

*Full Paper***Insufficient Expression of Cyclooxygenase-2 Protein Is Associated With Retarded Degradation of Aggregated Protein in Diabetic Glomeruli**Yasushi Hirasawa^{1,2,*}, Aiko Muramatsu¹, Yoshio Suzuki¹, and Tadashi Nagamatsu¹¹Department of Pharmacobiology and Therapeutics, Faculty of Pharmacy, Meijo University,
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Abstract. To elucidate the involvement of cyclooxygenase (COX) in degradation of aggregated protein in diabetic glomeruli, we used streptozotocin (STZ)-induced diabetic mice and aggregated bovine serum albumin (a-BSA) as a model protein. There was a higher deposition of a-BSA in diabetic glomeruli compared to normal glomeruli 18 h after a-BSA injection at 4 and 8 weeks after STZ. Degradation of a-BSA was confirmed using isolated glomeruli. Diabetic glomeruli produced prostaglandin E₂ (PGE₂) more than normal glomeruli in the basal level at 8 weeks. a-BSA caused further increase of PGE₂ production in normal glomeruli, but not in diabetic glomeruli. Niflumic acid, a selective COX-2 inhibitor, reduced PGE₂ production of normal glomeruli in the a-BSA loading group, but not that in the control group. In diabetic glomeruli, niflumic acid reduced PGE₂ production in both the control group and a-BSA loading group. In normal glomeruli, a-BSA increased expressions of both COX-2 mRNA and protein. However, in diabetic glomeruli, a-BSA increased COX-2 mRNA expression but not COX-2 protein expression. These results suggest that retarded degradation of aggregated protein in diabetic glomeruli is associated with lack of further expression of COX-2 protein and further production of PGE₂ in response to aggregated protein.

Keywords: cyclooxygenase-2, aggregated bovine serum albumin, streptozotocin, diabetic, glomeruli

Introduction

Diabetic nephropathy results from hyperglycemia that is caused by abnormal carbohydrate metabolism; it has been clarified from the DCCT (Diabetic Control and Complication Trial Research Group) report (1). Hyperglycemia causes the glycation of glomerular basement membrane, leading to the deposition of immunoglobulin G with a high affinity for advanced glycation end products and to the formation of aggregated proteins (2). The deposition of glycated albumin and glycated immunoglobulin G has actually been reported in the glomeruli of diabetic nephropathy (3). Such glomerular

deposits are essentially eliminated by mesangial cells, which are one of the structural components of the glomerulus, and resident macrophages (4, 5). We consider that hyperglycemia induces dysfunction of the mesangial cells followed by excessive accumulation of aggregated protein in the glomeruli, and this is one of the causal factors underlying progression of diabetic nephropathy and a decrease in renal function.

Streptozotocin (STZ)-induced diabetic rats have been demonstrated increased cyclooxygenase (COX)-2 protein expression in macula densa and glomerular epithelial cells (6). Another report indicates increased conversion of exogenous arachidonic acid into eicosanoids in the glomeruli of STZ-diabetic rat compared to normal rats (7). We have already reported that in the presence of aggregate proteins, COX-2 expression increased in the glomeruli of normal mice

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(8). Furthermore, it has been reported that prostaglandin E_2 (PGE_2) promotes the degradation of aggregated protein deposited in the glomeruli (9). COX is involved in the production of various types of eicosanoids using arachidonic acid released from phospholipids of cell membranes (10, 11). Two isozymes of COX have been identified based on the primary structure, namely, COX-1 with strong homology in approximately 60% of amino acids and COX-2 (12, 13). Of these, COX-2 is the inducible form whose expression is increased when there is inflammation or cells are exposed to pre-inflammatory cytokines (14, 15). We have demonstrated that hereditarily type 2 diabetic mice accumulate more aggregated protein and display delayed disappearance of aggregated protein in glomeruli compared to normal mice (16). The aim of this study is to clarify whether the accumulation of more aggregated protein and delayed disappearance of aggregated protein are common phenomena in diabetic glomeruli and how COX-1 and COX-2 are involved in the degradation of aggregated protein in diabetic glomeruli, by using STZ-induced type 1 diabetic mice and aggregated bovine serum albumin (a-BSA) as an aggregated protein (17, 18). The present results demonstrate the following: 1) There is more a-BSA and retarded degradation of a-BSA in the glomeruli of type 1 diabetic mice, 2) no increase in PGE_2 production by a-BSA, and 3) no increase in COX-1 by a-BSA and an increase in COX-2 mRNA followed by impaired COX-2 protein expression.

Materials and Methods

Animals

ICR:CrJ:CD-1 mice (ICR) obtained from Charles River Japan (Yokohama) were used in the experiment. The animals were housed in an animal room kept at 20°C – 26°C and lighting condition of 12 h each of light and darkness. Water and food were given ad libitum.

