Candesartan reduced advanced glycation end-products accumulation and diminished nitro-oxidative stress in type 2 diabetic KK/Ta mice

Qiuling Fan, Jie Liao, Michimasa Kobayashi, Michifumi Yamashita, Leyi Gu, Tomohito Gohda, Yusuke Suzuki, Li Ning Wang1, Satoshi Horikoshi and Yasuhiko Tomino

Division of Nephrology, Department of Internal Medicine, Juntendo University School of Medicine, Tokyo, Japan; and Division of Nephrology, the First Affiliated Hospital of China Medical University, Shenyang, China

Abstract

Background. Angiotensin-II induces nitro-oxidative stress in patients with diabetic nephropathy. Peroxy-nitrite and reactive oxide species can accelerate formation of advanced glycation end-products (AGEs). We investigated the effects of candesartan, an angiotensin-II type 1 receptor blocker (ARB), on the formation of AGEs and nitro-oxidative stress in type 2 diabetic KK/Ta mouse kidneys.

Methods. KK/Ta mice were divided into three treatment groups: an early treatment group receiving 4 mg/kg/day candesartan from 6 to 28 weeks of age, a late treatment group receiving the same candesartan dose from 12 to 28 weeks of age and a group receiving the vehicle for candesartan. BALB/c mice treated with vehicle were used as controls. We evaluated at 28 weeks the renal expressions of carboxymethyllysine, the receptor for AGE (RAGE), the p47phox component of NADPH oxidase, endothelial nitric oxide synthase (eNOS), induced nitric oxide synthase (iNOS) and 8-OHdG and nitrotyrosine by immunohistochemistry and/or by competitive RT–PCR.

Results. Kidneys from KK/Ta mice showed increased formation of AGEs, nitro-oxidative stress and RAGE expression and these were attenuated by candesartan treatment. Protein and mRNA expressions of p47phox and iNOS were upregulated in KK/Ta kidneys, which also showed increased immunostaining intensities of 8-OHdG and nitrotyrosine. Treatment with candesartan attenuated all of these changes and prevented significant albuminuria. There were no significant differences in the expression of eNOS among the four groups.

Conclusions. These findings suggest that candesartan, an ARB, reduces AGE accumulation and subsequent albuminuria by down-regulating the NADPH oxidase p47phox component and iNOS expression and by attenuating RAGE expression in type 2 diabetic KK/Ta mouse kidneys.

Keywords: advanced glycation end-products; angiotensin-II-receptor antagonist; KK/Ta mouse; nitro-oxidative stress; NADPH oxidase p47phox; RAGE

Introduction

Increasing evidence indicates that advanced glycation end-products (AGEs) contribute to the pathogenesis of diabetic nephropathy. AGEs exert chemical, cellular and tissue effects through the formation of protein cross-links that alter the structure and function of extracellular matrix or that interact with specific receptors [1]. The best-characterized receptor for AGE, designated as RAGE (receptor for AGE), is a multiligand member of the immunoglobulin superfamily [1]. The binding of RAGE by AGEs activates several intracellular signalling pathways, including mitogen-activated protein kinase (MAPK), NF-κB and AP-1, and increases the production of cytokines, including TGF-β, VEGF and TNF-α [2]. Unless interrupted, this cascade of events leads to albuminuria and mesangial expansion and results in glomerular sclerosis. The AGE–RAGE pathway, therefore, represents a candidate molecular target for prevention and treatment of diabetic nephropathy.

Oxidative and nitrosative stresses are widely recognized as key factors in the development of diabetic nephropathy [3]. AGEs have long been associated with increased oxidative and nitrosative stresses in both in vitro [4] and in vivo [5] studies. Carboxymethyllysine (CML) is formed from sequential glycation and oxidation reactions (so-called glycoxidation).
Alternatively, interactions between AGEs and RAGE induce cellular oxidative stress and upregulate renal inducible nitric oxide synthase (iNOS) expression [4,6]. Thus, the crosstalk between nitro-oxidative stress and the AGE–RAGE pathway establishes an amplification loop leading to further production of free radicals and AGEs.

Angiotensin-II-receptor blockers (ARB) and angiotensin-converting enzyme inhibitors (ACEI) have been reported to reduce the in vitro formation of AGEs [7]. A recent study demonstrated that the ARB olmesartan significantly reduced kidney pentosidine content in spontaneously hypertensive/NH-corpulent rats [8]. Although this study did not explore the mechanism of AGE-lowering by ARB, the drug may have caused decreased oxidative stress. An additional study recently showed that ACEI reduced the accumulation of AGEs and nitrotyrosine in experimental diabetic nephropathy [9].

