alb/μmol creat)	3.70 ± 0.70	7.41 ± 0.70**	3.95 ± 0.50	16.00 ± 3.57**,#	0.0225	0.0002
Blood glucose (mg per 100 ml)	131 ± 8	545 ± 34***	127 ± 11	589 ± 11***	0.33	$P<0.0001$
Plasma creatinine (μmol/l)	11.9 ± 1.0	17.8 ± 0.9**	13.7 ± 0.6	15.0 ± 1.7	0.66	0.0027
Creatinine clearance (ml/24h)	316 ± 46	273 ± 19	252 ± 24	330 ± 50	0.64	0.92
Kidney weight						
Absolute (mg)	239 ± 10	319 ± 12***	249 ± 8	341 ± 13***	0.11	<0.0001
Relative (mg/10 g body weight)	104 ± 4	185 ± 10***	109 ± 3	181 ± 6***	0.96	<0.0001
Glomerular surface area (μm^2)	4543 ± 245	5096 ± 103**	4523 ± 133	5267 ± 187**	0.79	0.0028
Glomerular tuft surface area (μm^2)	3149 ± 143	3489 ± 64**	2991 ± 56	3526 ± 155**	0.60	0.034
Bowman's capsule surface area (μm^2)	398 ± 10	416 ± 14	347 ± 10	429 ± 8**	0.13	0.0004

ANOVA, analysis of variance; CONT, control groups; DIAB, diabetic groups; TK-null, tissue kallikrein–deficient mice.
Results are means ± s.e.m., *n*=7–8/group.
Fisher *post hoc* test: DIAB vs CONT, **$P<0.01$, ***$P<0.0001$; TK-null vs wild type, #$P<0.05$, ##$P<0.0001$.

1.43 ± 0.08, TK-null CONT: 0.76 ± 0.02, TK-null DIAB: 1.16 ± 0.11) but the diabetes effect disappeared at 2 months (WT CONT: 1.00 ± 0.07, WT DIAB: 1.02 ± 0.07, TK-null CONT: 0.69 ± 0.06, TK-null DIAB: 0.79 ± 0.04). We observed that gene expression of kidney injury molecule-1, a protein known to be induced in the proximal tubule during acute

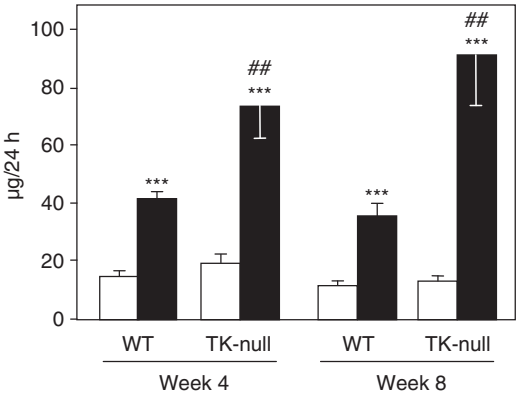

Figure 1 | Urinary albumin excretion in wild-type (WT) and tissue kallikrein–deficient (TK-null) mice 1 and 2 months after induction of diabetes (series B). Black bars: diabetic groups, white bars: control groups. Results are means ± s.e.m. $n = 7$–8/group. Analysis of variance followed by Fisher PLSD *post hoc* test, diabetic vs control: $^{***}P < 0.001$; TK-null vs WT: $^{\#\#}P < 0.01$.

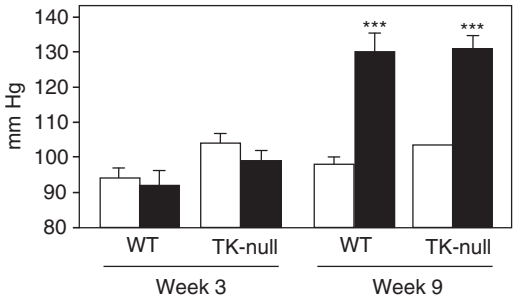

Figure 2 | Systolic blood pressure in wild-type (WT) and tissue kallikrein–deficient (TK-null) mice 1 and 2 months after induction of diabetes (series B). Black bars: diabetic groups, white bars: control groups. Results are means ± s.e.m. $n = 7$–8/group. Analysis of variance followed by Fisher PLSD *post hoc* test, diabetic vs control: $^{***}P < 0.001$, TK-null vs WT: nonsignificant.

kidney injury, is markedly increased by diabetes at 1- and 2 months (20- and 60-fold, respectively) with no effect of genotype.