Reagents

The following reagents were used in the experiments: crystallized BSA (Bayer, Kankakee, IL, USA); iron oxide (Fe_3O_4 ; Aldrich Chemical, Milwaukee, WI, USA); Glucose C-II Test (Wako Pure Chemical Industries, Osaka); SEA BLOCK™ (Techno Chemical, Tokyo); horseradish-peroxidase-conjugated streptavidin (Zymed Laboratories, San Francisco, CA, USA); ABTS peroxidase substrate and ABTS stop solution (KPL, Gaithersburg, MD, USA); horseradish-peroxidase-conjugated goat anti-rabbit immunoglobulin G (Vector Laboratories, Burlingame, CA, USA); strong lysis solution (CytoSignal, Irvine, CA, USA). Indomethacin, protease inhibitor cocktail, rabbit anti-actin antibody,

monoclonal anti-BSA antibody (clone BSA-33), biotin-conjugated anti-mouse immunoglobulin G antibody, RPMI 1640, collagenase, deoxyribonuclease, arachidonic acid, and STZ were obtained from Sigma Chemical (St. Louis, MO, USA). Rabbit anti-mouse COX-1 antibody and random 9'mers were obtained from Takara Bio (Otsu). 5-(4-Chlorophenyl)-(4-methoxyphenyl)-3-(trifluoromethyl)-1*H*-pyrazone (SC-560), 2[[3-(trifluoro-methyl) phenyl] amino]-3-pyridine-carboxylic acid (niflumic acid), and rabbit anti-mouse COX-2 antibody were obtained from Cayman Chemical (Ann Arbor, MI, USA). Fetal bovine serum (FBS), insulin-transferrin-selenium-A, non-essential amino acid solution, sodium bicarbonate solution, and DTT were obtained from Gibco (Grand Island, NY, USA). First standard buffer, 0.5 mM dNTP mix, Super Script II™ RT, and RNase H were obtained from Invitrogen (Carlsbad, CA, USA). AmpliTaq Gold™ DNA polymerase, Gold buffer™, dNTP Mix, and $MgCl_2$ were obtained from Roche Molecular Systems (Branchburg, NJ, USA). DC protein assay, biotinylated SDS-PAGE standards high range, horseradish-peroxidase-conjugated anti-rabbit immunoglobulin G antibody, and horseradish-peroxidase-conjugated streptavidin were obtained from Bio Rad Laboratories (Hercules, CA, USA). PGE_2 enzyme immunoassay (EIA) system, enhanced chemiluminescence (ECL) Western blotting detection reagents, and Hyperfilm were obtained from Amersham Pharmacia Biosciences (Buckinghamshire, UK).

Preparation of reagents

STZ was dissolved in 0.05 M citric acid buffer. a-BSA was prepared according to the method by Ford (18) and Nagamatsu et al. (17, 19). BSA was dissolved in physiological saline to 30 mg/mL and the pH was adjusted to 10 with 0.1 M NaOH. This BSA solution was incubated at 70°C for 20 min and then at 79°C for a further 15 min. After cooling down the solution to room temperature, the pH was adjusted to 7.5 with 0.1 M HCl. The solution was then centrifuged at 4°C, 3,500 rpm for 30 min to remove the insoluble substances and kept at –20°C until used. Arachidonic acid was dissolved in dimethyl sulfoxide. Indomethacin, SC-560, and niflumic acid were dissolved in dimethyl sulfoxide followed by dilution with culture medium to the required concentration.

Induction of diabetes in mice

STZ solution (STZ: 200 mg/kg) was administered i.v. to mice to induce type I diabetes (20). After 4 or 8 weeks of STZ injection, blood glucose level was measured by the Glucose C-II Test. After hyperglycemia was confirmed, the mice were used in the experiment. Glucose

levels were measured in accordance with the protocol provided by the manufacturer.

Isolation of the a-BSA-loaded glomeruli of mice

Glomeruli were isolated according to the method of Nagamatsu et al. (16). Briefly, a-BSA solution at the dose of 0.02 mL/g of body weight was administered twice i.v. to the mice at 0 and 3 h. In addition, physiological saline was injected to mice in the control group. At 6 or 18 h after the first a-BSA injection, the kidneys were perfused with phosphate-buffered saline (PBS) containing 1 mg/mL of ferric oxide and then isolated. After fragmenting the kidneys, they were passed through a 90- μ m mesh screen and the glomeruli-rich fraction was collected on a 38- μ m mesh screen. The fraction was suspended in PBS and a magnet used to collect only the glomeruli containing ferric oxide. The purity of the glomeruli was confirmed to be >95% by light microscopy.

Determination of a-BSA in a-BSA-loaded glomeruli

The glomeruli were isolated and suspended in PBS at 3,000 glomeruli/mL. The glomeruli were destructed by sonication and the resulting solution used as the samples for ELISA as previously reported (16–19). Additionally, in the experiment using isolated glomeruli, the isolated glomeruli were washed several times with RPMI 1640 [insulin-transferrin-selenium-A, 0.1 mM non-essential amino acid solution, 7.5% (w/v) sodium bicarbonate solution, pH 7.2] containing 20% FBS and suspended to a concentration of 3,000 glomeruli/mL. Aliquots (1 mL) from this suspension were transferred to a 48-well culture plate and incubated under an environment of 5% CO₂ at 37°C. Glomeruli were collected at the start of the incubation and after 1, 3, and 6 h of incubation. They were then washed twice in PBS using centrifuge operations and a magnet. Finally, the glomeruli were suspended in 1 mL of PBS, crushed by sonication, and the resulting solution used as samples. The amount of a-BSA was determined by ELISA.