The inbred KK/Ta mouse strain, established in Japan, is a diabetic strain that spontaneously exhibits type 2 diabetes associated with fasting hyperglycaemia, glucose intolerance, hyperinsulinaemia, mild obesity, dyslipidaemia and albuminuria [10]. Renal lesions in KK/Ta mice closely resemble those in human diabetic nephropathy [11]. Glomeruli from diabetic KK/Ta mice show diffuse-type and/or nodular-type hyperplasia of mesangial areas with mesangial cell proliferation. Immunohistochemical studies show an intense, specific fluorescence for albumin and γ-globulin along the glomerular capillary walls. As in human disease, albumin excretion in diabetic KK/Ta mice shows an increase at several weeks after the establishment of hyperglycaemia and this presumably represents intact leaking nephrons without overt reduction in the single nephron glomerular filtration rate [12]. Therefore, KK/Ta mice are considered to provide a suitable model for type 2 diabetes and the early phase of diabetic nephropathy in humans. Recently, we carried out a genome-wide linkage analysis of KK/Ta alleles and identified a susceptible KK/Ta locus (U-A-I) responsible for the development of albuminuria on chromosome 2 [13,14]. In the present study, we focused on the interaction between the AGE–RAGE pathway and local nitro-oxidative stress in kidneys of the KK/Ta mouse in order to elucidate the in vivo AGE-lowering mechanism of ARB.

Subjects and methods

Animals and drug treatment

Male diabetic KK/Ta and non-diabetic BALB/c mice were purchased from CLEA Japan Inc. (Tokyo, Japan). The BALB/c mouse was found to be an appropriate control for the KK/Ta mouse in our previous study [13]. The mice were individually housed in plastic cages and were given free access to food (rodent pellet diet CE-2; 342.2 kcal/100 g containing 4.4% crude fat) and water or water treated with candesartan throughout the experimental periods. Fluid intake was measured every day. The dosages of candesartan were adjusted via the drinking water.

Clinical characteristics of animals

Body weight (BW), fasting glucose, systolic blood pressure (SBP), serum creatinine concentration and urinary albumin excretion of each mouse were serially monitored every 4 weeks. Glucose tolerance was assessed using the intraperitoneal glucose tolerance test (IPGTT). IPGTT was performed by injecting glucose (2 g/kg in 20% solution) intraperitoneally in overnight-fasted mice. Glucose levels in blood obtained from the retro-orbital sinus were measured using Glustest E (Kyoto Daichi Kagaku, Kyoto, Japan) at 0 (fasting blood glucose level) and 120 min after glucose injection. SBP was measured by a non-invasive tail cuff and pulse transducer system (BP-98A; Softron, Tokyo, Japan). Serum creatinine concentrations were enzymatically determined by an autoanalyzer (Fuji Dry-Chem 5500; Fuji Film, Tokyo, Japan). The concentrations of urinary albumin were examined by Exocell’s immunspecific enzyme-linked immunosorbent assay (Albwell M kit; Exocell Inc., Philadelphia, PA, USA) and all samples were individually adjusted for creatinine excretion (Creatinine Companion; Exocell Inc., Philadelphia, PA, USA).

Immunohistochemistry

Renal tissues were snap-frozen in optimum cutting temperature compound and cut into 3-μm-thick sections. Immunohistochemical studies were performed with the following commercially available antibodies: peroxidase-conjugated anti-CML monoclonal antibody (Transgenic, Kumamoto, Japan), anti-RAGE goat polyclonal antibody (Chemicon, Temecula, CA, USA), anti-nitrotyrosine rabbit polyclonal antibody (Upstate Biotechnology, Lake Placid, NY, USA), anti-8-OHdG mouse monoclonal antibody (NOF Corp., Tokyo, Japan), anti-NADPH oxidase p47phox rabbit polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-iNOS and endothelial nitric oxide synthase (eNOS) rabbit polyclonal antibodies (BD Transduction Laboratories, Lexington, KY, USA).

Frozen cryostat sections were fixed in acetone for 10 min and air dried. Endogenous peroxidase activity was quenched by incubation in 3% hydrogen peroxide/methanol for 15 min. The sections were then blocked by blocking solution (2% fetal bovine serum, 0.2% fish gelatin and 10% normal serum in phosphate-buffered saline). After incubation with primary antibodies at 4°C overnight, the sections were incubated with anti-rabbit or anti-mouse Envision+ polymer reagents (DAKO, Carpinteria, CA, USA), or anti-goat polymer reagents (Histofine, Tokyo, Japan), at room temperature for 30 min. Bound antibody was visualized by light microscopy with diaminobenzidine. The omission of primary antibodies
served as negative controls for each antibody in this study. Quantification of immunostaining for 8-OHdG in glomeruli was calculated by a modification of the method described by Toyokuni et al. [15].