DISCUSSION

In addition to being the earliest clinical manifestation of nephropathy, albuminuria is an independent risk factor and a marker for renal as well as cardiovascular disease for type 1 or type 2 diabetic patients. The level of albuminuria proved to be one of the major factors that predict renal outcome. The main finding of our study is that diabetic mice with inactivation of the TK gene displayed a higher albumin excretion rate after diabetes induction compared with diabetic WT mice. The effect of TK deficiency on micro-albuminuria was observed as early as 1 month after diabetes induction, and not linked to blood pressure which remains normal at that stage. At 2 months, in the presence of hypertension, urinary albumin excretion was still elevated in TK-deficient mice, whereas blood pressure was not

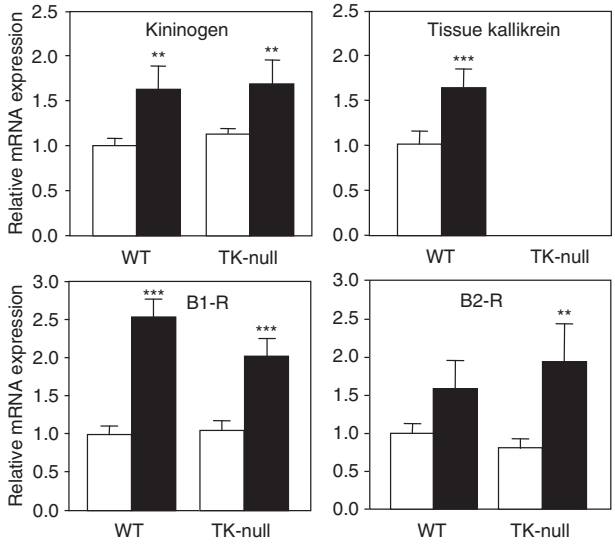

Figure 3 | Gene expression of the components of the renal kallikrein–kinin system in wild-type (WT) and tissue kallikrein–deficient (TK-null) mice 1 month after induction of diabetes (series A). Black bars: diabetic groups, white bars: control groups. Results are means ± s.e.m. $n = 10$–12/group. Results for each gene are expressed relative to the mean of the corresponding WT control group. Analysis of variance followed by Fisher PLSD *post hoc* test, diabetic vs control: $^{***}P < 0.0001$, $^{**}P < 0.01$, TK-null vs WT: nonsignificant.

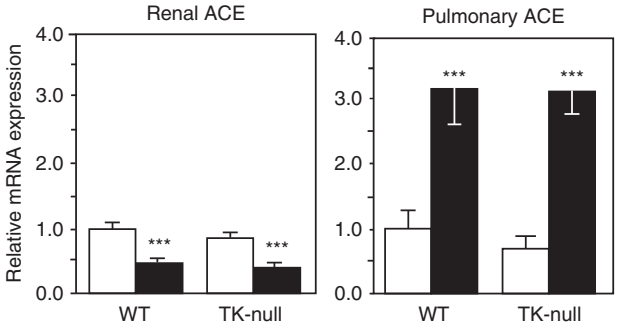

Figure 4 | Gene expression of ACE in kidney and lung of wild-type (WT) and tissue kallikrein–deficient (TK-null) mice 1 month after induction of diabetes (series A). Black bars: diabetic groups, white bars: control groups. Results are means ± s.e.m. $n = 10$–12/group. Results for each gene are expressed relative to the mean of the corresponding WT control group. Analysis of variance followed by Fisher PLSD *post hoc* test, diabetic vs control: $^{***}P < 0.0001$, TK-null vs WT: nonsignificant. ACE, angiotensin I–converting enzyme.

influenced by this deficiency. This suggests a direct effect of TK at the glomerular or tubular levels. The finding of increased urinary albumin excretion in TK-deficient mice together with the observation of increased gene expression of TK and other KKS components in the kidney after induction of diabetes, strongly suggests that TK protects against kidney dysfunction caused by diabetes mellitus.

In contrast to its effect on albuminuria, TK deficiency did not influence the hyperfiltration and kidney hypertrophy induced by diabetes. This confirms that hyperfiltration is

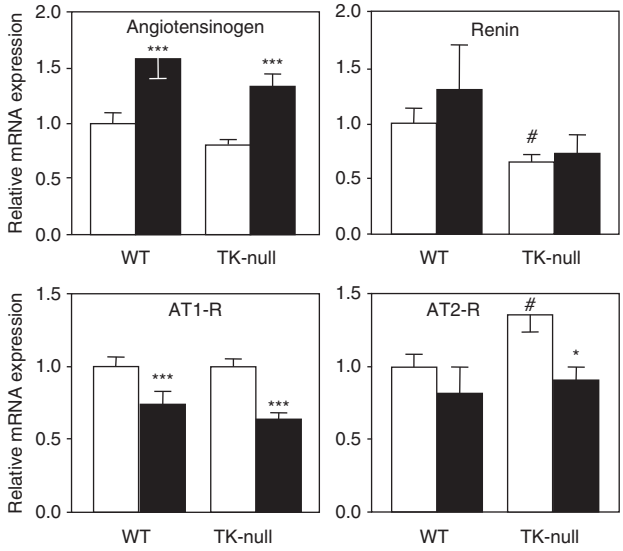

Figure 5 | Gene expression of the components of the renal renin–angiotensin system in wild-type (WT) and tissue kallikrein-deficient (TK-null) mice 1 month after induction of diabetes (series A). Black bars: diabetic groups, white bars: control groups. Results are means ± s.e.m. $n = 10$–12/group. Results for each gene are expressed relative to the mean of the corresponding WT control group. Analysis of variance followed by Fisher PLSD *post hoc* test, diabetic vs control: ***$P < 0.0001$, *$P < 0.05$, TK-null vs WT; #$P < 0.05$.

