

# Renal effects of a neutralising RAGE-antibody in long-term streptozotocin-diabetic mice

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## Abstract

Advanced glycation endproducts (AGEs) have been implicated in the pathogenesis of diabetic kidney disease. The actions of AGEs are mediated both through a non-receptor mediated pathway and through specific receptors for AGEs (e.g. RAGE). To explore a potentially specific role for RAGE in renal changes in type 1 diabetes, we examined the renal effects of a neutralising murine RAGE-antibody (ab) in streptozotocin (STZ)-diabetic mice, a model of type 1 diabetes. One group of STZ-diabetic mice was treated for two months with the RAGE-ab, while another STZ-diabetic group was treated for the same period with an irrelevant immunoglobulin G (IgG). Two groups of non-diabetic NMRI mice were treated with either RAGE-ab or isotype-matched IgG for two months. Placebo-treated STZ-diabetic mice showed an increase in kidney weight, glomerular volume, basement membrane thickness (BMT), urinary albumin

excretion (UAE) and creatinine clearance (CrCl), when compared with non-diabetic controls. In RAGE-ab-treated STZ-diabetic mice, the increase in kidney weight and UAE was reduced, while the increase in CrCl was abolished. RAGE-ab administration in NMRI mice caused a reduction in liver weight and an increase in BMT. Renal messenger RNA (mRNA) for connective tissue growth factor and collagen IV $\alpha$ 1 was increased in placebo-treated diabetic animals. RAGE-ab treatment had no impact on the expression of these factors. The renal effects of RAGE-ab administration in STZ-diabetic mice were seen without impact on body weight, blood glucose or food consumption. In conclusion, the present data support the hypothesis that RAGE is an important pathogenic factor in the renal changes in an animal model of type 1 diabetes.

*Journal of Endocrinology* (2006) **188**, 493–501

## Introduction

Diabetic nephropathy develops in 15–25% of all patients with type 1 diabetes and is one of the leading causes of end-stage renal failure. Accordingly, development of diabetic nephropathy is associated with a considerable increase in morbidity and mortality. Among the many mechanisms responsible for the development of diabetic kidney disease, advanced glycation endproducts (AGEs) have been suggested to have measurable effects on the development of diabetic kidney changes. Furthermore, the receptor for AGE (RAGE) has been proposed to play a key role in the development of diabetic renal changes in experimental diabetes (Yamamoto *et al.* 2001). Accordingly, streptozotocin (STZ)-diabetic mice, a model of type 1 diabetes, over-expressing RAGE, showed a pronounced increase in renal damage when compared with changes seen in non-transgenic diabetic animals

(Yamamoto *et al.* 2001). In addition, administration of soluble RAGE (sRAGE), a truncated form of RAGE, was shown to blunt the renal changes seen in a mouse model of type 2 diabetes (i.e. the *db/db* mouse) (Wendt *et al.* 2003). Finally, administration of a neutralising RAGE-antibody (ab) in the same mouse strain attenuated or normalised long-term renal changes (Flyvbjerg *et al.* 2004).

The aim of the present study was to explore the role of RAGE in the development of renal changes in type 1 diabetes. Accordingly, a specific neutralising murine RAGE-ab was administered for 2 months in STZ-diabetic mice. Renal functional and morphological parameters were recorded. In addition, the renal gene expressions (mRNA) of transforming growth factor  $\beta$  (TGF- $\beta$ ), connective tissue growth factor (CTGF), collagen IV $\alpha$ 1 and fibronectin were measured as markers of renal matrix accumulation, while mRNAs for vascular endothelial

growth factor A (VEGF-A), VEGF receptor 2 (VEGFR-2) and nephrin were used as markers for renal permeability.

## Materials and Methods

### Animals

Adult female NMRI mice (Taconic M&B, Ry, Denmark) with an initial body weight of 25 g were used. Diabetes was induced by a single i.v. injection of STZ (Upjohn, Kalamazoo, MI, USA) in a dose of 150 mg/kg body weight. Some animals were re-injected on day 2 with an STZ dose of 30 mg/kg body weight i.p. to obtain the appropriate degree of diabetes. Only animals with a blood glucose level above 17 mM and without ketonuria or massive body weight loss were included in the study.

The mice were housed 6 to 8 per cage in a room with a 12 h:12 h artificial light cycle (lights on 0700 h to 1900 h), a temperature of  $21 \pm 1$  °C, and a humidity of  $55 \pm 5\%$ . The animals had free access to standard chow (Altromin #1324; Altromin, Lage, Germany) and tap water throughout the experiment. The study complied with Danish regulations for the care and use of laboratory animals.

### Study design

The STZ-diabetic mice were randomised into two groups. One group of STZ-diabetic mice was treated with ip injections of a neutralising murine RAGE-ab, while the other half was treated with an isotype-matched, irrelevant immunoglobulin G (IgG) (Sigma). NMRI mice were randomised into two groups to receive either RAGE-ab or irrelevant IgG. The RAGE-ab and irrelevant IgG were administered i.p. in an initial bolus dose of 300 µg followed by doses of 100 µg three times weekly. The RAGE-ab and irrelevant IgG were dissolved in 0.154 mol/l NaCl and injected in a volume of 0.5 ml. A full characterisation of the RAGE-ab and documentation of its neutralising activity are described below.

Body weight, food consumption and blood glucose levels were determined at the initiation of the experiment and every 2 weeks. Blood glucose was measured in tail-vein blood as described below. After 8 weeks, the mice were placed in metabolic cages to collect 24-h urine samples for urinary albumin excretion (UAE) and urinary creatinine determinations. Urine samples were stored at  $-20$  °C until assay was performed. At the time of death, mice were anaesthetised with pentobarbital (50 mg/kg i.p.) and non-fasting blood samples were drawn from the retro-orbital venous plexus using heparinised capillary tubes. Serum samples were stored at  $-80$  °C until analysis was performed. In all animals, the right and left kidneys were removed and weighed. The middle piece of the right kidney (including the papilla) was fixed in 4%

paraformaldehyde for determination of glomerular volume by light microscopy (LM) (see details below). The middle piece of the left kidney (including the papilla) was fixed in 0.1 M cacodylate buffer with 1% glutaraldehyde and 2% paraformaldehyde for later determination of basement membrane thickness (BMT) by electron microscopy (EM) (see details below). Cortical renal tissue obtained from the left kidney poles was snap-frozen in liquid nitrogen for later mRNA measurements by quantitative Real-Time PCR (RT-PCR). In addition, liver and heart were removed, weighed, and snap-frozen in liquid nitrogen.

