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 animals 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 IVt1 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.


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 β (TGF-β), connective tissue growth factor (CTGF), collagen IVt1 and fibronectin were measured as markers of renal matrix accumulation, while mRNAs for vascular endothelial

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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 12:12 h artificial light cycle (lights on 0700 h to 1900 h), a temperature of 21 ± 1°C, and a humidity of 55 ± 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-umbilical 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 (Bjercke 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 (IgG3) and light chain composition (k) of the ab were determined as described previously (Tilton et al. 1997).

Characterisation of RAGE-ab neutralising activity

A nuclear factor kappa B (NFkB) reporter-gene assay using Nε-(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 × 10⁶ 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-αb 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 Combur5 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 cross-sectional area (AG) at a magnification of ×400 as previously described (Weibel 1979, Gundersen et al. 1988, Pagtalunan et al. 1995). About 60 measurements were performed per glomerulus and BMT is given as a harmonic mean.

**Quantitive 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 ethidium 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 a size distribution coefficient (Weibel 1979, Gundersen et al. 1988, Pagtalunan et al. 1995).
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: \((1/2)^\Delta\Delta CT\), 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 ± 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 compared to 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 (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 ± 9–0 vs 158±5 ± 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 ± 6–0 vs 222±4 ± 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±0 ± 0 vs 1±0 ± 7 × 10⁻⁵ μm³, NS), as was the case between the two STZ-diabetic groups (2±4 ± 0 vs 2±3 ± 7 × 10⁻⁵ μm³, NS) (Fig. 1). In placebo-treated STZ-diabetic mice, the BMT was greater than in placebo-treated NMRI mice (189±7 ± 6–0 vs 174±6 ± 3–1 nm, P<0.04). RAGE-ab administration in NMRI mice was followed by a modest but significant increase in BMT (186±5 ± 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 ± 37–7 μg/24 h) vs placebo-treated STZ-diabetic mice (1067±5 ± 184–0 μg/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 ± 20–1 vs 88±8 ± 18–5 μg/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 ± 1–0 ml/h vs 7±0 ± 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 ± 0–3 ml/h, P<0.001). No effect of RAGE-ab treatment was seen in NMRI animals (Fig. 2).

**Renal gene transcripts**

TGF-β mRNA levels in placebo-treated NMRI mice and placebo-treated STZ-diabetic mice were comparable
The new finding of the present study is a specific e

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 (Soulis 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).
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-β 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-β (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-β and CTGF mRNA and protein in diabetic kidney disease (Tsuchida et al. 1999, Twigg et al. 2002). Furthermore, overproduction of TGF-β 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 IVα1 mRNA, but not TGF-β mRNA, when compared with placebo-treated NMRI mice. In the STZ–diabetic mice, RAGE-ab administration had no impact on the
increased renal CTGF and collagen IV mRNA levels, indicating that the renal effects of RAGE blockade may not be mediated through CTGF and may not alter the production of collagen IV. 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 3 Transforming growth factor-β (TGF-β), connective tissue growth factor (CTGF) and collagen IV 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.

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.
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