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Involvement of Prostaglandin E_2 in Clearance of Aggregated Protein via Protein Kinase A in Glomeruli

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ABSTRACT—Recently we immunohistochemically demonstrated that prostaglandin E_2 (PGE_2) promoted the clearance of aggregated bovine serum albumin (a-BSA) deposited in glomeruli. Herein, we investigated the role of PGE_2 and its signal transduction in the disposal of macromolecules in glomeruli. EP_2 and EP_4 receptor mRNA was detected in glomeruli by RT-PCR analysis. A-BSA was injected twice into mice. Glomeruli were then isolated and incubated. A-BSA gradually disappeared from isolated glomeruli. PGE_2 increased the intracellular cyclic AMP and decreased a-BSA level in glomeruli. Additionally, 8-bromo-cyclic AMP evoked a loss of a-BSA in isolated glomeruli. The effect of 8-bromo-cyclic AMP on the clearance of a-BSA was abolished by KT 5720 in glomeruli. PGE_2 and 8-bromo-cyclic AMP also prompted disposal of a-BSA in cultured mesangial cells. These findings indicate that PGE_2 positively regulates the removal of macromolecules via cyclic AMP and protein kinase A in glomeruli, and they provide insight into how to prevent the development of glomerulonephritis and glomerulosclerosis.

Keywords: Glomeruli, Prostaglandin E_2, Aggregated protein, Cyclic AMP, Protein kinase A

Recent studies have emphasized that mesangial cells not only regulate the glomerular blood flow but have a variety of physiological functions such as production of cytokines (1 – 3) and the extracellular matrix (4). Mesangial cells are also known to engulf large molecules (5), and they are reported to endocytose neutrophils that cause apoptosis in nephritic glomeruli (6, 7). Several macromolecules and aggregated protein deposited in the mesangial area are thought to be eliminated by two pathways, the mesangial channels that run from the peripheral mesangial area to the lacis area at the vascular pole of the glomerulus (8) and endocytosis by mesangial cells (9). Despite the importance of this process in maintaining the glomerular function, the regulatory mechanisms for the clearance of aggregated protein in glomeruli are still unclear.

Lee and Vernier observed the distribution of aggregated human albumin in mesangial matrix channels and endosome of mesangial cells using immunoelectron microscopy (10). They emphasized that the mesangial channels act as a pathway for the disposal of aggregated protein from glomeruli (10). Previously, by in vivo experiments, we demonstrated that aggregated bovine serum albumin (a-BSA) was accumulated in glomeruli and cleared within 48 h, and that prostaglandin E_2 (PGE_2) and prostaglandin E_1 (PGE_1) accelerated the disposal of a-BSA in glomeruli (11, 12). On the other hand, PGE_1 did not accelerate the clearance of carbon particles that were drained through the mesangial channels over several weeks (13). This difference was possibly due to the property of the substances. Therefore, we considered that PGE_2 up-regulates the disposal process of aggregated protein by mesangial cells.

In the present study, we attempted to elucidate 1) expression of mRNA of PGE_2 receptors in glomeruli and 2) the accelerating effect of PGE_2 and its mechanisms on the disposal process in mesangial cells using cultured glomeruli and cultured mesangial cells. We demonstrate that mouse glomeruli express mRNA for the EP_2 and EP_4 receptor subtype of PGE_2 and that PGE_2 accelerates the disposal of aggregated protein in glomeruli and mesangial cells via the cyclic AMP - protein kinase A system.

MATERIALS AND METHODS

Animals

Male ICR strain mice, 4-week-old and weighing 15 –

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20 g, and male Sprague Dawley rats, weighing 100 – 200 g, were purchased from Nihon Clea (Tokyo) and housed in an air-conditioned room at 22 ± 2°C with a 12 h-light-dark cycle until used. They had access to water and standard mouse chow ad libitum.