### Neutralising monoclonal RAGE-ab

The preparation and characterisation of the neutralising monoclonal RAGE-ab followed procedures previously described in detail for the preparation of other neutralising monoclonal antibodies (Tilton *et al.* 1997). The human RAGE extracellular domain encompassing residues 23–340 (sRAGE) was expressed and purified from *Escherichia coli* using the pET thioredoxin system (Novagen, Madison, WI, USA). Female 8-week-old Balb/c mice were immunised, boosted 3 times 21 days apart, by i.p. and s.c. injections of 100 µg sRAGE protein in Complete Freund's adjuvant for the primary immunisation and an additional 50 µg sRAGE protein in Incomplete Freund's adjuvant for secondary immunisations. The mouse with the highest serum titre to sRAGE, measured by ELISA, was injected i.v. with an additional 30 µg immunogen in PBS, 21 days after the last immunisation. Three days later, spleen cells were harvested for production of hybridomas to sRAGE using previously described techniques (Bjerkke *et al.* 1986). The hybridoma cell line with the highest ab titre and neutralising ab activity was selected after cloning 3–4 times by limiting dilution in 96-well microtiter plates, grown in a Cellmax Bioreactor (Spectrum, Rancho Dominguez, CA, USA) using DMEM culture media. Purified IgG was prepared by Protein-A chromatography. The isotype (IgG<sub>3</sub>) and light chain composition (κ) of the ab were determined as described previously (Tilton *et al.* 1997).

### Characterisation of RAGE-ab neutralising activity

A nuclear factor kappa B (NFκB) reporter-gene assay using N<sup>ε</sup>-(carboxymethyl)-lysine-modified human serum albumin (CML-HSA) as a ligand for RAGE was used to measure the neutralising activity of the monoclonal ab. Details of the preparation of CML-HSA as well as the reporter-gene assay have been published previously (Yeh *et al.* 2001). THP-1 cells were seeded at  $5 \times 10^6$  cells per 100-mm dish in 10 ml serum-free medium (SFM) the day before transfection. Transient transfection was performed using the DEAE-dextran method as described previously (Shirakawa *et al.* 1993). Cells were washed once with SFM and re-suspended in 1 ml of the same medium containing

2 µg NFκB-Luc reporter plasmid (Clontech, Palo Alto, CA, USA) and 200 mg/l DEAE-dextran (Promega). The cell-DNA mixture was incubated at room temperature for 20–30 min prior to washing, centrifugation, and resuspension into fresh SFM. Transfected cells were seeded into 96-well plates at 70 000 cells/well for recovery. After 24 h, cells were pretreated with 10 to 100 mg/l RAGE-ab for 1 h and then treated with 200–600 mg/l CML-modified albumin for 1–6 h before the reporter assay. Equivalent amounts of cell lysates, normalised for total protein (Bradford protein assay; Bio-Rad), were used for measurement of luciferase activity. Luciferase assays were performed using the Steady-Glo luciferase assay system according to the manufacturer's instructions (Promega), and luminescence was detected in a TopCount microplate scintillation counter using a single-photon monitor program (Packard Instrument Company, Meriden, CT, USA).

#### Determination of blood glucose, UAE and creatinine clearance (CrCl)

Blood glucose was measured at day 0 and every 2 weeks in tail-vein blood by Precision Xtra Plus (Abbott Laboratories, MediSence Products), and the urine was tested for glucose and ketone bodies by Combur<sup>5</sup> Test D (Roche Diagnostics). Urinary albumin concentration was determined by a commercially available kit, Mouse ELISA Quantification Kit (Bethyl Laboratories Inc., Montgomery, TX, USA). Serum and urinary creatinine concentrations were measured by an automated technique adapted from the method of Jaffé and corrected for the prevailing glucose content due to interference in the Jaffé reaction. The CrCl was expressed in ml/h. The intra- and interassay coefficients of variation were <5% and <10% for both assays.

#### Estimation of glomerular volume

The middle part of the right kidney (containing the papilla) was embedded in paraffin for LM examination. Two micron-thick sections were cut on a Rotary microtome HM350 (Microm, Walldorf, Germany) and stained with periodic acid-Schiff (PAS) and haematoxylin. The mean glomerular tuft volume ( $V_G$ ) was determined from the mean glomerular cross-sectional area ( $A_G$ ) at a magnification of  $\times 400$  as previously described (Weibel 1979, Gundersen *et al.* 1988, Pagtalunan *et al.* 1995). The areas were determined with a 2D-version of the nucleator (CAST, Olympus, Copenhagen, Denmark) (Weibel 1979) as the average area of a total of 40–50 glomerular profiles (tuft omitting the proximal tubular tissue within the Bowman capsule).  $V_G$  was calculated as:  $V_G = \beta/k \times (A_G)^{3/2}$  where  $\beta = 1.38$ , which is the shape coefficient for spheres (the idealised shape of glomeruli), and  $k = 1.1$ ,

which is a size distribution coefficient (Weibel 1979, Gundersen *et al.* 1988, Pagtalunan *et al.* 1995).

#### Estimation of BMT

Small blocks of cortical tissue were embedded in Epon 825 (Taab, Reading, Berks, UK) for EM examination. Thin sections were cut on a Reichert Ultracut (Leica, Vienna, Austria) and stained with uranyl acetate and lead citrate. From an EM (Tecnai 12; Phillips, Eindhoven, Holland), images covering the whole glomerular profile were recorded with a MegaView video camera (Soft Imaging System, Münster, Germany) onto a monitor. For measurements of BMT, randomised fields were recorded at a magnification of  $\times 30\,000$  from the same sections described above. BMT was measured applying the orthogonal intercept method as previously described (Jensen *et al.* 1979). About 60 measurements were performed per glomerulus and BMT is given as a harmonic mean.