For immunofluorescent staining, the sections were incubated with anti-CML or anti-nitrotyrosine antibody overnight using the same methods as described above. For CML immunostaining, the sections were exposed to Alexa Fluor 488 tyramide (Molecular Probes, Eugene, OR, USA) for 30 min according to the manufacturer’s instructions. For nitrotyrosine immunostaining, the sections were exposed to Alexa Fluor 488 goat anti-rabbit immunoglobulin G (Molecular Probes, Eugene, OR, USA) at room temperature for 2 h. The omission of primary antibodies served as negative controls for each antibody in this study. Immunofluorescent images were viewed with an Olympus BX50 microscope (Olympus, Tokyo, Japan) equipped with a cooled (−25°C) charge-coupled device camera (PXL; Photometrics Ltd, Tucson, AZ, USA). Fluorescence intensities in 10 glomeruli from each mouse were analysed with IPLab Spectrum v. 3.0 software (Signal Analytics, Vienna, VA, USA).

**Competitive RT–PCR**

Kidneys were dissected and snap frozen in liquid nitrogen for total RNA extraction. RNA was extracted with Trizol (Total RNA Isolation Reagent; Life Technologies, Rockville, MD, USA) and the purity was checked by spectrophotometry and agarose gel electrophoresis. Competitive reverse transcription–polymerase chain reaction (RT–PCR) was carried out using the Quantum RNA kit according to manufacturer’s instructions (Ambion Inc., Austin, TX, USA). Briefly, 1 μg total RNA was reverse transcribed with Superscript II RNase H–reverse transcriptase (Life Technologies, Rockville, MD, USA) using random hexamers as downstream primers. The obtained cDNA was further amplified by PCR. The sequences of the primers used for the amplifications were as follows:

- **RAGE:** (sense) 5’-CAG GGT CAC AGA AAC CGG-3’ (antisense) 5’-ATT CAG CTC TGC ACG TTC CT-3’
- **NADPH** oxidase p47phox: (sense) 5’-TCC TGG TTA AGT GGC AGG AC-3’ (antisense) 5’-CCA TGA GGC CGT TGA AGT AT-3’
- **eNOS:** (sense) 5’-GAC CCT CAC CGC TAC AAC AT-3’ (antisense) 5’-CTG GCC TTC TGC TCA TTT TC-3’
- **iNOS:** (sense) 5’-GAG GGA AGG AGG TCA AGT CC-3’ (antisense) 5’-AAG GTA GGA TGG GTG GTT CC-3’

The PCR parameters were 30 s denaturation at 95°C, 30 or 90 s annealing at 52–60°C and 60 or 90 s extension at 72°C for 33–35 cycles. A mixed ratio of 18S ribosomal RNA primers and competimers (usually primer:competimer ratios from 1:9 to 2:8, depending on the genes) was used to amplify rRNA as an internal control under the same conditions as the genes of interest. The PCR products were resolved on 2% agarose gel, stained with ethidium bromide and analysed by the VersaDocTM Imaging System (Bio-Rad Laboratories Inc., Hercules, CA, USA).

**Statistical analysis**

Data are expressed as means ± SEM. Statistical significance was determined by analysis of variance using Stat View 4.0. Differences with *P*-values of <0.05 were considered statistically significant.

**Results**

**Candesartan reduced elevations in urinary albumin excretion**

In untreated KK/Ta mice, urinary albumen excretion was markedly higher than in BALB/c mice at 28 weeks of age. Candesartan significantly reduced albuminuria in KK/Ta mice. There were no significant differences in urinary albumin excretion between the two candesartan groups (Figure 1).

Mean BW and fasting as well as 120 min blood glucose levels during IPGTT in KK/Ta mice were significantly higher than in age-matched BALB/c mice. Candesartan treatment did not affect BW or blood glucose levels. SBP in both the early and late treatment groups was markedly decreased (*P < 0.05*). Mean levels of serum creatinine were not significantly different among the four groups at 28 weeks of age (Table 1).

**Candesartan reduced CML accumulation and RAGE expression**

Immunofluorescent staining revealed that 28-week-old KK/Ta mice had enhanced accumulation of CML, especially in the glomerular mesangium. Candesartan treatment markedly reduced this accumulation, but there were no differences between the early and late treatment groups (Figure 2).