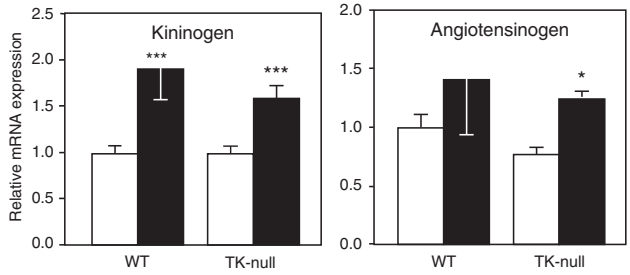

Figure 6 | Gene expression of precursors of the kallikrein–kinin and renin–angiotensin system in the liver of wild-type (WT) and tissue kallikrein-deficient (TK-null) mice 1 month after induction of diabetes (series A). Black bars: diabetic groups, white bars: control groups. Results are means ± s.e.m. $n = 10$–12/group. Results for each gene are expressed relative to the mean of the corresponding WT control group. Analysis of variance followed by Fisher PLSD *post hoc* test, diabetic vs control: ***$P < 0.0001$, *$P < 0.05$, TK-null vs WT; nonsignificant.

not invariably linked to albuminuria.[28] During this early phase of diabetic nephropathy glomerular hypertrophy was present, but no glomerulosclerosis was observed, which is consistent with the relative resistance of mouse strain C57Bl/6J to renal lesions.[28]

These observations suggest that the higher albuminuria observed in TK-null mice could arise from an increase in glomerular permeability. As podocytes play a central role in glomerular filtration barrier, we studied two physiologically important components of these cells, nephrin and ezrin. Nephrin is an essential component of the slit diaphragms of the glomerulus. Gene expression of nephrin was shown to decrease in the proteinuric phase but not in the early pre-proteinuric phase in rats made diabetic by streptozotocin.[29] Ezrin is concentrated along the apical membrane of the foot processes of podocytes. It belongs to a family of plasma membrane-cytoskeleton linking, actin-binding proteins that have been identified as binding proteins for advanced glycation end products,[30] and implicated in diabetic complications. Ezrin was also shown to be modulated during podocyte injury and regeneration.[31] In this study, we found no change in nephrin gene expression in the entire kidney at the early stage of diabetes. However, hypertrophy does not develop in all glomeruli concurrently, and recent data suggest that nephrin gene could be differentially expressed according to glomerular size.[32] A defect in ezrin mRNA level was observed in this study in TK-deficient mice. This defect may be indicative of podocyte dysfunction in these mice.

Kidney injury molecule (Kim-1), a type 1 transmembrane glycoprotein, was shown to be markedly induced after ischemia/reperfusion injury and exposure to nephrotoxicants,[33] in model of acute massive proteinuria[34] and in non-diabetic proteinuric patients.[35] We show here for the first time that Kim-1 is also associated with microalbuminuria in diabetic nephropathy, thus extending the concept that Kim-1 is a biomarker not only for acute but also for chronic tubular damage. The absence of genotype effect on Kim-1 expression in diabetic mice may suggest that the difference of albuminuria observed in absence and presence of TK was not due to difference in proximal tubule damage.

Our data revealed gene and tissue- and time-specific effect of diabetes on the expression of the KKS and renin–angiotensin system genes. To our knowledge, no study has addressed mRNA quantification of components of both peptide systems in major organs (the kidney, heart, lung, and liver) during the hyperfiltrating phase of diabetes. The present experiment is the first documenting the upregulation of kallikrein–kinin system in the kidney at early stage of diabetes, with increased gene expression of both factors involved in kinin formation, TK and kininogen, and those involved in kinin action, B1 and B2 receptor that is consistent with increased availability of kinins, at least in some renal compartments. The B1-R and B2-R gene expression was increased by diabetes in both WT and TK-null mice, which rules out the possibility of ligand-induced receptor upregulation. This upregulation may be due to a direct effect of hyperglycemia, as suggested by the effect of glucose on B1-R and B2-R gene expression in cultured rat endothelial[36] and vascular smooth muscle cells.[37] These observations suggest that the nephroprotective role of TK in the context of diabetes is mediated by kinins, although other non-kinin-mediated actions of TK may also be involved.[38,39]

Concerning the renin–angiotensin system, angiotensinogen gene expression is increased in liver, kidney, and heart and this can result in increased angiotensin II production.[40] On the other hand, AT1-R mRNA is decreased. The decrease in AT1-R mRNA may be secondary to elevated angiotensin II concentration in the diabetic kidney, or reflect damage in the

Table 3 | Gene expression in wild-type and TK-null mice 2 months after induction of diabetes (series B)