#### Quantitative Real-Time PCR (RT-PCR)

After homogenisation of kidney tissue by the use of an MM301 Mixer Mill (Retsch, Haan, Germany), total cellular RNA was extracted from renal cortical tissue using a 6100 Nucleic Acid PrepStation (Applied Biosystems, Foster City, CA, USA). The quality of rRNA was estimated by agarose gel electrophoresis by the appearance of two distinct bands visible by fluorescence of ethide bromide representing intact rRNA. The amounts of RNA extracted were quantified by measuring the absorbance at 260 nm by spectrophotometry. Reverse transcription from RNA to DNA was performed with a Multiscribe Reverse Transcriptase kit from Applied Biosystems under the following conditions: 25 °C for 10 min, 48 °C for 30 min and 94 °C for 29 s. The PCR was performed in triplicates of each sample in a volume of 25 µl in each well containing RNA, TaqMan Universal PCR MasterMix and a primer of the target, i.e. TGF-β (Mm 00441724), CTGF (Mm 00515790), collagen IVα1 (185088267A), fibronectin (1546736), VEGF-A (Mm 00437304), VEGFR-2 (Mm 00440099) or nephrin (Mm 497828), and a primer of the housekeeping gene, 18S (4319413), all purchased from Applied Biosystems. Liver RNA was used as a negative control. Each RT-PCR reaction ran at 50 °C for 2 min, 95 °C for 10 min and in 40 cycles changing between 95 °C for 15 s and 60 °C for 1:30 min.

#### PCR data analysis

Data were analysed with the ABI Prism 7000 Sequence Detector Software (Applied Biosystems). The output of amplification was measured in the exponential phase of the reaction as the threshold cycle/Ct-value, which is

**Table 1** Mean body weight, blood glucose, food consumption, liver weight and heart weight at day 60 in placebo-treated NMRI mice, RAGE-antibody (ab)-treated NMRI mice, placebo-treated STZ-diabetic mice and RAGE-ab-treated STZ-diabetic mice. Data are expressed as means  $\pm$  S.E.M.,  $n=8-11$  in each group

	Bodyweight (g)	Blood glucose (mM)	Food consumption (g)	Liver weight (mg)	Heart weight (mg)
NMRI, placebo	30.8 $\pm$ 0.6	6.6 $\pm$ 0.1	5.3 $\pm$ 0.2	1398 $\pm$ 59	131.8 $\pm$ 7.7
NMRI, RAGE-ab	30.6 $\pm$ 0.6	6.1 $\pm$ 0.3	5.5 $\pm$ 0.3	1218 $\pm$ 41*	118.8 $\pm$ 2.6
STZ-diabetic, placebo	23.4 $\pm$ 0.6*	23.4 $\pm$ 1.2*	9.1 $\pm$ 0.9*	1520 $\pm$ 62	96.2 $\pm$ 2.5*
STZ-diabetic, RAGE-ab	23.5 $\pm$ 0.6*	25.2 $\pm$ 0.9*	8.9 $\pm$ 1.0*	1491 $\pm$ 62	92.8 $\pm$ 2.9*

\* $P<0.01$  vs non-diabetic control groups.

defined as the cycle number at which amplification products are detected, corresponding to the point where fluorescent intensity exceeds the background fluorescent intensity, which is 10 times the standard deviation of the baseline. The average of triplicates from each sample was used. The relative quantification of target gene was calculated using the formula:  $(1/2)^{Ct\text{-target gene} - Ct\text{-housekeeping gene}}$ , which is described in the Users Bulletin 2 1997 from Perkin-Elmer (Perkin-Elmer Cetus, Norwalk, CT, USA) (Lihn *et al.* 2004).

#### Statistical analysis

ANOVA for repeated measurements was used to evaluate differences with Student's *t*-test for unpaired comparisons. A *P*-value of less than 0.05 was considered statistically significant. For data not following a normal distribution, the Mann-Whitney Rank Sum test was used. All data are expressed as means  $\pm$  S.E.M., with *n* indicating the number of mice studied. Statistical analysis was performed using the statistical package STATA for Windows (Stata Corp LP, Texas, USA).

## Results

#### Body weight, blood glucose, food consumption, liver and heart weight

STZ-diabetic mice had a lower body weight ( $P<0.001$ ) and higher food consumption than the non-diabetic controls ( $P<0.01$ ) (Table 1). Mean blood glucose levels were approximately 23–25 mM in STZ-diabetic mice throughout the study, and 6–7 mM in NMRI mice (Table 1). There was no difference in liver weight between placebo-treated NMRI mice and placebo-treated STZ-diabetic mice (not significant (NS)) (Table 1). The heart weight of placebo-treated NMRI mice was increased when compared with placebo-treated STZ-diabetic mice ( $P<0.001$ ) (Table 1). With the exception of liver weights in NMRI mice ( $P<0.03$ ), RAGE-ab administration did not affect any of the above parameters in either NMRI or STZ-diabetic mice (Table 1).

#### Kidney weight, glomerular volume and BMT

The kidney weight of placebo-treated STZ-diabetic mice was increased when compared with placebo-treated