Co-localization experiments using anti-synaptophysin immunoglobulin G revealed that RAGE expression was increased in the glomerular podocytes of KK/Ta mice compared with BALB/c mice (data not shown). RAGE expression was not found in other glomerular
intrinsic cells. In mice treated with candesartan, only podocytes with glomeruli showed weak RAGE expression (Figure 3A–E).

Competitive RT-PCR demonstrated significant overexpression of RAGE in KK/Ta mouse kidneys. Candesartan markedly suppressed this overexpression to produce levels similar to those in BALB/c control mice (Figure 3F).

Candesartan treatment attenuated nitro-oxidative stress

To explore effects of candesartan on nitro-oxidative stress, expressions of nitrotyrosine and 8-OHdG were evaluated by immunohistochemistry. 8-OHdG, a marker of oxidative DNA damage, was overexpressed in the nuclear region of glomerular and tubular cells in KK/Ta mice (Figure 4). An accumulation of nitrotyrosine, an index of the nitrosylation of protein by peroxynitrite and/or superoxide, was found in the glomeruli and particularly within the mesangium of KK/Ta mice (Figure 5). Candesartan treatment significantly decreased the accumulation of both 8-OHdG and nitrotyrosine (Figures 4 and 5).

Candesartan treatment attenuated the enhanced expression of NADPH oxidase p47phox and iNOS, but not eNOS

The p47phox cytosolic component of NADPH oxidase was expressed in podocytes, mesangial cells and in the basolateral membrane of tubules in BALB/c mice. Its expression at these sites was elevated in KK/Ta mice compared with BALB/c mice and these elevations were attenuated by candesartan treatment. Candesartan also caused a consistent attenuation of p47phox mRNA expression (Figure 6).
Fig. 3. Representative immunohistochemical staining and competitive RT–PCR for RAGE at 28 weeks of age. (A) BALB/c, (B) KK/Ta, (C) early treatment KK/Ta, (D) late treatment KK/Ta, (E) negative control. Original magnification: ×400. (F) The upper bands are 18S competitors (489 bp) and the lower bands are RAGE (246 bp). Lane 1, 100 bp ladder marker; lane 2, BALB/c; lane 3, KK/Ta; lane 4, early treatment KK/Ta; lane 5, late treatment KK/Ta. Each bar represents means±SEM from six mice in each group. †P < 0.005 vs BALB/c; *P < 0.005 vs KK/Ta; §P < 0.05 vs KK/Ta.

Fig. 4. Representative immunohistochemical staining for 8-OHdG at 28 weeks of age. (A) BALB/c, (B) KK/Ta, (C) early treatment KK/Ta, (D) late treatment KK/Ta, (E) negative control. Original magnification: ×400. (F) Glomerular 8-OHdG index was calculated as [positive area (μm²)/glomerular total area (μm²)]. Each bar represents means±SEM from six mice in each group and 10 glomeruli from each mouse. †P < 0.005 vs BALB/c; *P < 0.005 vs KK/Ta.
Both the expressions of iNOS mRNA and protein were induced in KK/Ta mouse kidneys at 28 weeks of age. Administration of candesartan reduced the overexpression of iNOS at transcription and translation levels (Figure 7F). However, there were no significant differences in eNOS mRNA (Figure 7G) or protein (data not shown) expression among the four groups.

Discussion

In the present study, the three KK/Ta mouse groups showed mild glomerular mesangial expansion at 12 weeks of age. By 28 weeks, the untreated KK/Ta group exhibited moderate glomerular mesangial expansion with glomerular hypertrophy. However, these changes were markedly reduced in the candesartan groups. Renal tissues from the control BALB/c mice showed normal histological findings [12]. CML accumulation and RAGE expression were increased in KK/Ta mouse kidneys and these were attenuated by candesartan treatment. This attenuation occurred in the context of reductions in both glomerular and tubular 8-OHdG and nitrotyrosine, which are markers of oxidative and nitrosative stress. Although these effects of candesartan tended to be stronger in the early treatment group, the differences did not attain statistical significance.