Reagents

Prostaglandin E_2 (PGE_2) was purchased from Cayman Chemical (Ann Arbor, MI, USA); KT 5720 (a selective protein kinase A inhibitor) from Biomol (Plymouth Meeting, PA, USA); 8-bromo-cyclic AMP and 3-isobutyl-1-methylxanthine (IBMX, a phosphodiesterase inhibitor) from Sigma Chemical Co. (St. Louis, MO, USA); and indomethacin from Wako Pure Chemical (Osaka). The following regents were also used in the present experiments: crystallized bovine albumin (Bayer Co., Kankakee, IL, USA); iron oxide (Aldrich Chemical Company, Inc., Milwaukee, WI, USA); RPMI 1640 (Nissui Pharmaceutical, Tokyo); dimethyl sulfoxide (Wako Pure Chemical); affinity purified rabbit anti-BSA antibody (Yagai Co., Yamagata); peroxidase-conjugated rabbit anti-BSA antibody (Cappel, Durham, NC, USA); *o*-phenylenediamine (Sigma Chemical Co.); random 9 mers (Takara Biomedicals, Tokyo); reverse transcriptase (Super Script II; Life Technologies, Inc., Rockville, MD, USA); and Ampli Taq Gold polymerase (Perkin Elmer, Branchburg, NJ, USA).

Experimental protocol of isolated glomeruli

A-BSA was prepared according to a procedure reported previously (12). Mice (n = 10) were injected with 0.8 mg/g body weight of a-BSA, followed by the reinjection of a-BSA after 3 h. Glomeruli were isolated using iron oxide, sieves and a magnet (14) 6 h after the first injection of a-BSA. Glomerular suspension (3000 glomeruli/ml RPMI 1640 per well) was moved to a 48-well tissue culture plate (Corning Inc., Corning, NY, USA). Aliquots of glomeruli were frozen in medium to measure a-BSA before incubation. Two or three wells were assigned to vehicle and each concentration of reagent. PGE_2 was added to medium at $10^{-7} – 10^{-9}$ M, 8-bromo-cyclic AMP at $10^{-7} – 10^{-9}$ M, and KT 5720 at $10^{-6} – 10^{-8}$ M in the presence of 10^{-5} M indomethacin to prevent the production of PGE_2 in glomeruli. They were incubated for 30 min in a CO_2-incubator. After the incubation, glomeruli were frozen in fresh medium at −20°C until measured. Glomeruli were thawed and disrupted by sonication. A-BSA in the lysate was determined in duplicate by ELISA. The levels of a-BSA are shown as a percentage of the mean a-BSA of glomeruli before the incubation (0 min). The experiment was performed 3 or 4 times. In additional experiments, a-BSA was determined regarding glomeruli and culture medium before and after the 3 h-incubation with PGE_2 plus indomethacin (Table 1), and regarding glomeruli 12 h after the incubation with KT 5720 in the absence of indomethacin (Fig. 5B).

Experimental protocol of cultured mesangial cells

Because the culturing procedure for rat mesangial cells is established, mesangial cells were obtained from glomeruli that were isolated from rat kidneys by conventional sieving methods (15). All experiments were performed with the cells between the 5th and the 10th passages. Mesangial cells (1×10^4 cells/well) were cultured until subconfluent. Mesangial cells were incubated in serum-free medium for 12 h and then incubated for 1 h for uptake of biotinylated a-BSA, which had been labeled by a biotinylation kit (American Qualex, San Clemente, CA, USA). After washing with PBS, mesangial cells were treated with each agent for 30 min in the presence of 10^{-5} M indomethacin. They were then washed, and permeabilized with 120 μl of 0.5% NP-40. Biotinylated a-BSA in the cytosol fraction was determined in duplicate by ELISA. The experiment was performed 4 times. The levels of a-BSA in mesangial cells were obtained as the optical density (O.D.) in absorbance at 490 nm. Results are shown as a percentage of the mean O.D. of the vehicle-treated group.

RNA isolation and RT-PCR detection of EP_2 and EP_4 receptor mRNA

Total RNA was isolated from 1.5×10^4 glomeruli of 10 mice using a Fast RNA Green Kit (BIO 101; Joshna Way, Vista, CA, USA) and Fast Prep FP 120 (Savant Instrument, Inc., Holbrook, NY, USA). Reverse transcription was performed according to the protocol of manufacturer (Life Technologies, Inc.). The resulting cDNA was amplified using a PCR Thermal Cycler Personal (Takara Biomedicals) as described by Arakawa et al. (16).