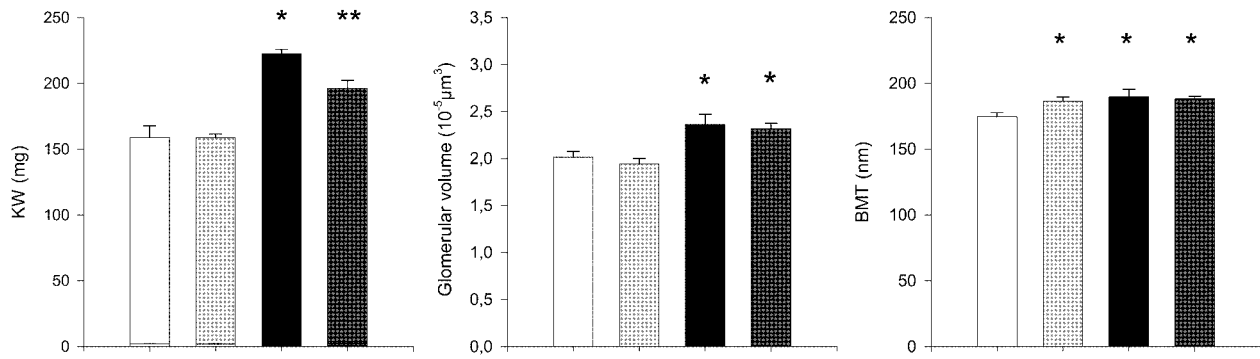
NMRI mice ( $P<0.001$ ). RAGE-ab administration had no effect on kidney weight in NMRI animals (158.9  $\pm$  9.0 vs 158.5  $\pm$  3.0 mg, NS). However, the kidney weight of RAGE-ab-treated STZ-diabetic mice was decreased when compared with placebo-treated STZ-diabetic mice (196.3  $\pm$  6.0 vs 222.4  $\pm$  3.0 mg,  $P<0.002$ ), although the kidney weight was still higher than that seen in placebo-treated NMRI animals ( $P<0.002$ ) (Fig. 1). The glomerular volume of placebo-treated STZ-diabetic mice was increased when compared with that of placebo-treated NMRI mice ( $P<0.03$ ). No difference in glomerular volume was seen between the two NMRI groups (2.02  $\pm$  0.1 vs 2.0  $\pm$  0.7  $10^{-5}$   $\mu\text{m}^3$ , NS), as was the case between the two STZ-diabetic groups (2.4  $\pm$  0.1 vs 2.3  $\pm$  0.7  $10^{-5}$   $\mu\text{m}^3$ , NS) (Fig. 1). In placebo-treated STZ-diabetic mice, the BMT was greater than in placebo-treated NMRI mice (189.7  $\pm$  6.8 vs 174.6  $\pm$  3.1 nm,  $P<0.04$ ). RAGE-ab administration in NMRI mice was followed by a modest but significant increase in BMT (186.5  $\pm$  3.2 nm), when compared with placebo-treated NMRI-mice ( $P<0.03$ ). RAGE-ab administration had no effect on BMT in the STZ-diabetic group (Fig. 1).

#### UAE and CrCl

A pronounced increase in UAE was observed in placebo-treated STZ-diabetic mice at day 60 when compared with placebo-treated NMRI mice ( $P<0.001$ ). UAE was significantly lower in RAGE-ab-treated STZ-diabetic mice (326.1  $\pm$  37.7  $\mu\text{g}/24$  h) vs placebo-treated STZ-diabetic mice (1067.5  $\pm$  184.0  $\mu\text{g}/24$  h,  $P<0.003$ ), but still the level was higher than in placebo-treated NMRI mice ( $P<0.002$ ). No difference in UAE was seen between the NMRI groups (97.4  $\pm$  20.1 vs 88.8  $\pm$  18.5  $\mu\text{g}/24$  h, NS) (Fig. 2). Placebo-treated STZ-diabetic mice showed almost a doubling in CrCl when compared with placebo-treated NMRI mice (11.9  $\pm$  1.0 ml/h vs 7.0  $\pm$  2.9,  $P<0.02$ ) (Fig. 2). RAGE-ab administration in STZ-diabetic mice decreased CrCl to below the level of non-diabetic animals (3.6  $\pm$  0.3 ml/h,  $P<0.001$ ). No effect of RAGE-ab treatment was seen in NMRI animals (Fig. 2).

#### Renal gene transcripts

TGF- $\beta$  mRNA levels in placebo-treated NMRI mice and placebo-treated STZ-diabetic mice were comparable



**Figure 1** Kidney weight (KW), glomerular volume and basement membrane thickness (BMT) on day 60 in placebo-treated NMRI mice (open bars), RAGE-antibody (RAGE-ab)-treated NMRI mice (light grey bars), placebo-treated streptozotocin (STZ)-diabetic mice (black bars) and RAGE-ab-treated STZ-diabetic mice (dark grey bars). Data are means  $\pm$  S.E.M.,  $n=8-12$  in each group. \* $P<0.04$  vs placebo-treated NMRI mice; \*\* $P<0.002$  vs placebo-treated STZ-diabetic mice.

( $8.7 \pm 0.9$  vs  $8.2 \pm 0.6 \times 10^{-6}$ , NS). No difference in TGF- $\beta$  mRNA levels were seen between the two STZ-diabetic groups (NS), although TGF- $\beta$  mRNA levels were lower in RAGE-ab-treated NMRI mice ( $6.1 \pm 0.6 \times 10^{-6}$ ) when compared with placebo-treated mice ( $P<0.03$ ) (Fig. 3). CTGF mRNA levels were increased in placebo-treated STZ-diabetic mice when compared with placebo-treated NMRI mice ( $1.8 \pm 0.1$  vs  $0.7 \pm 0.1 \times 10^{-4}$ ,  $P<0.001$ ). RAGE-ab treatment had no effect on CTGF mRNA levels either in NMRI or in STZ-diabetic animals (Fig. 3). Collagen IV $\alpha$ 1 mRNA was higher in placebo-treated STZ-diabetic mice when compared with placebo-treated NMRI mice ( $2.6 \pm 0.2$  vs  $1.7 \pm 0.1 \times 10^{-3}$ ,  $P<0.003$ ). RAGE-ab administration lowered the level of collagen IV $\alpha$ 1 mRNA in NMRI mice ( $P<0.02$ ), but had no effect in the STZ-diabetic group (Fig. 3). No difference in fibronectin mRNA between the four groups was seen. The level of VEGF-A mRNA was lower in placebo-treated STZ-diabetic mice than in placebo-treated NMRI mice ( $4.3 \pm 0.2$  vs  $6.4 \pm 0.3 \times 10^{-4}$ ,  $P<0.001$ ). No effect of RAGE-ab administration on VEGF-A mRNA levels was seen between the NMRI animals or between the STZ-diabetic animals (Fig. 4). VEGFR-2 and nephrin mRNA levels were comparable between NMRI mice and STZ-diabetic mice with no impact of RAGE-ab administration (Fig. 4).