NADPH oxidase and nitric oxide synthase (NOS) are the major sources of superoxide production in the kidney. Angiotensin-II has been reported to upregulate the in vitro synthesis of NADPH oxidase and NOS in various cell types, including glomerular endothelial cells, mesangial cells, podocytes and tubular epithelial cells. NADPH oxidase consists of a membrane-associated cytochrome b558 composed of one p22phox subunit and one gp91phox subunit and at least four cytosolic subunits that include p47phox, p67phox, p40phox and the small GTPase rac1 or rac2. Superoxide is generated after phosphorylation of the cytosolic p47phox subunit, which plays a crucial role in agonist (angiotensin-II, PMA and TNF-α)-induced NADPH oxidase activation [16]. All three NOS isoforms are found in the kidney. eNOS is typically expressed in endothelial cells along the renal vascular tree. iNOS is induced by various cytokines in mesan-
gial and tubular cells. High levels of neuronal NOS (nNOS) are located in the macula densa [17]. Increased expressions of renal p47phox and eNOS have been reported after 2 weeks of streptozotocin (STZ)-induced diabetes and these were attenuated by either quinapril or candesartan [3]. In the present study, the expressions of p47phox and iNOS were increased in kidneys of type 2 diabetic KK/Ta mice, which is consistent with findings in rats having long-term diabetes [6]. However, eNOS expression was not detected in KK/Ta mouse kidneys at 28 weeks of age. Since prior increases in eNOS expression have been associated with glomerular hyperfiltration at the early stage of experimental diabetic nephropathy [3], the expression pattern of NOS may relate to differences in the type of diabetes, existence of hyperinsulinaemia, long duration of hyperglycaemia or hyperglycaemia-induced endothelial damage [18]. iNOS mediates the synthesis of large (nM) amounts of nitric oxide (NO) following stimulation by inflammatory cytokines or disturbances in the cellular milieu. NO has a high affinity for the superoxide anion and their interaction forms peroxynitrite (ONOO). Therefore, blockade of AT1 receptors by candesartan may decrease the generation of superoxide and ONOO by down-regulating p47phox and iNOS expression in diabetic KK/Ta mouse kidneys.

Oxidation influences AGE formation at different stages, such as before and after the formation of Amadori products [6]. Recent observations demonstrated that ONOO can induce CML formation through oxidative cleavage of Amadori products and also through generation of reactive α-oxoaldehydes from glucose [19]. Horie et al. [5] found that CML and pentosidine accumulate in expanded glomerular mesangial matrices and nodular lesions in diabetic nephropathy and that they co-localize with malondialdehyde-lysine, a lipoxidation product. They suggested that local oxidative stress contributes to the in situ generation of AGE in diabetic nephropathy [5]. In our present study, the effect of AGE inhibition might have depended, at least in part, on the prevention of local oxidative stress, even though candesartan treatment did not influence hyperglycaemia.

CML is a major AGE in diabetic nephropathy and its accumulation upregulates RAGE expression on podocytes. The interaction between AGEs and RAGE activates NADPH oxidase species in podocytes and mesangial cells, thereby triggering signal transduction
mechanisms, such as p21ras, erk 1/2 MAP kinases and NF-\(\kappa\)B in an oxidant-sensitive manner [2]. Moreover, Sugimoto et al. [6] demonstrated that both CML and iNOS were detected in the glomerular mesangial areas in STZ-induced diabetic rats at 52 weeks of age and that they decreased after treatment with aminoguanidine, an inhibitor of AGE formation. They concluded that CML may enhance the expression of iNOS by stimulating TNF-\(\alpha\) via RAGE [6]. These findings suggest that RAGE contributes to sustained generation of nitro-oxidative stress, which in turn accelerates the formation of AGE. Candesartan may down-regulate AGE-induced overexpression of RAGE by attenuating the activities of NF-\(\kappa\)B and the JAK/STAT pathway since the RAGE gene promoter contains NF-\(\kappa\)B and STAT binding sites that can be activated by angiotensin-II via the AT1 receptor [20].

In summary, the present findings demonstrated that nitro-oxidative stress and the production of AGEs are enhanced in the kidneys of type 2 diabetic KK/Ta mice. The AT1 receptor antagonist candesartan decreased nitro-oxidative stress by down-regulating NADPH oxidase p47phox and iNOS expression. Candesartan also modified the interaction between AGEs and RAGE by attenuating RAGE expression, which contributed to the reduction of AGE accumulation and subsequent albuminuria. Further studies are ongoing to elucidate the effects of ARB on other target molecules activated by the AGE–RAGE pathway.

Acknowledgements. The authors thank Ms T. Shibata and Ms T. Shigihara for their skilful technical support. The authors also acknowledge all the groups that have conducted similar experiments on different diabetic strains. This study was supported in part by a High Technology Research Center Grant from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

Conflict of interest statement. None declared.

References
5. Hori K, Miyata T, Maeda K et al. Immunohistochemical colocalization of glycoxidation products and lipid peroxidation...


20. Huang JS, Guh JY, Chen HC *et al.* Role of receptor for advanced glycation end-product (RAGE) and the JAK/STAT-signaling pathway in AGE-induced collagen production in NRK-49F cells. *J Cell Biochem* 2001; 81: 102–113

Received for publication: 28.1.04
Accepted in revised form: 28.6.04