Measurement of cyclic AMP

To evaluate generation of cyclic AMP in response to PGE_2, 3000 glomeruli of mice were incubated with PGE_2 ($10^{-7} – 10^{-9}$ M) and IBMX (5×10^{-6} M) in 1 ml of medium for 30 min, and then 500 μl of trichloroacetic acid were added. Glomeruli were then homogenized, and a half volume of the homogenate was centrifuged at 5000 rpm for 20 min. Then trichloroacetic acid was removed with water-saturated ether from the supernatant. Cyclic AMP in the supernatant was measured by a cyclic AMP assay kit (Yamasa, Chiba). The remaining half of the homogenate was dissolved by the same volume of 1 N NaOH. The protein content of the homogenate was measured by Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, CA, USA).

Statistical analyses

The results obtained are expressed as means ± S.E.M. The data were analyzed by the Bartlett test and then by a

one-way analysis of variance (ANOVA) or the Kruskal-Wallis test. To determine the significance of differences among the groups, the Bonferoni or Cocklan procedure was used.

RESULTS

Time course of a-BSA in isolated glomeruli (Fig. 1)

Glomeruli were isolated from mice after the last injection of a-BSA. The glomeruli contained 14.4 ± 2.3 ng /3000 glomeruli of a-BSA (n = 11) immediately after the isolation (0 min). Thereafter, the glomerular a-BSA levels gradually decreased over the incubation, being 85% and 72% of the preincubation levels after 30 min and 3 h, respectively.

EP_2 and EP_4 receptor mRNA in glomeruli (Fig. 2)

The yield of total RNA was 14 μg. The cDNAs for EP_2 and EP_4 receptors of mice were subjected to amplification by PCR. When PCR products were analyzed on 2% agarose gels, each single ethidium bromide staining band was observed. A 401-bp EP_2 cDNA and a 423-bp EP_4 cDNA were generated, respectively.

Effect of PGE_2 on production of cyclic AMP and on clearance of a-BSA in isolated glomeruli (Fig. 3 and Table 1)

When a-BSA-loaded glomeruli were exposed to PGE_2, PGE_2 significantly increased the production of cyclic AMP compared to vehicle. On the other hand, the glomerular a-BSA levels were decreased by PGE_2, and the effect was dependent on the concentration. Furthermore, as shown in Table 1, PGE_2 did not increase a-BSA in the culture medium more than that with vehicle in spite of the decrease of glomerular a-BSA by the treatment of PGE_2.

Effect of 8-bromo-cyclic AMP on clearance of a-BSA in isolated glomeruli (Fig. 4)

A significant decrease of glomerular a-BSA was observed even with 10^{-9} M 8-bromo-cyclic AMP. The treatment of 10^{-7} M 8-bromo-cyclic AMP resulted in a diminution of glomerular a-BSA, which was approximately 40% less than that of the vehicle group.

Involvement of protein kinase A in clearance of a-BSA in isolated glomeruli (Fig. 5)

Glomerular a-BSA was decreased to 65% of that of the vehicle group with 10^{-8} M 8-bromo-cyclic AMP. This diminution was abolished by the coincubation with KT 5720, a selective protein kinase A inhibitor. In additional experiments, a-BSA-loaded glomeruli were incubated with KT 5720 for 12 h without 8-bromo-cyclic AMP. In vehicle-treated glomeruli, a-BSA decreased to 53% of a-BSA at 0 min. KT 5720 (10^{-6} M)-treated glomeruli had

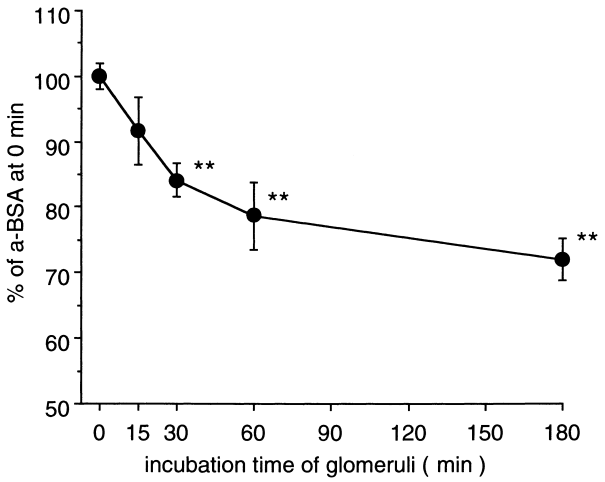

Fig. 1. Time course of a-BSA in isolated glomeruli. A-BSA was injected twice at a 3 h-interval. Glomeruli were isolated 3 h after the last injection of a-BSA and incubated in serum-free RPMI-1409 medium at 37°C without indomethacin. Results are expressed as means ± S.E.M. of 6 experiments. $^{**}P<0.01$, compared with the a-BSA level at 0 min.