## Discussion

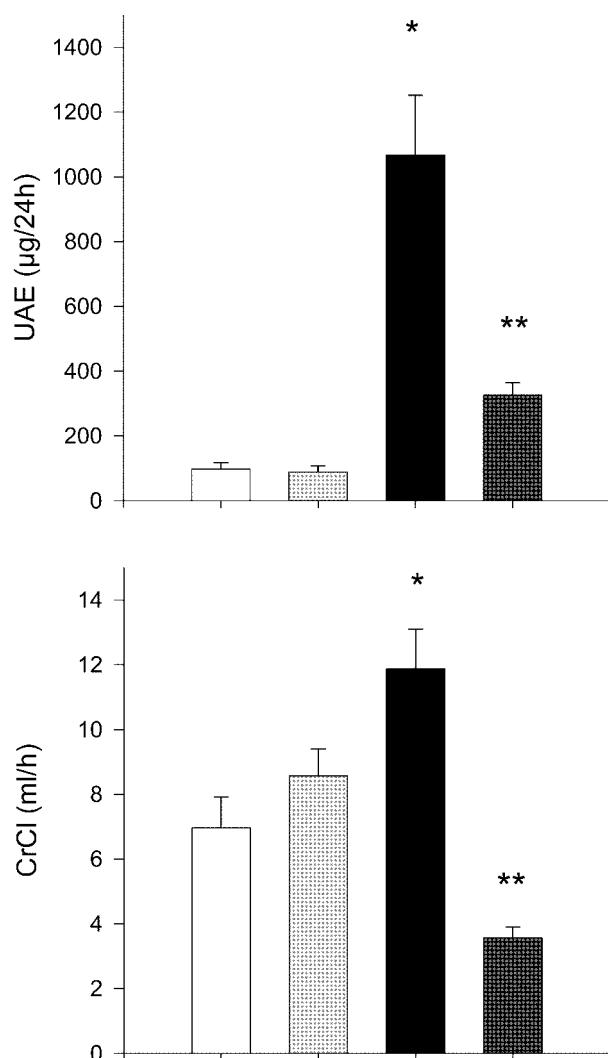
The new finding of the present study is a specific effect of RAGE-ab administration on several features of renal dysfunction in STZ-diabetic mice. RAGE-ab administration attenuated the increase in kidney weight and UAE and decreased CrCl to below normal. RAGE-ab administration had few renal effects in non-diabetic animals. Further, the renal effects in diabetic mice were seen without any effect on metabolic control, body weight

or food consumption. These data indicate that RAGE plays a pathogenic role in the development of renal changes seen in a model of type 1 diabetes.

Non-enzymatic glycation occurs through a series of biochemical reactions between glucose and other reactive carbonyl compounds, proteins, lipids or nucleic acids (Singh *et al.* 2001). AGEs interact with specific receptors, the best characterised being RAGE (Schmidt *et al.* 2000). In addition, AGEs also act through a non-receptor-mediated pathway by formation of cross-linked proteins (Schmidt *et al.* 2000). Through both mechanisms, AGEs may activate signal transduction pathways that involve intracellular factors, cytokines and growth factors (Schrijvers *et al.* 2004). The co-localisation of AGEs and RAGE in diabetic kidneys suggests that this ligand-receptor interaction may represent an important mechanism in the pathogenesis of diabetic complications (Souliis *et al.* 1997).

The overall involvement of AGEs in the pathogenesis of renal damage is supported by a study in non-diabetic rats, where AGE infusion enhanced serum and renal AGEs, and furthermore was followed by an aggravation in kidney morphology and function (Vlassara *et al.* 1994). Further, AGE injections in non-diabetic mice increased the levels of gene transcripts of growth factors and extracellular matrix (ECM) components, classically linked to the development of diabetic kidney disease (Striker & Striker 1996). These studies indicate that AGEs have an impact on the kidney independent of hyperglycaemia. In STZ-diabetic rats, an increase in renal AGEs was seen in parallel with morphologic kidney alterations (Bendayan 1998). Further, in OLETF rats (a model of type 2 diabetes), renal AGEs levels were increased, which, in addition, correlated with impaired kidney function (Nakamura *et al.* 2003).

Evidence for a role of RAGE in renal changes in STZ-diabetic mice was seen in a study using STZ-diabetic mice overexpressing human RAGE (Yamamoto *et al.* 2001).



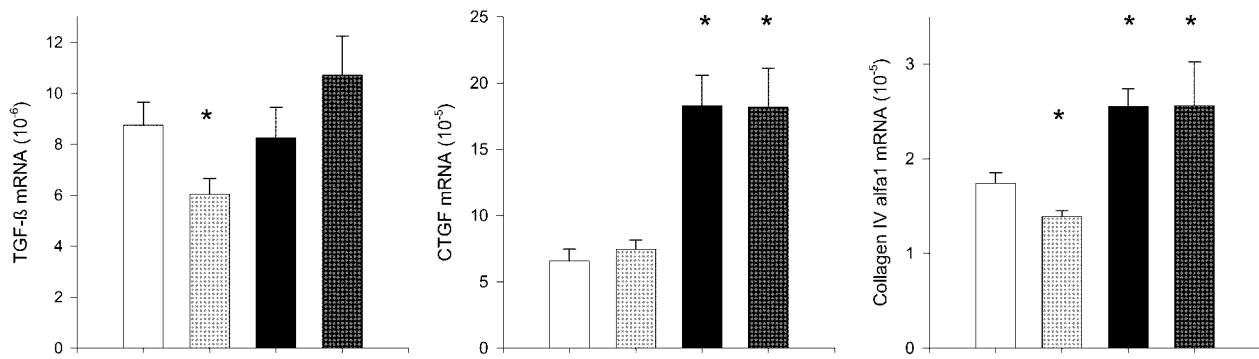
**Figure 2** Urinary albumin excretion (UAE) and creatinine clearance (CrCl) on day 60 in placebo-treated NMRI mice (open bars), RAGE-antibody (RAGE-ab)-treated NMRI mice (light grey bars), placebo-treated streptozotocin (STZ)-diabetic mice (black bars) and RAGE-ab-treated STZ-diabetic mice (dark grey bars). Data are means  $\pm$  S.E.M.,  $n=8-12$  in each group. \* $P<0.02$  vs placebo-treated NMRI mice; \*\* $P<0.003$  vs placebo-treated STZ-diabetic mice.

