Angiotensin II augments advanced glycation end product-induced pericyte apoptosis through RAGE overexpression

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Abstract Advanced glycation end product (AGE)-their receptor (RAGE) and angiotensin II (AII) are implicated in diabetic retinopathy. However, a crosstalk between the two is not fully understood. In vivo, AGE injection stimulated RAGE expression in the eye of spontaneously hypertensive rats, which was blocked by an AII-type 1 receptor blocker, telmisartan. In vitro, AII-type 1 receptor-mediated reactive oxygen species generation elicited RAGE gene expression in pericytes through NF-κB activation. Further, AII augmented AGE-induced pericyte apoptosis, the earliest hallmark of diabetic retinopathy. Our present study may implicate a crosstalk between AGE-RAGE system and AII in diabetic retinopathy.

Keywords: Advanced glycation end products; Angiotensin II; Diabetic retinopathy; Oxidative stress; Pericyte loss; Receptor for AGEs

1. Introduction

There is a growing body of evidence that hypertension is an independent risk factor for the incidence and progression of diabetic retinopathy and that strict blood pressure (BP) control achieves a clinically important reduction in the risk of progression of diabetic retinopathy [1–4]. Furthermore, recent clinical studies suggest active participation of the renin–angiotensin system (RAS) in the pathogenesis of diabetic retinopathy [5,6]. Indeed, interruption of the RAS with angiotensin-coverting enzyme inhibitors or angiotensin II (AII)-type 1 receptor blockers (ARBs) has been reported to reduce renal or retinal disease progression in diabetic patients with hypertension [7–9].

Non-enzymatic modification of proteins by reducing sugars, a process that is also known as Maillard reaction, progress at an extremely accelerated rate under diabetes, leading to the formation of advanced glycation end products (AGEs) in vivo [10,11]. Recent understandings of this process have revealed that the AGE-their receptor (RAGE) system plays a central role in the pathogenesis of diabetic vascular complication as well [12–15]. Engagement of RAGE by AGEs activates its downstream signaling and subsequently evokes oxidative stress and inflammatory responses in vascular wall cells, thus, contributing to the development and progression of diabetic retinopathy [16–19]. However, the crosstalk between the AGE-RAGE system and the RAS in diabetic retinopathy is not fully understood. In this study, we first investigated whether AII-type 1 receptor interaction was involved in RAGE expression in the eye of spontaneously hypertensive rats (SHR). Then we examined whether AII upregulated RAGE mRNA levels in cultured retinal pericytes in vitro and the way that it might achieve this effect. We further studied here whether AII could augment the cytopathic effects of AGEs on pericytes through RAGE overexpression.

2. Materials and methods

2.1. Materials

AII, diphenylene iodonium (DPI), an inhibitor of NADPH oxidase and *N*-acetylcysteine (NAC) were purchased from Sigma (St. Louis, MO, USA). Telmisartan, an ARB, was generously gifted from Boehringer Ingelheim (Ingelheim, Germany). GeneAmp RNA PCR Core Kit was from Applied Biosystems (Branchburg, NJ, USA).

2.2. Preparation of AGE-proteins

AGE-bovine serum albumin (BSA) was prepared as described previously [20]. Briefly, BSA was incubated under sterile conditions with D-glyceraldehyde for 7 days. Then unincorporated sugars were removed by dialysis against phosphate-buffered saline. Control non-glycated BSA was incubated in the same conditions except for the absence of reducing sugars. Preparations were tested for endotoxin using Endospecy ES-20S system (Seikagaku Co., Tokyo, Japan); no endotoxin was detectable. The extent of chemical modification was determined with 2,4,6-trinitrobenzenesulfonic acid and reported as the difference between lysine residues of modified and unmodified protein preparations [20]. The extent of lysine modification (%) of modified BSA preparations was 65% for AGE-BSA.