Determination of PGE₂ in the a-BSA-loaded glomeruli

The glomeruli were isolated at 0 and 8 h of the a-BSA load. After the isolation, they were suspended in Krebs-Henseleit bicarbonate buffer (KRB) containing 25 U/mL collagenase and 10 μ g/mL deoxyribonuclease and incubated at 37°C for 10 min. The glomeruli were then suspended in KRB to a concentration of 10,000 glomeruli/mL. Aliquots (1 mL) of this suspension were transferred to a centrifuge tube and incubated at 37°C for 60 min with mixing (21). The suspension was then centrifuged at 4°C and 5,000 rpm for 5 min, and 800 μ L of the supernatant was collected. PGE₂ in the

culture medium was determined according to the manual of the PGE₂ enzyme immunoassay system kit and indicated as the amount per 10,000 glomeruli (fmol/10,000 glomeruli).

Effects of COX inhibitors on the production of PGE₂ by a-BSA-loaded glomeruli of diabetic mice

The glomeruli were isolated 8 h after the a-BSA load. The glomeruli were suspended in KRB containing 25 U/mL collagenase and 10 μ g/mL deoxyribonuclease, and incubated in a shaker at 37°C for 10 min. The glomeruli were then washed twice in KRB and counted using a microscope. The glomeruli were suspended in KRB to a concentration of 5,000 glomeruli/mL. Arachidonic acid (final concentration of 30 μ M) was added to the culture medium in order to confirm the inhibitory effect of the COX inhibitor by the method of Nagamatsu et al. (8). Each of the COX inhibitors (final concentration of 0, 10⁻⁷, 10⁻⁶, and 10⁻⁵ M) were respectively added to the medium and incubated at 37°C under an environment of 5% CO₂ for 1 h. After the incubation, the culture medium was centrifuged and the glomeruli were removed. The supernatant was used as the samples. PGE₂ in the sample was measured by EIA and indicated as the amount per 2,500 glomeruli (pg/2,500 glomeruli). PGE₂ was determined according to the manufacturer's protocol of the measurement kit.

RT-PCR analysis for the expressions of COX-1 and COX-2 mRNAs in the a-BSA-loaded glomeruli

The glomeruli were isolated 6 h after the a-BSA injection, and RNA was extracted from the glomeruli using FastRNA™ Kit-Green (BIO 101, La Jolla, CA, USA). The RNA was dissolved in DEPC-treated water. Random 9mers solution at 2.5 μ M was added to the solution containing 3 μ g of this RNA and then DEPC-treated water added up to a total volume of 14 μ L. The reaction below was performed by using PCR Thermal Cycler Personal™ (Takara Bio). The RNA solution was heated for 10 min at 70°C and quickly cooled. Master A mix [final concentration: 1 \times first stand buffer, 0.01 M DTT, 0.5 mM dNTPmix] was added and the solution incubated at 25°C for 5 min. Super Script II™ RT (200 units) was added and the mixture incubated at 25°C for 10 min and then at 42°C for 50 min. Finally, RNase H (2.2 units) was added and the mixture heated at 37°C for 20 min and then at 70°C for 15 min. This was used as the cDNA sample. The sequences of primers were as follows: 3-phosphate dehydrogenase (GAPDH) sense primer, 5'-AGC CGC ATC TTC TTG TGC A-3', anti-sense primer, 3'-TCA TAC TGA GGT GAG TGC CG-5'; COX-1 sense primer, 5'-TAA CAC TTC TAT GCT GGT GGA CTA TGG GG-3', antisense primer, 3'-TCA GGA

ACT ACA CCC AAA AGC ACC G-5'); COX-2 sense primer, 5'-GAC TAG ATG ACA TTA ACC CTA CAG TAC-3', antisense primer: 3'-CTT AAT CGA ATT GTT CTT GGT TCC TT-5'. Reaction mixture (25 μ L) consisted of sense and antisense primer (0.2 pmol), cDNA sample (1 μ L) 1 \times PCR Gold bufferTM, dNTP Mix (0.2 mM), MgCl₂ (1.5 mM), and AmpliTaq GoldTM DNA polymerase (1.25 U) and heated at 94°C for 10 min. The reaction mixture for GAPDH was then heated at 94°C for 30 s, at 58°C for 30 s, and 72°C for 30 s for 35 cycles; COX-1 heated at 94°C for 30 s, at 65°C for 30 s, and 72°C for 30 s for 35 cycles; COX-2 heated at 94°C for 30 s, at 60°C for 30 s, and 72°C for 30 s for 35 cycles. After that, they were heated at 72°C for 5 min and quickly cooled to 4°C. PCR substances were analyzed by electrophoresis using agarose gel. After the electrophoresis, the gel was stained with ethidium bromide and analyzed using NIH Image software.