These animals developed renal/glomerular hypertrophy, increased albuminuria, mesangial expansion, glomerulosclerosis, and increased serum creatinine, robustly exceeding the levels seen in diabetic non-transgenic littermates (Yamamoto *et al.* 2001). Furthermore, induction of STZ-diabetes in homozygous RAGE null mice (i.e. animals with a global deletion of RAGE) was followed by a diminished increase in renal hypertrophy, glomerular and mesangial area, and BMT when compared with diabetic wild-type animals (Wendt *et al.* 2003).

Several studies have investigated the role of AGE blockade in diabetic kidney disease, especially in rodents (Degenhardt *et al.* 2002, Davis *et al.* 2004) and some in humans (Bolton *et al.* 2004, McGill *et al.* 2004), but only a few studies have focused on blockade of RAGE. In a study using *db/db* mice, administration of sRAGE for 19 weeks ameliorated the increase in glomerular area, mesangial area, UAE and BMT (Wendt *et al.* 2003). Furthermore, VEGF antigen and TGF- $\beta$  mRNA were reduced (Wendt *et al.* 2003). A recent study showed that RAGE-ab administration for 2 months in *db/db* mice was followed by an attenuation of the diabetes-associated increase in renal weight, glomerular volume, mesangial expansion and UAE, and further by prevention of the increase in CrCl and BMT (Flyvbjerg *et al.* 2004). Both these studies support the role of RAGE in diabetic kidney disease in models of type 2 diabetes.

In the present study, administration of a neutralising RAGE-ab for 2 months in a mouse model of type 1 diabetes was followed by effects on early features of diabetic kidney disease, i.e. a reduction or normalisation of renal enlargement, UAE and CrCl. No effect of RAGE-ab administration was seen on glomerular volume or BMT, which is in contrast to the study mentioned above where *db/db* mice were treated with the same RAGE-ab as the one used in the present study. The reason for these slight discrepancies might reflect that each study was performed at a single time point and, consequently, at different points in disease progression. Clearly, future studies evaluating the time course of the effect of RAGE in the amelioration of structural and functional changes will be essential. Furthermore, these differences might reflect fundamentally different roles of RAGE in the structural and functional changes in type 1 and type 2 models.

Several studies have investigated the role of growth factors and cytokines in the development of diabetic kidney disease (Schrijvers *et al.* 2004). Two promising candidates to play a role in the pathogenesis of diabetic kidney disease are TGF- $\beta$  (Sharma *et al.* 1996) and CTGF (Riser *et al.* 2000, Twigg *et al.* 2002), as the renal expression of these factors has been shown to be increased in various animal models of diabetes. The production of these growth factors is thought, in part, to be regulated by AGEs, as inhibitors of AGEs and RAGE have been shown to decrease TGF- $\beta$  and CTGF mRNA and protein in diabetic kidney disease (Tsuchida *et al.* 1999, Twigg *et al.* 2002, Wendt *et al.* 2003). Furthermore, overproduction of TGF- $\beta$  and CTGF has been linked to the increase in renal ECM formation i.e. collagen and fibronectin, classically seen in diabetes (Riser *et al.* 2000, Twigg *et al.* 2002). In the present study, placebo-treated diabetic animals presented with increased renal levels of CTGF and collagen IV $\alpha$ 1 mRNA, but not TGF- $\beta$  mRNA, when compared with placebo-treated NMRI mice. In the STZ-diabetic mice, RAGE-ab administration had no impact on the

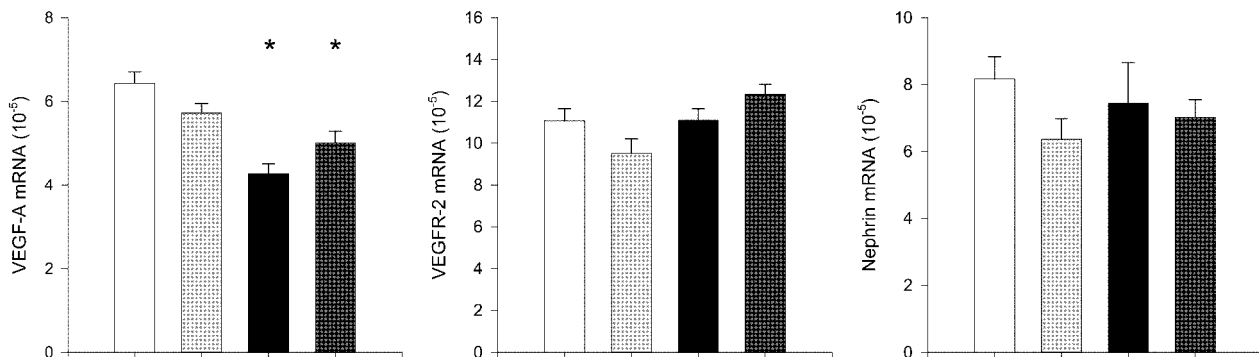


**Figure 3** Transforming growth factor-β (TGF-β), connective tissue growth factor (CTGF) and collagen IVα1 mRNA on day 60 in placebo-treated NMRI mice (open bars), RAGE-antibody (RAGE-ab)-treated NMRI mice (light grey bars), placebo-treated streptozotocin (STZ)-diabetic mice (black bars) and RAGE-ab-treated STZ-diabetic mice (dark grey bars). Data are means ± s.e.m.,  $n=8-12$  in each group. \* $P<0.03$  vs placebo-treated NMRI mice.