	Wild-type		TK-null		ANOVA (*P*-value)	
	CONT	DIAB	CONT	DIAB	Genotype	Diabetes
Liver						
Kininogen	1.00 ± 0.10	1.39 ± 0.28**	0.72 ± 0.07	1.24 ± 0.11**	0.24	0.017
Angiotensinogen	1.00 ± 0.07	1.03 ± 0.16	0.74 ± 0.15	1.37 ± 0.13**	0.76	0.016
Lung						
ACE	1.00 ± 0.16	0.96 ± 0.21	0.85 ± 0.14	1.24 ± 0.15	0.82	0.16
Kidney						
Kininogen	1.00 ± 0.18	1.61 ± 0.41	1.01 ± 0.13	1.75 ± 0.29**	0.77	0.022
B1 receptor	1.00 ± 0.17	1.44 ± 0.26	1.21 ± 0.24	1.80 ± 0.31*	0.25	0.04
B2 receptor	1.00 ± 0.15	2.20 ± 0.24***	1.32 ± 0.16	2.03 ± 0.25	0.72	$P<0.0001$
TK	1.00 ± 0.28	0.60 ± 0.22	0 ± 0	0 ± 0	0.0003	0.31
ACE	1.00 ± 0.11	0.47 ± 0.11***	0.93 ± 0.08	0.75 ± 0.04*	0.26	0.0006
Angiotensinogen	1.00 ± 0.07	1.64 ± 0.14***	1.06 ± 0.06	1.55 ± 0.14	0.92	$P<0.0001$
AT1 receptor	1.00 ± 0.21	0.66 ± 0.15	1.05 ± 0.08	0.48 ± 0.07**	0.64	0.003
AT2 receptor	1.00 ± 0.16	0.76 ± 0.17	1.16 ± 0.11	0.90 ± 0.15	0.33	0.11
Renin	1.00 ± 0.18	0.93 ± 0.31	0.65 ± 0.11	0.71 ± 0.08	0.17	0.96
Nephrin	1.00 ± 0.09	0.91 ± 0.10	0.85 ± 0.05	0.87 ± 0.06	0.24	0.65
Ezrin	1.00 ± 0.07	1.05 ± 0.07	0.70 ± 0.07	0.82 ± 0.04	0.0003	0.21
Kim-1	1.00 ± 0.21	65.0 ± 13.2***	0.80 ± 0.16	57.8 ± 9.1***	0.65	0.0001

ACE, angiotensin-converting enzyme; ANOVA, analysis of variance; CONT, control groups; DIAB, diabetic groups; Kim-1, kidney injury molecule-1; TK-null, tissue kallikrein–deficient mice.
Results are means ± s.e.m., n=7–8/group.
Values for each gene are expressed relative to the mean of the corresponding wild-type control.
Fisher *post hoc* test: DIAB vs CONT, *$P<0.05$, **$P<0.02$, ***$P<0.0001$.

glomerulus and proximal tubule, the major site of AT1-R synthesis.[41] In any case these results document divergent regulation of the two peptide systems at the receptor level, presumably resulting in imbalance in their activity.

Angiotensin I-converting enzyme availability is a critical factor for the activity of KKS and renin–angiotensin system, especially in the renal circulation, which has very low level of endothelial ACE.[42] Diabetes induced a marked but transient increase in ACE gene expression in the lung, an endothelium-rich organ, but a decrease in the kidney. This is consistent with previous findings.[12] Induction of ACE gene expression may also occur in endothelial cells of the kidney, contributing to kinin depletion in the vascular compartment, but this cannot be documented by mRNA measurement. Indeed, the major site of ACE gene expression in the kidney is the proximal tubule and renal ACE mRNA is mainly of tubular origin. The diabetes-induced decrease in renal ACE mRNA is indicative of tubular dysfunction,[43,44] as also suggested by induction of Kim-1 expression. Nevertheless, ACE production by the endothelium is probably the most important contributor to the deleterious effect of this enzyme on diabetic nephropathy. There is some evidence that plasma ACE circulating through the kidney, which originates from endothelium, is increased in diabetes[12] and locally produces angiotensin II and metabolizes kinins.[42] Moreover, neo-expression of ACE by renal endothelial cells was observed in diabetic kidney.[45] All together, these observations are consistent with increased availability of ACE in vascular compartment in diabetes with deleterious consequences for the kidney, and decreased availability of ACE in the tubular compartment.

The pattern of KKS activation is not modified in TK-deficient mice. However, AT2-R mRNA in the kidney was slightly greater in control TK-null than WT mice, as previously observed in carotid arteries.[46] This may explain the downregulation of renin gene expression in TK-deficient mice (this study and Trabold *et al.*[27]) in agreement with the hypothesis that AT2-R regulates renin–angiotensin system activity by inhibition of renin synthesis.[47] As the AT2 receptor is functionally coupled to TK,[46,48] upregulation of this receptor in TK-null mice may be an adaptative attempt to counteract the lack of active KKS.