Western blotting for COX-1 and COX-2 protein in the a-BSA-loaded glomeruli

At 0 and 8 h after the a-BSA load, the glomeruli were isolated. After the isolation, a suspension containing 20,000 glomeruli was prepared in 1 mL strong lysis solution containing 10% protease inhibitor cocktail. The glomeruli were crushed at 4°C twice for 15 min by sonication, centrifuged at 4°C for 10 min at 16,000 rpm, and the supernatant used as the sample. The quantity of protein in the sample was measured by the Lowry procedure. The sample and biotinylated high molecular weight range SDS-PAGE standards were mixed with 2 \times SDS-sample buffer [0.125 M Tris-HCl (pH 6.8), 4% SDS, 20% glycerol, and 10% β -mercapto ethanol] and heated at 95°C for 5 min. These samples were then applied to a 10% acylamide gel and electrophoresis performed. After the electrophoresis, the gel was washed with Tris/Glycine-SDS buffer containing 20% methanol and transferred to HybondTM-ECLTM by a semi-dry procedure. The membrane was then washed with purified water and PBS-T (0.1% Tween-20). Blocking was performed with 5% nonfat dry milk. After incubating the membrane with primary antibodies (anti-COX-1 or anti-COX-2) at 4°C overnight, it was reacted with horseradish-peroxidase-conjugated secondary antibody for COX-2 or biotin-conjugated second antibody for COX-1 at room temperature for 60 min. The membrane for COX-1 was further reacted with horseradish-peroxidase-conjugated streptavidin for 60 min. Finally, it was reacted with luminescence ECL Western blotting detection reagents and photographed using Hyperfilm ECLTM. The photographed image was analyzed with NIH Image software.

Statistical analyses

The results are presented as the mean \pm S.E.M. The SAS pre-clinical package version 5.0 Stand Alone (SAS statistical processing system; SAS Institute Japan, Tokyo) was used to test significant differences and a risk rate of below 5% ($P < 0.05$) was significant. Variance ratio of F -test was used to test two groups and Student's t -test was used for equality of variance. The Aspin-Welch's t -test was performed for inequality of variance. For multiple groups, Bartlett's t -test was used to test between the groups; Dunnett's parametric test, for equality of variance; and Dunnett's non-parametric test, for inequality of variance.

Results

Time course for the amount of a-BSA in the glomeruli of diabetic mice

When a-BSA was intravenously injected into diabetic mice at 4 and 8 weeks after the injection of STZ, the glomeruli of diabetic mice and those of normal mice showed similar levels of a-BSA at 6 h after the a-BSA injection (Fig. 1: A and B). In the experiments at 4 and 8 weeks after the injection of STZ, the amount of a-BSA in the glomeruli of normal mice at 18 h after the a-BSA injection had decreased to almost half the quantity at 6 h after the injection (Fig. 1: A and B). In contrast to the glomeruli of normal mice, the amount of a-BSA in the glomeruli of diabetic mice at 18 h after the a-BSA injection was equivalent to that at 6 h after the injection.

In the next experiment, a-BSA was administered to diabetic mice at 8 weeks after the STZ injection and to the normal control mice; the glomeruli were then isolated 6 h after the injection of a-BSA and incubated in culture medium. As shown in Fig. 1C, the amount of a-BSA in the cultured diabetic glomeruli was about twofold that in the normal glomeruli at all measurement time points after the start of the incubation. In the cultured medium of the normal glomeruli, only a minute amount of the given dose of a-BSA was seen even though the amount of a-BSA in the glomeruli was decreased (Fig. 1D). These results suggest that some process interferes with degradation of aggregated a-BSA in the glomeruli of diabetic mice.

Involvement of COX in the degradation of aggregated protein in the glomeruli

In diabetes, COX-2 expression in the renal cortex is increased (6). We reported that clearance of aggregated protein from the glomeruli was accelerated by exogenous PGE₂ (8, 9). We therefore investigated PGE₂ production in the glomeruli isolated from STZ-induced type 1 diabetic mice 3 h after the injection of a-BSA