2.3. Animal study

Seven-week-old normoglycemic SHR (Charles River Breeding Laboratories, Yokohama, Japan) were used in this study. After a 1-week adaptation period, rats were given tail vein injections with 2 mg

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Abbreviations: RAS, renin–angiotensin system; AII, angiotensin II; ARBs, AII-type 1 receptor blockers; AGEs, advanced glycation end products; RAGE, receptor for AGEs; SHR, spontaneously hypertensive rats; DPI, diphenylene iodonium; NAC, *N*-acetylcysteine; BSA, bovine serum albumin; ROS, reactive oxygen species; RT-PCR, reverse transcription-polymerase chain reaction; BP, blood pressure

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AGE-BSA ($n = 4$) or non-glycated BSA ($n = 4$) or with 2 mg AGE-BSA followed by oral administration of telmisartan (2 mg/kg/day) ($n = 4$) every day for up to 10 days. Then, the rats were sacrificed 1–2 h after injection on the final day. This AGE administration significantly ($P < 0.01$) increases serum AGE levels, but telmisartan treatment did not affect the AGE levels; the serum AGE levels of non-glycated BSA-treated, AGE-BSA-treated, and AGE-BSA plus telmisartan-treated SHR were 21.7 ± 0.3, 26.0 ± 0.3, and 25.3 ± 0.3 μg/ml, respectively. All animal procedures were conducted according to the guidelines provided by the Kurume University Institutional Animal Care and Use Committee under an approved protocol.

2.4. Western blotting analysis

Proteins were extracted from enucleated eyes, and then separated by SDS–polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Biorad, Hercules, CA, USA) as described previously [21]. Membranes were probed with 1:1000 dilution of anti-serum against RAGE [22] or 1:5000 dilution of monoclonal antibody against α-tubulin (Sigma), and then the immune complexes were visualized with an enhanced chemiluminescence detection system (ECL; Amersham Bioscience, Buckinghamshire, United Kingdom).

2.5. Cells

Pericytes were isolated from bovine retina and maintained in Dulbecco's modified Eagle's medium containing 5.5 mM glucose (Sigma) supplemented with 20% of fetal bovine serum (ICN Biomedicals Inc., Aurora, OH, USA) as described previously [23]. AGE or AII treatments were carried out in a medium containing 2% fetal bovine serum.

2.6. Measurement of intracellular ROS generation

Pericytes were incubated with or without 100 nM AII in the presence or absence of 100 nM telmisartan or 50 nM DPI for the indicated time periods. Then the intracellular formation of reactive oxygen species (ROS) was measured using the fluorescent probe CM–H_2DCFDA (Molecular Probes, Eugene, OR, USA) [24].

2.7. Measurement of luciferase activity

Plasmid, containing the NF-κB promoter attached upstream to the luciferase reporter gene, was kindly provided by Dr. T. Fujita, Department of Tumor Cell Biology, Tokyo Metropolitan Institute of Medical Science. Luciferase activity was measured as described previously [25].

2.8. Primers and probes

Sequences of the upstream and downstream primers used in the semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) were 5′-ATGGAAACTGAACACAGGCC-3′ and 5′-CACACATGTCCCCACCTTAT-3′ for detecting bovine RAGE mRNA [26]. Sequences of the primers for human β-actin mRNAs were the same as described previously [20].

2.9. Semi-quantitative RT-PCR

Poly(A)$^+$RNAs were isolated from pericytes treated with or without 100 nM AII in the presence or absence of 100 nM telmisartan or 1 mM NAC for 4 h, and then analyzed by RT-PCR as described previously [20]. The amounts of poly(A)$^+$RNA templates (30 ng) and cycle numbers (37 cycles for RAGE gene and 22 cycles for β-actin gene) for amplification were chosen in quantitative ranges, where reactions proceeded linearly, which had been determined by plotting signal intensities as functions of the template amounts and cycle numbers [26].

2.10. Measurement of growth of pericytes

Pericytes were preincubated in the presence or absence of 100 nM AII for 4 h. After washing with phosphate-buffered saline once, the cells were treated with various concentrations of AGE-BSA or 100 μg/ml non-glycated BSA for 2 days, and then the number of viable cells was determined as described previously [27].