The contribution of kinins to the development of diabetic nephropathy was assessed recently in two models of type 1 diabetes[18,22] involving B2-R deletion. Kakoki *et al.*[18] showed that 6-month-old male Akita diabetic mice, with hypofiltrating kidney,[49] developed overt albuminuria in the absence of bradykinin B2 receptor, whereas WT diabetic littermates remained microalbuminuric, suggesting a protective role of KKS against the development of diabetic nephropathy. In contrast, Tan *et al.*[22] reported that deletion of B2 receptor protects against microalbuminuria and the development of diabetic nephropathy. The discrepancy between these studies may be due, at least in part, to differences in genetic background, extent of B1-receptor activation, and severity of hyperglycemia. In the Tan *et al.* study, diabetic B2-null mice developed, for unknown reasons, slightly lower hyperglycemia level during the first 2 months than the WT mice.

Our study clarifies the issue by showing that for similar levels of severe hyperglycemia, TK-null mice with drastically reduced renal levels of kinins developed more severe microalbuminuria than their WT littermates. One limitation of the study relies on the fact that diabetic nephropathy develops slowly in C57bl6 mice.[28] After 2 months of diabetes, mortality by metabolic complications increased sharply (personal observation). This did not allow studying the effect of TK deficiency on established diabetic nephropathy, renal fibrosis, and glomerulosclerosis. Nevertheless, our results support the concept that KKS has a net protective effect at least in the early phase of diabetic nephropathy. Albuminuria was also higher in diabetic mice with three copies of the ACE gene than WT 4 weeks after diabetes induction and worsened thereafter.[12] This study suggests that kinins deficiency is involved in the harmful impact of genetically high ACE levels in the diabetic kidney, in both mice and men. Genetic polymorphism of ACE levels is an established risk factor for diabetic nephropathy.[8–11] Partial genetic TK deficiency occurs in man as a consequence of an active site mutation (R53H) present in the heterozygote state in 7% of white and 14% of black individuals.[50] Carriers of the R53H allele similar to those of the high ACE level allele may be at increased risk of nephropathy in chronic hyperglycemia, although this remains to be demonstrated in clinical studies.

MATERIALS AND METHODS

Animals

Two- to three-month-old female TK-null mice[26] on C57Bl/6J background, and their WT littermates were used. Four groups of mice, WT control, WT diabetic, TK-null control, and TK-null diabetic were studied. Diabetes was induced by 5–7 daily intraperitoneal injections of low-dose of streptozotocin without nephrotoxic effect (80 μg/g body weight).[12] Control mice (CONT) received vehicle (0.05 mol/l sodium citrate, pH 4.5). Only diabetic mice (DIAB) with hyperglycemia $\geqslant$200 mg per 100 ml 10 days after the first injection were included in the study. The mice were housed with a 12 h light/dark cycle, and had free access to standard chow (A03, Safe, Augy, France) and demineralized drinking water. Two series of mice were studied, one was killed 5 weeks after diabetes induction (series A, $n = 10$–12 mice/group) and the other at 9 weeks (series B, $n = 7$–8 mice/group). A time point of 1 month diabetes duration was chosen to examine the effects of TK on early changes in diabetic kidney, without the potentially confounding effect of hypertension or fibrosis. However, in a second study, mice were followed for 2 months for studying the temporal effect of TK deficiency during diabetes evolution. All experiments were conducted in accordance with the European regulations for the care and use of laboratory animals (L 358-86/609/EEC).

Functional study

Four weeks (series A and B) and 8 weeks (series B) after inducing diabetes, 24 h urine collections were obtained from individual mice that were housed temporarily in metabolic cages (Techniplast, Limonest, France). Blood from the retro-orbital sinus was collected at killing. Plasma and urine concentrations of sodium, potassium, glucose, urea and creatinine were assayed with an automatic analyzer (Konelab 20I, Orthoclinical Diagnostics, Thermo electron Corporation, Courtaboeuf, France). Urinary albumin concentration was determined by immunonephelemetry (series A) (Behring laser-nephelemeter, Marburg, Germany) or ELISA (series B) (Albuwell M, Exocell, Philadelphia, PA). Urinary osmolality was measured using a freezing point osmometer (Roebling, Germany). Urinary ketones and pH were determined in spontaneously voided urine using the Keto-Diabur-Test 5000 (Accu-Chek, Roche Diagnostic, Meylan, France) and Duotest (Macherey-Nagel, Oensingen, Switzerland), respectively. Blood β-hydroxybutyrate, the major blood ketone, was determined with Optium Xceed (MediSense, ABOTT, Rungis, France). Urinary kallikrein activity was measured by means of a chromogenic substrate (S-226, Chromogenix, Biogenix, Maurin, France).