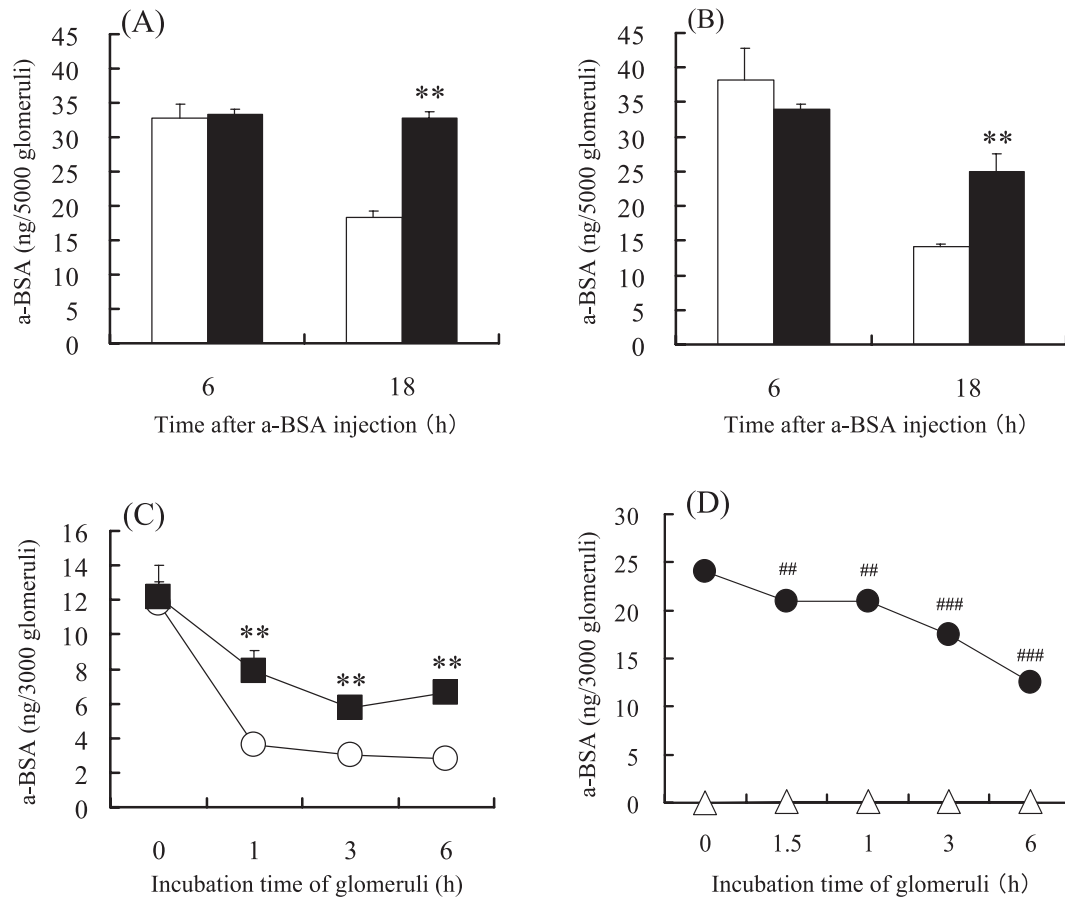


Fig. 1. Clearance of a-BSA in diabetic glomeruli. A and B: Mice were injected twice at a interval of 3 h with a-BSA at 4 weeks (A) and 8 weeks (B) after the injection of STZ, respectively. Glomeruli were isolated at 6 and 18 h after the injection of a-BSA on each week. White column shows the Normal group, and black column shows the DM group. Results are expressed as the means \pm S.E.M. of 5 mice. ** $P < 0.01$, compared with the Normal group. Panel C shows the clearance of a-BSA in isolated diabetic glomeruli. Experiments were performed 8 weeks after the injection of STZ. a-BSA-loaded glomeruli were isolated and incubated in RPMI-1640 containing 20% FBS for each period. White circle indicates the normal glomeruli. Black square indicates the diabetic glomeruli. Results are expressed as the means \pm S.E.M. of 5 mice. ** $P < 0.01$, compared with the Normal group. Panel D shows the time course of clearance of a-BSA in isolated normal glomeruli and a-BSA in their culture medium. a-BSA-loaded glomeruli were isolated 6 h after the first injection of a-BSA and incubated in serum-free medium for each period. Black circle and white triangle indicate glomerular a-BSA and supernatant-a-BSA, respectively. $n = 5$. Results are expressed as the means \pm S.E.M. ### $P < 0.01$ and #### $P < 0.001$, compared with a-BSA levels at 0 h.

and compared it with that in the normal glomeruli. As shown in Fig. 2, a-BSA-induced PGE₂ production in the glomeruli of normal mice increased two-fold compared to that before the a-BSA injection. On the other hand, PGE₂ production in the glomeruli of diabetic mice prior to the a-BSA injection was already increased by 1.6-fold compared to the glomeruli of normal mice. However, contrary to the glomeruli of normal mice, the amount of PGE₂ in the glomeruli 3 h after the a-BSA injection was equivalent to that before the injection. We previously reported that a-BSA increases COX-2 expression in the glomeruli of normal mice (8). Thus, we investigated whether COX-1 or COX-2 is involved in the PGE₂ production in the glomeruli of diabetic mice using

selective COX-inhibitors in the presence of exogenous arachidonic acid in the culture medium. In the presence of exogenous arachidonic acid, the production of PGE₂ was increased by a-BSA load in the glomeruli of both normal and diabetic mice (Fig. 3). As shown in Fig. 3, SC-560, a selective COX-1 inhibitor, inhibited PGE₂ production in the glomeruli with a-BSA load and without a-BSA load in the both normal and diabetic mice (normal, 50% and diabetes, 30%). However, niflumic acid, a selective COX-2 inhibitor, at 10^{-7} and 10^{-6} M inhibited PGE₂ production in a concentration-dependent manner in the a-BSA-loaded glomeruli of the normal mice, but not in the non-a-BSA group. Niflumic acid at 10^{-5} M completely eliminated PGE₂ production that was