2.11. Measurement of apoptotic cell death in pericytes

Pericytes were preincubated in the presence or absence of 100 nM AII for 4 h. After washing with phosphate-buffered saline once, the cells were treated with various concentrations of AGE-BSA or 100 μg/ml non-glycated BSA for 2 days. Then the cells were lysed and the supernatant analyzed in an enzyme-linked immunosorbent assay for DNA fragments (Cell Death Detection enzyme-linked immunosorbent assay, Roche Molecular Biochemicals, Mannheim, Germany) according to the manufacturer's instructions. The amount of apoptosis is shown as the relative absorbance value of the control cells [28].

2.12. Statistical analysis

All values were presented as means ± S.E. Unless otherwise indicated, one-way ANOVA followed by the Scheffe F test was performed. For statistical comparisons of the values between no treatment and AII pretreatment (Fig. 5), unpaired t test was performed; $P < 0.05$ was considered significant.

3. Results

3.1. Effects of telmisartan on RAGE expression in the eye of SHR

We first investigated whether AII-type 1 receptor interaction was involved in RAGE expression in the eye of SHR. For this, we examined effects of an ARB, telmisartan on RAGE expression in the eye of AGE-injected SHR. As shown in Fig. 1, telmisartan blocked the AGE-induced RAGE expression in the eye, decreasing its level below that of non-glycated BSA-injected SHR. Telmisartan treatment for 10 days significantly decreased BP level; systolic BP in non-glycated BSA-treated, AGE-BSA-treated, and AGE-BSA plus telmisartan-treated SHR were 194.8 ± 3.7, 195.0 ± 3.6, and 182.8 ± 1.5 mmHg, respectively. The serum creatinine levels of non-glycated BSA-treated, AGE-BSA-treated, and AGE-BSA plus telmisartan-treated rats were 0.33 ± 0.02, 0.33 ± 0.02, and 0.35 ± 0.03 mg/dl, respectively.

3.2. Effects of AII on ROS generation in pericytes

We next investigated effects of AII on ROS generation in cultured pericytes. As shown in Fig. 2A, 100 nM AII significantly increased ROS generation in pericytes; 2- or 4-h incubation of 100 nM AII increased intracellular ROS generation by about 1.5-fold. Telmisartan or DPI, an inhibitor of NADPH oxidase was found to completely inhibit the AII-induced increase in ROS generation, thus, suggesting that AII-type 1 receptor interaction elicited ROS generation in cultured pericytes via NADPH oxidase activity (Fig. 1B). Although high glucose elicited the mitochondrial ROS generation in pericytes [29], we used here Dulbecco's modified Eagle's medium containing 5.5 mM glucose. Furthermore, there is a growing

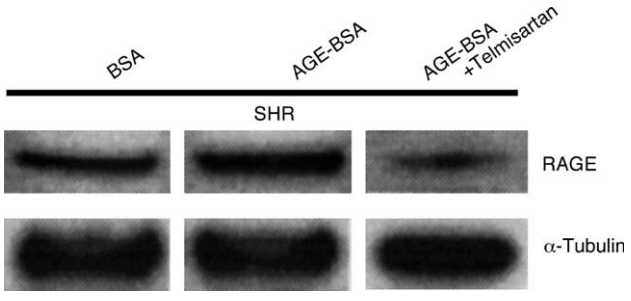

Fig. 1. Effects of oral administration of telmisartan on RAGE expression in the eye of SHR. SHR were given tail vein injections with 2 mg AGE-BSA ($n = 4$) or non-glycated BSA ($n = 4$) or with 2 mg AGE-BSA followed by oral administration of telmisartan (2 mg/kg/day) ($n = 4$) every day for up to 10 days. Then, RAGE expression in the eye was analyzed by Western blots. Similar results were obtained in three independent experiments.