Blood pressure and heart rate were measured for 3 consecutive days 4 and 8 weeks after inducing diabetes in conscious, restrained mice by the tail-cuff method (BP-2000, Visitech system, Bioseb, Vitrolles, France) (series B). Because subtle differences in blood pressure may be missed by this technique, telemetric measurements were performed before and after diabetes induction in another series of mice ($n = 4$/group) equipped with TA11PA-C10 device (Data Sciences International, St Paul, MN) as previously described.[51]

Heart and kidney histology and morphometry

One half of the right kidney and the heart were fixed in alcoholic Bouin's solution and 10% formaldehyde, respectively. Tissues were embedded in paraffin. For the kidney, 4 μm thick sections were stained with Masson's trichrome for qualitative assessment of mesangium expansion and glomerular, tubulointerstitial and vascular lesions, and with periodic acid-Schiff for morphometry. Glomerular surface area was measured using computerized image analysis as previously described.[12] For the heart, a transversal section just below mitral valve was stained with hematoxylin–eosin and Sirius red for qualitative assessment of myocyte necrosis, myocardial inflammation or fibrosis.

The remaining of the right kidney was used for electron microscopy. The kidney was minced into 1 mm^3 pieces and was fixed in 2.5% glutaraldehyde in phosphate buffer (pH 7.4, 0.1 M), postfixed in 2% OsO_4 in phosphate buffer, dehydrated in graded alcohol and embedded in Epon resin at 60°C during 72 h. Ultra-thin sections (52 nm) were stained with uranyl acetate and lead citrate and examined under JEOL-100CXII transmission electron microscopy (Jeol Ltd, Tokyo, Japan).

Quantification of mRNA abundance

Relative changes in gene expression of the renin–angiotensin system and KKS components, and three genes coding for tubular or structural glomerular proteins were quantified using real-time PCR. Total RNA was isolated from the left kidney, heart apex, and pieces of liver and lung (Qbiogen, MP Biomedicals, Illkirch, France). cDNA was synthetized from 2 μg total RNA using 0.3 μg random hexamers and 40U Superscript II MMLV-reverse transcriptase (Invitrogen, Cergy Pontoise, France), in presence of 200U RNase-OUT in a 20 μl final volume. Real-time PCR was performed on the ABI PRISM 7000 Sequence Detection System by using TaqMan Universal Master Mix and Assays-on-Demand Gene Expression Probes for kallikrein, kininogen, kinin B1 (B1-R) and B2 (B2-R) receptors, ACE, angiotensinogen, type 1 (AT1-R) and type 2 (AT2-R) angiotensin II receptors, renin, ezrin, nephrin, and kidney injury molecule (Kim-1) (AppliedBiosystems, Applera France, Courtaboeuf, France). Fifteen ng of reverse transcribed RNA was submitted to PCR in a 20-μl final volume. Each sample was tested in triplicate. DNA contamination was excluded by performing PCR amplification without the reverse transcription step for each RNA

sample, and blank without sample but with all reagents. Data were normalized to 18S rRNA. Changes in the target gene relative to the mean expression in the WT control group were calculated by the $2^{-\Delta\Delta CT}$ comparative method for each sample.[52]

Statistical analysis

Data are expressed as means ± s.e.m. Statistical analyses were performed using two-way analysis of variance followed, when appropriate, by the Fisher protected least significant *post hoc* test. Significance was set at $P<0.05$.

DISCLOSURE

All the authors declared no competing interests.

ACKNOWLEDGMENTS

We thank Véronique Baudrie, Martine Douheret, Laetitia Perret, Georges Salmon, and Georges Zadigue for expert technical assistance, and N Quellard, B Fernandez, and JM Goujon (Unité de Pathologie Ultrastructurale et Expérimentale, Service d'Anatomie et Cytologie Pathologiques, C.H.U La Milétrie, Poitiers, France) for electron microscopy photographies. This study was funded by INSERM, University Paris-Descartes, the National Research Agency (ANR-05-PCOD-027), and ALFEDIAM-LILLY, AFD, and French Society of Nephrology grants. This study emanates from the European Vascular Genomics Network (http://www.evgn.org), a Network of Excellence supported by the European Community's sixth Framework Program for Research Priority 1 'Life sciences, genomics and biotechnology for health' (Contract no. LSHM-CT-2003-503254).