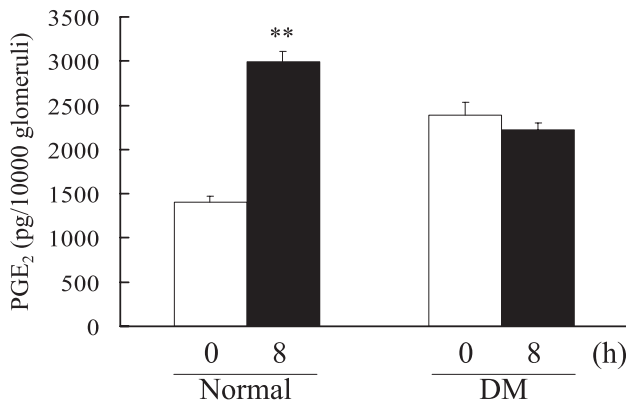


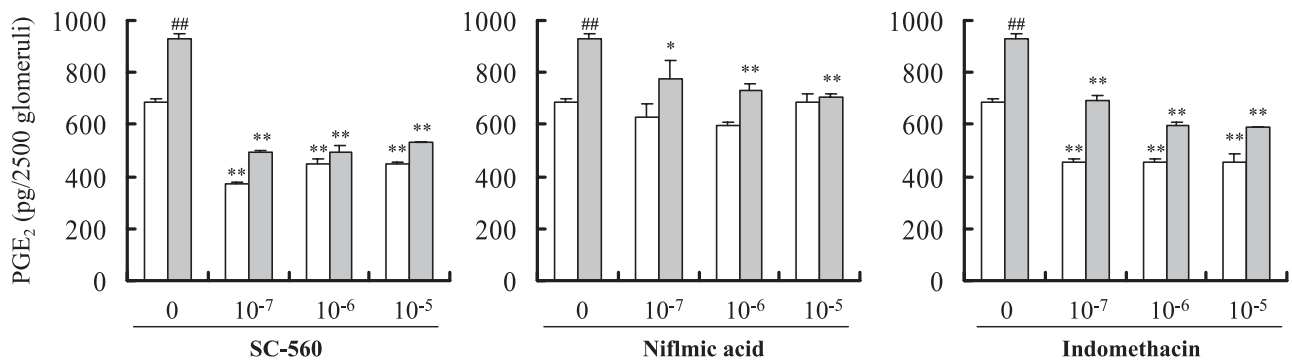
Fig. 2. PGE₂ production of glomeruli in mice after the injection of a-BSA. Experiments were performed 8 weeks after the injection of STZ. Mice were injected with a-BSA at 0 and 3 h. Glomeruli were isolated before the first injection of a-BSA and 8 h after the first injection and incubated with KRB for 60 min at 37°C. White column shows the levels of PGE₂ before the a-BSA injection. Black column shows the levels of PGE₂ after the a-BSA-injection. Results are expressed as the means \pm S.E.M. of 5 mice. ** P <0.01, compared with the levels before the injection of a-BSA.

increased by the a-BSA load. On the other hand, in the glomeruli of diabetic mice, niflumic acid inhibited PGE₂ production to the similar extent in both the a-BSA and non-a-BSA groups. In the diabetic glomeruli in the absence of a-BSA, indomethacin, an inhibitor of both COX-1 and COX-2, inhibited PGE₂ production in a concentration-dependent manner to the similar extent as SC-560. On the other hand, in the presence of a-BSA, it inhibited PGE₂ production in a concentration-dependent manner to an extent equivalent to the inhibition by niflumic acid. In the glomeruli of diabetic mice, indomethacin inhibited PGE₂ production more potently than niflumic acid in both the a-BSA and non-a-BSA groups.

Analysis of glomerular COX-1 and COX-2 mRNA expression by RT-PCR

As PGE₂ production in the glomeruli of diabetic mice was shown to increase, COX-1 and COX-2 mRNA expression in the glomeruli due to a-BSA load was investigated by RT-PCR analysis. As shown in Fig. 4,

Normal group



DM group

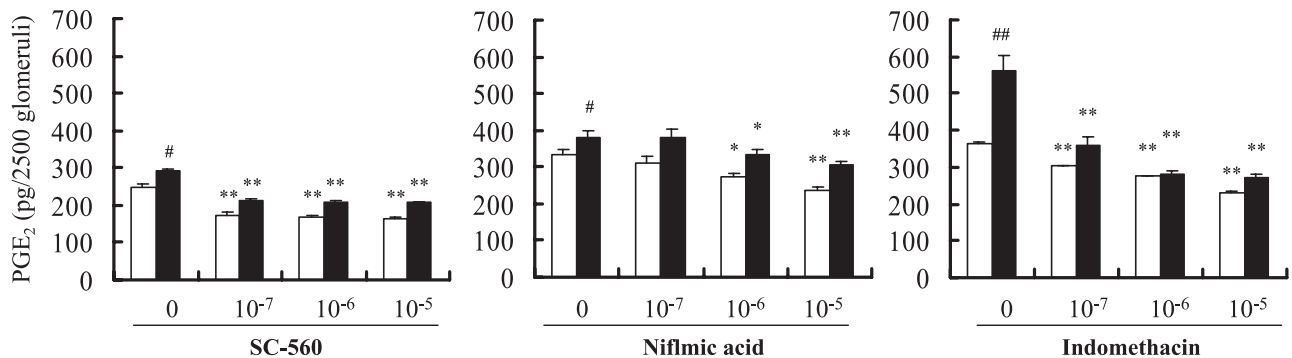


Fig. 3. Effects of COX inhibitors on PGE₂ production in glomeruli after loading a-BSA. Experiments were performed 8 weeks after the injection of STZ. Mice were injected with a-BSA or vehicle at 0 and 3 h. The glomeruli were isolated 8 h after the first injection of a-BSA and incubated with a inhibitor in the presence of 30 μ M arachidonic acid for 1 h at 37°C. Gray columns and black columns indicate the levels of PGE₂ of a-BSA loaded glomeruli. White columns show the levels of PGE₂ of non-a-BSA loaded glomeruli. Results are expressed as the means \pm S.E.M. of 5 mice. * P <0.05, ** P <0.01, compared with vehicle. # P <0.05, ## P <0.01, compared with non-a-BSA-loaded glomeruli.