increased renal CTGF and collagen IVα1 mRNA levels, indicating that the renal effects of RAGE blockade may not be mediated through CTGF and may not alter the production of collagen IVα1. VEGF is another growth factor that has been linked to the development of diabetic kidney disease by mediating vascular hyperpermeability. Increased renal VEGF levels in parallel with VEGFR-2 have been described in animal models of diabetes (Cooper *et al.* 1999, Tsuchida *et al.* 1999). Because the increases in VEGF mRNA and protein have been reduced by AGE and RAGE inhibitors in animal models of diabetes, it has been suggested that VEGF is controlled by the AGE-RAGE system (Tsuchida *et al.* 1999, Wendt *et al.* 2003). In the present study, the level of VEGF-A mRNA was higher in the placebo-treated NMRI mice than in the STZ-diabetic mice, whereas no difference between placebo-treated NMRI mice and STZ-mice was seen when measuring VEGFR-2 mRNA. Nephrin is a molecule located to the slit diaphragm in the glomerulus filtration barrier between the foot-processes of the podocytes, involved in the filtration of molecules into

Bowmans capsule (Hamano *et al.* 2002, Wartiovaara *et al.* 2004). In different kidney diseases, defects in the slit diaphragm are followed by a decrease in renal nephrin and proteinuria (Kawachi *et al.* 2002). This was also seen in STZ-diabetic rats, where the nephrin mRNA level was decreased after 32 weeks of diabetes (Kelly *et al.* 2002). Studies investigating the potential effect of AGEs on nephrin expression in the diabetic kidney are conflicting; in one study, an AGE inhibitor could ameliorate the reduction in renal nephrin protein in a model of diabetic kidney disease (Davis *et al.* 2003) while in another study no effect was seen (Kelly *et al.* 2002). In the present study, the nephrin mRNA level was unchanged in all four groups after diabetes duration of 8 weeks.

In conclusion, the present data support the hypothesis that RAGE is an important pathogenic factor in the development of early renal changes in diabetic kidney disease in type 1 diabetes. Future studies are warranted to further elucidate the role of RAGE in diabetic kidney disease and to explore how agents with specific RAGE blocking properties can be developed for clinical trials.



**Figure 4** Vascular endothelial growth factor A (VEGF-A), VEGF receptor 2 (VEGFR-2) and nephrin mRNA on day 60 in placebo-treated NMRI mice (open bars), RAGE-antibody (RAGE-ab)-treated NMRI mice (light grey bars), placebo-treated streptozotocin (STZ)-diabetic mice (black bars) and RAGE-ab-treated STZ-diabetic mice (dark grey bars). Data are means ± s.e.m.,  $n=8-12$  in each group. \* $P<0.004$  vs placebo-treated NMRI mice.

## Acknowledgements

The excellent technical assistance by Karen Mathiasen and Kirsten Nyborg is highly appreciated. Encysive Pharmaceuticals, Houston, Texas is thanked for the generous gift of the RAGE-ab. The work was supported by the Danish Medical Research Council, the Eva and Henry Fränkels Memorial Foundation, the Danish Diabetes Association and the Clinical Institute, University of Aarhus, Denmark. Louise J N Jensen was supported by a scholarship from the Danish Medical Research Council. The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