REFERENCES

1. Seaquist ER, Goetz FC, Rich S *et al.* Familial clustering of diabetic kidney disease. Evidence for genetic susceptibility to diabetic nephropathy. *N Engl J Med* 1989; **320**: 1161–1165.
2. Borch-Johnsen K, Norgaard K, Hommel E *et al.* Is diabetic nephropathy an inherited complication? *Kidney Int* 1992; **41**: 719–722.
3. Cambien F, Alhenc-Gelas F, Herbeth B *et al.* Familial resemblance of plasma angiotensin-converting enzyme level: the Nancy Study. *Am J Hum Genet* 1988; **43**: 774–780.
4. Costerousse O, Allegrini J, Lopez M *et al.* Angiotensin I-converting enzyme in human circulating mononuclear cells: genetic polymorphism of expression in T-lymphocytes. *Biochem J* 1993; **290**(Part 1): 33–40.
5. Rigat B, Hubert C, Alhenc-Gelas F *et al.* An insertion/deletion polymorphism in the angiotensin I-converting enzyme gene accounting for half the variance of serum enzyme levels. *J Clin Invest* 1990; **86**: 1343–1346.
6. Marre M, Bernadet P, Gallois Y *et al.* Relationships between angiotensin I converting enzyme gene polymorphism, plasma levels, and diabetic retinal and renal complications. *Diabetes* 1994; **43**: 384–388.
7. Doria A, Warram JH, Krolewski AS. Genetic predisposition to diabetic nephropathy. Evidence for a role of the angiotensin I – converting enzyme gene. *Diabetes* 1994; **43**: 690–695.
8. Marre M, Jeunemaitre X, Gallois Y *et al.* Contribution of genetic polymorphism in the renin-angiotensin system to the development of renal complications in insulin-dependent diabetes: Genetique de la Nephropathie Diabetique (GENEDIAB) study group. *J Clin Invest* 1997; **99**: 1585–1595.
9. Boright AP, Paterson AD, Mirea L *et al.* Genetic variation at the ACE gene is associated with persistent microalbuminuria and severe nephropathy in type 1 diabetes: the DCCT/EDIC Genetics Study. *Diabetes* 2005; **54**: 1238–1244.
10. Hadjadj S, Tarnow L, Forsblom C *et al.* Association between angiotensin-converting enzyme gene polymorphisms and diabetic nephropathy: case-control, haplotype, and family-based study in three European populations. *J Am Soc Nephrol* 2007; **18**: 1284–1291.
11. Costacou T, Ellis D, Fried L *et al.* Sequence of progression of albuminuria and decreased GFR in persons with type 1 diabetes: a cohort study. *Am J Kidney Dis* 2007; **50**: 721–732.
12. Huang W, Gallois Y, Bouby N *et al.* Genetically increased angiotensin I-converting enzyme level and renal complications in the diabetic mouse. *Proc Natl Acad Sci USA* 2001; **98**: 13330–13334.
13. Murphey LJ, Gainer JV, Vaughan DE *et al.* Angiotensin-converting enzyme insertion/deletion polymorphism modulates the human *in vivo* metabolism of bradykinin. *Circulation* 2000; **102**: 829–832.
14. Takahashi N, Hagaman JR, Kim HS *et al.* Minireview: computer simulations of blood pressure regulation by the renin-angiotensin system. *Endocrinology* 2003; **144**: 2184–2190.
15. Christopher J, Jaffa AA. Diabetes modulates the expression of glomerular kinin receptors. *Int Immunopharmacol* 2002; **2**: 1771–1779.
16. Mage M, Pecher C, Neau E *et al.* Induction of B1 receptors in streptozotocin diabetic rats: possible involvement in the control of hyperglycemia-induced glomerular Erk 1 and 2 phosphorylation. *Can J Physiol Pharmacol* 2002; **80**: 328–333.
17. Abdouh M, Khanjari A, Abdelazziz N *et al.* Early upregulation of kinin B1 receptors in retinal microvessels of the streptozotocin-diabetic rat. *Br J Pharmacol* 2003; **140**: 33–40.
18. 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.
19. Jaffa AA, Rust PF, Mayfield RK. Kinin, a mediator of diabetes-induced glomerular hyperfiltration. *Diabetes* 1995; **44**: 156–160.
20. 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.
21. Allard J, Buleon M, Cellier E *et al.* ACE inhibitor reduces growth factor receptor expression and signaling but also albuminuria through B2-kinin glomerular receptor activation in diabetic rats. *Am J Physiol Renal Physiol* 2007; **293**: F1083–F1092.
22. 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.
23. Lawson SR, Gabra BH, Nantel F *et al.* Effects of a selective bradykinin B1 receptor antagonist on increased plasma extravasation in streptozotocin-induced diabetic rats: distinct vasculopathic profile of major key organs. *Eur J Pharmacol* 2005; **514**: 69–78.
24. Duka I, Shenouda S, Johns C *et al.* Role of the B(2) receptor of bradykinin in insulin sensitivity. *Hypertension* 2001; **38**: 1355–1360.
25. 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.
26. Meneton P, Bloch-Faure M, Hagege AA *et al.* Cardiovascular abnormalities with normal blood pressure in tissue kallikrein-deficient mice. *Proc Natl Acad Sci USA* 2001; **98**: 2634–2639.
27. Trabold F, Pons S, Hagege AA *et al.* Cardiovascular phenotypes of kinin B2 receptor- and tissue kallikrein-deficient mice. *Hypertension* 2002; **40**: 90–95.
28. Qi Z, Fujita H, Jin J *et al.* Characterization of susceptibility of inbred mouse strains to diabetic nephropathy. *Diabetes* 2005; **54**: 2628–2637.
29. Kelly DJ, Aaltonen P, Cox AJ *et al.* Expression of the slit-diaphragm protein, nephrin, in experimental diabetic nephropathy: differing effects of anti-proteinuric therapies. *Nephrol Dial Transplant* 2002; **17**: 1327–1332.
30. McRobert EA, Gallicchio M, Jerums G *et al.* The amino-terminal domains of the ezrin, radixin, and moesin (ERM) proteins bind advanced glycation end products, an interaction that may play a role in the development of diabetic complications. *J Biol Chem* 2003; **278**: 25783–25789.
31. Hugo C, Nangaku M, Shankland SJ *et al.* The plasma membrane-actin linking protein, ezrin, is a glomerular epithelial cell marker in glomerulogenesis, in the adult kidney and in glomerular injury. *Kidney Int* 1998; **54**: 1934–1944.
32. Kim JJ, Li JJ, Jung DS *et al.* Differential expression of nephrin according to glomerular size in early diabetic kidney disease. *J Am Soc Nephrol* 2007; **18**: 2303–2310.
33. Bonventre JV. Kidney Injury Molecule-1 (KIM-1): a specific and sensitive biomarker of kidney injury. *Scand J Clin Lab Invest Suppl* 2008; **241**: 78–83.
34. van Timmeren MM, Bakker SJ, Vaidya VS *et al.* Tubular kidney injury molecule-1 in protein-overload nephropathy. *Am J Physiol Renal Physiol* 2006; **291**: F456–F464.
35. Waanders F, Vaidya VS, van Goor H *et al.* Effect of renin-angiotensin-aldosterone system inhibition, dietary sodium restriction, and/or diuretics on urinary kidney injury molecule 1 excretion in nondiabetic proteinuric kidney disease: a *post hoc* analysis of a randomized controlled trial. *Am J Kidney Dis* 2009; **53**: 16–25.
36. Rodriguez AI, Pereira-Flores K, Hernandez-Salinas R *et al.* High glucose increases B1-kinin receptor expression and signaling in endothelial cells. *Biochem Biophys Res Commun* 2006; **345**: 652–659.