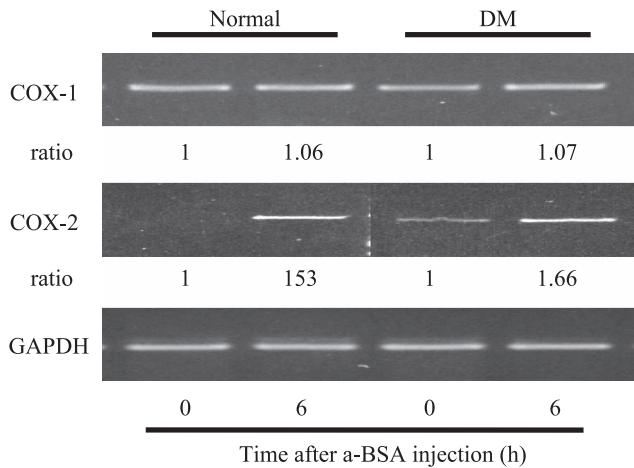


Fig. 4. RT-PCR analysis of COX mRNA expression in diabetic glomeruli after loading a-BSA. Experiments were performed 8 weeks after the injection of STZ. Mice ($n = 5$) were injected with a-BSA at 0 and 3 h. The glomeruli were isolated 0 and 6 h after the first injection of a-BSA. Stained area was standardized with the GAPDH expression. The numbers under the figure indicate the ratio to 0 h. Since the band of COX-2 mRNA at 0 h was hardly detected for the sample from normal glomeruli, we calculated the ratio of the COX-2 mRNA band at 6 h using 0.1 of approximate value for the 0 h-band.

amount of COX-1 mRNA expression in the glomeruli showed no difference between before and 6 h after the a-BSA injection in the normal and diabetic mice. Although normal mice showed negligible expression of COX-2 mRNA in the glomeruli without a-BSA load, 6 h after the injection of a-BSA the expression of COX-2 mRNA increased remarkably by up to 153-fold in the glomeruli compared to that of 0 h (Fig. 4). Diabetic mice showed clearly COX-2 mRNA expression in the glomeruli without a-BSA load compared to normal mice. However, a-BSA induced a 1.66-fold increase in COX-2 mRNA in the diabetic glomeruli 6 h after the injection of a-BSA (Fig. 4). While the increase-ratio on COX-2 mRNA expression of the normal glomeruli was quite larger than that of diabetic glomeruli, in a-BSA loading condition the amount of COX-2 expression was similar extent in the glomeruli of normal and diabetic mice (Fig. 4).

Analysis of glomerular COX-1 and COX-2 protein expression by Western blotting

Since in the glomeruli of diabetic mice, a-BSA increased COX-2 mRNA expression to the similar extent as in the glomeruli of normal mice, we next investigated COX-1 and COX-2 protein expression in the glomeruli by Western blotting. As shown in Fig. 5, the extent of COX-1 protein expression was equivalent in the normal and diabetic glomeruli, and there was no increase under the a-BSA loading condition. The amount

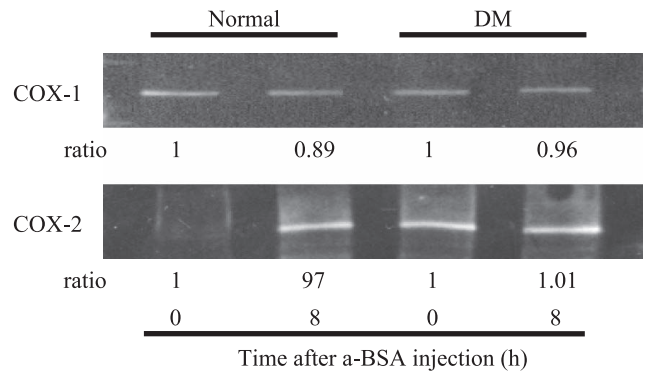


Fig. 5. Western blotting analysis of COX in diabetic glomeruli. Experiments were performed 8 weeks after the injection of STZ. Mice ($n = 5$) were injected with a-BSA at 0 and 3 h. The glomeruli were isolated 0 and 8 h after the first injection of a-BSA, and protein was extracted from 20,000 of the glomeruli. Samples were applied to a SDS-PAGE. The numbers under the figure indicate the ratio to 0 h. Since the obvious band of COX-2 protein at 0 h was not observed for the sample from normal glomeruli, we calculated the ratio of COX-2 protein at 8 h using the background level instead of the 0 h-COX-2 protein band.

of COX-2 protein expression in the normal glomeruli was markedly increased 8 h after the a-BSA injection at 97-fold. However, in the glomeruli of diabetic mice, the amount of the protein expression showed no increase 8 h after the a-BSA injection compared to that before the injection (1.01-fold) (Fig. 5).