## References

- Bendayan M 1998 Immunocytochemical detection of advanced glycosylated end products in rat renal tissue as a function of age and diabetes. *Kidney International* **54** 438–447.
- Bjerveck RJ, Cook G, Rychlik N, Gjika HB, Van Vunakis H & Langone JJ 1986 Stereospecific monoclonal antibodies to nicotine and cotinine and their use in enzyme-linked immunosorbent assays. *Journal of Immunological Methods* **90** 203–213.
- Bolton WK, Catran DC, Williams ME, Adler SG, Appel GB, Cartwright K, Foiles PG, Freedman BI, Raskin P, Ratner RE *et al.* 2004 Randomised trial of an inhibitor of formation of advanced glycation end products in diabetic nephropathy. *American Journal of Nephrology* **24** 32–40.
- Cooper ME, Vranes D, Youssef S, Stacker SA, Cox AJ, Rizkalla B, Casley DJ, Bach LA, Kelly DJ & Gilbert RE 1999 Increased renal expression of vascular endothelial growth factor (VEGF) and its receptor VEGFR-2 in experimental diabetes. *Diabetes* **48** 2229–2239.
- Davis BJ, Johnston CI, Burrell LM, Burns WC, Kubota E, Cao Z, Cooper ME & Allen TJ 2003 Renoprotective effects of vasopeptidase inhibition in an experimental model of diabetic nephropathy. *Diabetologia* **46** 961–971.
- Davis BJ, Forbes JM, Thomas MC, Jerums G, Burns WC, Kawachi H, Allen TJ & Cooper ME 2004 Superior renoprotective effects of combination therapy with ACE and AGE inhibition in the diabetic spontaneously hypertensive rat. *Diabetologia* **47** 89–97.
- Degenhardt TP, Alderson NL, Arrington DD, Beattie RJ, Basgen JM, Steffes MW, Thorpe SR & Baynes JW 2002 Pyridoxamine inhibits early renal disease and dyslipidemia in the streptozotocin-diabetic rat. *Kidney International* **61** 939–950.
- Flyvbjerg A, Denner L, Schrijvers BF, Tilton RG, Mogensen TH, Paludan SR & Rasch R 2004 Long-term renal effects of a neutralising RAGE antibody in obese type 2 diabetic mice. *Diabetes* **53** 166–172.
- Gundersen HJ, Bagger P, Bendtsen TF, Evans SM, Korbo L, Marcussen N, Møller A, Nielsen K, Nyengaard JR & Pakkenberg B 1988 The new stereological tools: disector, fractionator, nucleator and point sampled intercepts and their use in pathological research and diagnosis. *APMIS* **96** 857–881.
- Hamano Y, Grunkemeyer JA, Sudhakar A, Zeisberg M, Cosgrove D, Morello R, Lee B, Sugimoto H & Kalluri R 2002 Determinants of vascular permeability in the kidney glomerulus. *Journal of Biological Chemistry* **277** 31154–31162.
- Jensen EB, Gundersen HJ & Østerby R 1979 Determination of membrane thickness distribution from orthogonal intercepts. *Journal of Microscopy* **115** 19–33.
- Kawachi H, Koike H & Shimizu F 2002 mAb 5–1–6 nephropathy and nephrin. *Microscopy Research Technique* **57** 236–240.
- Kelly DJ, Aaltonen P, Cox AJ, Rumble JR, Langham R, Panagiotopoulos S, Jerums G, Holthofer H & Gilbert RE 2002 Expression of the slit-diaphragm protein, nephrin, in experimental diabetic nephropathy: differing effects of anti-proteinuric therapies. *Nephrology, Dialysis, Transplantation* **17** 1327–1332.
- Lihn AS, Bruun JM, He G, Pedersen SB, Jensen PF & Richelsen B 2004 Lower expression of adiponectin mRNA in visceral adipose tissue in lean and obese subjects. *Molecular Cellular Endocrinology* **219** 9–15.
- McGill JB, Williams ME, Degenhardt TP, Szabo JR & Scotzinger RJ A 2004 Phase 2 clinical investigation of pyridoxamine (pyridorin) in type 1 and type 2 diabetic patients with overt diabetic nephropathy (PYR-205/207) (Abstract). *Journal of the American Society of Nephrology* **15** 565A.
- Nakamura S, Tachikawa T, Tobita K, Aoyama I, Takayama F, Enomoto A & Niwa T 2003 An inhibitor of advanced glycation end product formation reduces N epsilon-(carboxymethyl) lysine accumulation in glomeruli of diabetic rats. *American Journal of Kidney Diseases* **41** S68–S71.
- Pagalunan ME, Rasch R, Rennke HG & Meyer TW 1995 Morphometric analysis of effects of angiotensin II on glomerular structure in rats. *American Journal of Physiology* **268** F82–F88.
- Riser BL, Denichilo M, Cortes P, Baker C, Grondin JM, Yee J & Narins RG 2000 Regulation of connective tissue growth factor activity in cultured rat mesangial cells and its expression in experimental diabetic glomerulosclerosis. *Journal of the American Society of Nephrology* **11** 25–38.
- Schmidt AM, Yan SD, Yan SF & Stern DM 2000 The biology of the receptor for advanced glycation end products and its ligands. *Biochimica et Biophysica Acta* **1498** 99–111.
- Schrijvers BF, De Vriese AS & Flyvbjerg A 2004 From hyperglycemia to diabetic kidney disease: the role of metabolic, hemodynamic, intracellular factors and growth factors/cytokines. *Endocrine Reviews* **25** 971–1010.
- Sharma K, Jin Y, Guo J & Ziyadeh FN 1996 Neutralization of TGF-beta by anti-TGF-beta antibody attenuates kidney hypertrophy and the enhanced extracellular matrix gene expression in STZ-induced diabetic mice. *Diabetes* **45** 522–530.
- Shirakawa F, Saito K, Bonagura CA, Galson DL, Fenton MJ, Webb AC & Auron PE 1993 The human prointerleukin 1 beta gene requires DNA sequences both proximal and distal to the transcription start site for tissue-specific induction. *Molecular Cellular Biology* **13** 1332–1344.
- Singh R, Barden A, Mori T & Beilin L 2001 Advanced glycation end-products: a review. *Diabetologia* **44** 129–146.
- Soulis T, Thallas V, Youssef S, Gilbert RE, McWilliam BG, Murray-McIntosh RP & Cooper ME 1997 Advanced glycation end products and their receptors co-localise in rat organs susceptible to diabetic microvascular injury. *Diabetologia* **40** 619–628.
- Striker LJ & Striker GE 1996 Administration of AGEs *in vivo* induces extracellular matrix gene expression. *Nephrology Dialysis Transplantation* **11** S62–S65.
- Tilton RG, Kawamura T, Chang KC, Ido Y, Bjerveck RJ, Stephan CC, Brock TA & Williamson JR 1997 Vascular dysfunction induced by elevated glucose levels in rats is mediated by vascular endothelial growth factor. *Journal of Clinical Investigation* **99** 2192–2202.
- Tsuchida K, Makita Z, Yamagishi S, Atsumi T, Miyoshi H, Obara S, Ishida M, Ishikawa S, Yasumura K & Koike T 1999 Suppression of transforming growth factor beta and vascular endothelial growth factor in diabetic nephropathy in rats by a novel advanced glycation end product inhibitor, OPB-9195. *Diabetologia* **42** 579–588.
- Twigg SM, Cao Z, McLennan SV, Burns WC, Brammar G, Forbes JM & Cooper ME 2002 Renal connective tissue growth factor induction in experimental diabetes is prevented by aminoguanidine. *Endocrinology* **143** 4907–4915.



- Vlassara H, Striker LJ, Teichberg S, Fuh H, Li YM & Steffes M 1994 Advanced glycation end products induce glomerular sclerosis and albuminuria in normal rats. *PNAS* **91** 11704–11708.
- Wartiovaara J, Ofverstedt LG, Khoshnoodi J, Zhang J, Makela E, Sandin S, Ruotsalainen V, Cheng RH, Jalanko H, Skoglund U *et al.* 2004 Nephritin strands contribute to a porous slit diaphragm scaffold as revealed by electron tomography. *Journal of Clinical Investigation* **114** 1475–1483.
- Weibel ER 1979 Stereologic methods: practical methods for biological morphometry, pp 51–57. London, UK: Academic Press.
- Wendt TM, Tanji N, Guo J, Kislinger TR, Qu W, Lu Y, Bucciarelli LG, Rong LL, Moser B & Schmidt AM 2003 RAGE drives the development of glomerulosclerosis and implicates podocyte activation in the pathogenesis of diabetic nephropathy. *American Journal of Pathology* **162** 1123–1137.
- Yamamoto Y, Kato I, Doi T, Yonekura H, Ohashi S, Takeuchi M, Watanabe T, Yamagishi S, Sakurai S, Takasawa S *et al.* 2001 Development and prevention of advanced diabetic nephropathy in RAGE-overexpressing mice. *Journal of Clinical Investigation* **108** 261–268.
- Yeh C-H, Sturgis L, Haidacher J, Zhang XN, Sherwood SJ, Bjercke RJ, Juhasz O, Crow MT, Tilton RG & Denner L 2001 Requirement for p38 and p44/p42 mitogen-activated protein kinases in RAGE-mediated nuclear factor-kappa B transcriptional activation and cytokine secretion. *Diabetes* **50** 1495–1504.

Received 29 November 2005

Accepted 16 December 2005