37. Christopher J, Velarde V, Zhang D *et al.* Regulation of B(2)-kinin receptors by glucose in vascular smooth muscle cells. *Am J Physiol Heart Circ Physiol* 2001; **280**: H1537–H1546.
38. Picard N, Van Abel M, Campone C *et al.* Tissue kallikrein-deficient mice display a defect in renal tubular calcium absorption. *J Am Soc Nephrol* 2005; **16**: 3602–3610.
39. Biyashev D, Tan F, Chen Z *et al.* Kallikrein activates bradykinin B2 receptors in absence of kininogen. *Am J Physiol Heart Circ Physiol* 2006; **290**: H1244–H1250.
40. Kim HS, Krege JH, Kluckman KD *et al.* Genetic control of blood pressure and the angiotensinogen locus. *Proc Natl Acad Sci USA* 1995; **92**: 2735–2739.
41. Cheng HF, Burns KD, Harris RC. Reduced proximal tubule angiotensin II receptor expression in streptozotocin-induced diabetes mellitus. *Kidney Int* 1994; **46**: 1603–1610.
42. Alhenc-Gelas F, Baussant T, Hubert C *et al.* The angiotensin converting enzyme in the kidney. *J Hypertens Suppl* 1989; **7**: S9–S13; discussion S14.
43. Anderson S, Jung FF, Ingelfinger JR. Renal renin-angiotensin system in diabetes: functional, immunohistochemical, and molecular biological correlations. *Am J Physiol* 1993; **265**: F477–F486.
44. Wysocki J, Ye M, Soler MJ *et al.* ACE and ACE2 activity in diabetic mice. *Diabetes* 2006; **55**: 2132–2139.
45. Metzger R, Bohle RM, Pauls K *et al.* Angiotensin-converting enzyme in non-neoplastic kidney diseases. *Kidney Int* 1999; **56**: 1442–1454.
46. Bergaya S, Hilgers RH, Meneton P *et al.* Flow-dependent dilation mediated by endogenous kinins requires angiotensin AT2 receptors. *Circ Res* 2004; **94**: 1623–1629.
47. Siragy HM, Xue C, Abadir P *et al.* Angiotensin subtype-2 receptors inhibit renin biosynthesis and angiotensin II formation. *Hypertension* 2005; **45**: 133–137.
48. Messadi-Laribi E, Griol-Charhbili V, Pizard A *et al.* Tissue kallikrein is involved in the cardioprotective effect of AT1-receptor blockade in acute myocardial ischemia. *J Pharmacol Exp Ther* 2007; **323**: 210–216.
49. Haseyama T, Fujita T, Hirasawa F *et al.* Complications of IgA nephropathy in a non-insulin-dependent diabetes model, the Akita mouse. *Tohoku J Exp Med* 2002; **198**: 233–244.
50. Slim R, Torremocha F, Moreau T *et al.* Loss-of-function polymorphism of the human kallikrein gene with reduced urinary kallikrein activity. *J Am Soc Nephrol* 2002; **13**: 968–976.
51. Baudrie V, Laude D, Elghozi JL. Optimal frequency ranges for extracting information on cardiovascular autonomic control from the blood pressure and pulse interval spectrograms in mice. *Am J Physiol Regul Integr Comp Physiol* 2007; **292**: R904–R912.
52. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(−Delta Delta C(T)) method. *Methods* 2001; **25**: 402–408.