Discussion

In the present study we demonstrated that in the glomeruli of STZ-induced diabetic mice, 1) the degradation of aggregate protein is delayed, 2) PGE₂ production is not further increased by a-BSA, 3) a-BSA causes an increase of expression of COX-2 mRNA, and 4) a-BSA does not induce an increase of expression of COX-2 protein.

Since we have reported that the clearance of aggregate proteins was retarded in the glomeruli of obese diabetic KK-A^y mice (16), delayed clearance of a-BSA in the glomeruli of STZ-induced diabetic mice was not due to STZ. We propose that such a phenomenon occurs commonly in the diabetic glomeruli in an earlier phase of diabetes. The present results in the normal glomeruli are consistent with the previous findings implicating COX-2 but not COX-1 in the further increase in PGE₂ production due to a-BSA load (8). In addition, in an experiment on isolated glomeruli, there was negligible increase in proteins that were recognized by anti-BSA antibody in culture medium, implying that the clearance in glomerular aggregated protein in vivo is not merely due to elimination though the reticuloendothelial system

but also by a glomerular mechanism whereby aggregate protein is degraded.

In this experiment, the STZ-induced diabetic glomeruli had an enhanced level of PGE₂ basal production compared to the normal glomeruli (Fig. 2). This finding is consistent with data obtained using the glomeruli isolated from STZ-induced diabetic rats (7). Although normal glomeruli increased PGE₂ production in response to a-BSA in our previous observations (8), and did in this study as well, the diabetic glomeruli showed no response regarding PGE₂ production induced by a-BSA (Fig. 2). Intriguingly, when a large amount of exogenous arachidonic acid was added to the incubation medium for the glomeruli, a-BSA-loaded diabetic glomeruli produced more PGE₂ than the control diabetic glomeruli, as did the normal glomeruli under the same conditions (Fig. 3). This result with arachidonic acid conflicts with that without arachidonic acid in the diabetic glomeruli. The causes of increased basal production of PGE₂ in the diabetic glomeruli are considered to be enhanced arachidonic acid availability through the phospholipase A₂ (PLA₂) pathway (22) and enhanced COX-2 expression (6, 7). Both of these factors could contribute to the enhanced production of PGE₂ in the diabetic glomeruli under basal conditions, but they do not account for the enhanced production in the a-BSA-loaded diabetic glomeruli. Because we observed an increase in COX-2 expression in response to a-BSA in the normal glomeruli (8), we investigated the involvement of COX-1 and COX-2 in the a-BSA loaded diabetic glomeruli by pharmacological manipulation. In these experiments, we could not clearly demonstrate using COX inhibitors how COX-1 and COX-2 are involved in the production of PGE₂ due to aggregated protein and their subsequent degradation.

There are many reports stating that eicosanoid production, particularly PGE₂, is enhanced in the glomeruli of diabetic mice and mesangial cells cultured in high glucose medium (6, 23–25). It is well known that in high glucose conditions, protein kinase C (PKC) is activated in mesangial cells (26, 27) followed by the phosphorylation and subsequent activation of cPLA₂ (28) and arachidonic acid release. Thus, it was speculated that since COX-2 expression was already increased even before a-BSA was injected in the diabetic glomeruli, there may be little room to enhance the production of PGE₂ by additional stimuli, a-BSA deposition. In order to confirm this speculation, the RT-PCR was used to analyze whether a-BSA increased levels of COX-1 and COX-2 mRNA expressions, and Western blotting was used to analyze the protein expressions. Although COX-2 mRNA expression was increased, the protein expression was not increased. Both

transcription and post-transcription regulations are always involved in protein expression. Part of the post-transcription regulation of COX-2 depends on the sequence included in the 3'-untranslated region (UTR) of COX-2 mRNA, especially the adenosine-, uridine-rich elements (AREs) that are rich in a repeated AUUA sequence conserved across species, (29). Furthermore, it has recently been reported that the expression of the IL-1 β -dependent gene in MC is involved in the post-transcriptional regulation of gene expression of the mitogen-activated protein kinase (MAPK) pathway (30), indicating that the activation of the MAPK pathway is required to stabilize the message at the post-transcription level though 3'-UTR. Although the precise mechanisms of COX-2 expression have not been elucidated in the processing of aggregated protein in the glomeruli, most often it entails an intracellular signaling pathway in which enzymes phosphorylate proteins. Activation of the PKC-MAPK route is thought to play an important role in the progress of diabetic nephropathy (31–33). These findings suggest that in diabetes, the MAPK system is already activated and so is probably not further activated by aggregated protein in the glomeruli. Further investigations using cultured mesangial cells are required to elucidate the involvement of intracellular signal transduction in regulation of COX-2 expression by aggregated proteins and of the degradation of aggregated protein in the glomeruli.

In this study, the processing of aggregated protein was retarded in the glomeruli of STZ-induced diabetic mice. This retardation was possibly associated with the instability of post-transcriptional mRNA followed by disability of COX-2 protein expression. In addition, the impaired processing of aggregate protein in the diabetic glomeruli is probably related to the progress of diabetic nephropathy.

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