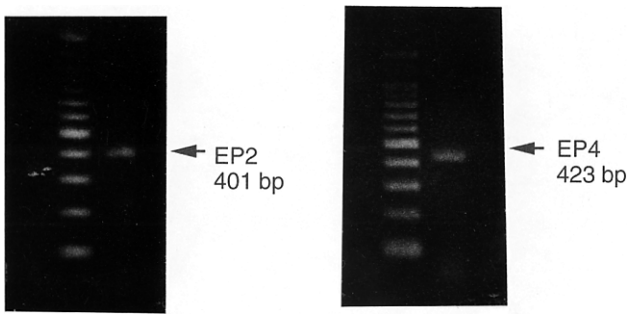

Fig. 2. Reverse transcription-polymerase chain reaction (RT-PCR) analysis demonstrating the presence of EP_2 and EP_4 receptor mRNA in mouse glomeruli. The expression of EP_2 and EP_4 receptor mRNA was determined by RT-PCR as detailed in the Methods and in the text.

20% more a-BSA than the vehicle control. KT 5720 caused a delay of clearance of a-BSA in glomeruli.

Effect of PGE_2 and 8-bromo-cyclic AMP on clearance of a-BSA in cultured mesangial cells (Fig. 6)

PGE_2 reduced concentration-dependently the content of a-BSA in mesangial cells after the incubation as well as in isolated glomeruli. Mesangial cells treated with 8-bromo-cyclic AMP indicated significantly less a-BSA than that with vehicle. It was 60% decreased with 10^{-6} M 8-bromo-cyclic AMP.

DISCUSSION

In the present study, a-BSA gradually decreased in iso-

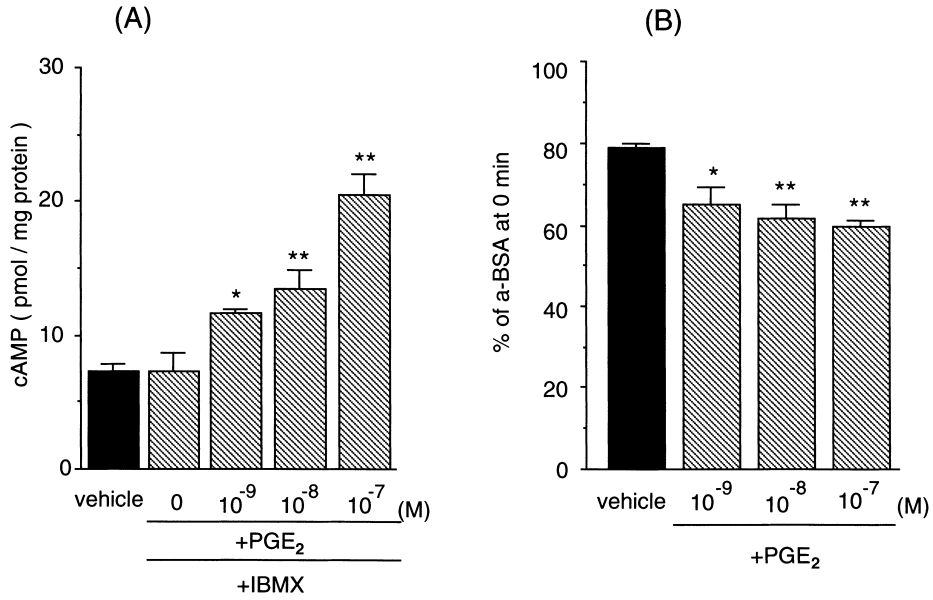

Fig. 3. Effect of PGE_2 on production of cAMP (A) and on clearance of a-BSA (B) in isolated glomeruli. A-BSA-loaded glomeruli were isolated after the last injection and incubated with vehicle, IBMX, or IBMX plus PGE_2 (A), and vehicle or PGE_2 (B). In experiment B, vehicle-treated glomeruli showed 16.0 ± 2.4 ng/3000 glomeruli (n = 6), and 10^{-7} M PGE_2-treated glomeruli showed 12.0 ± 2.0 ng/3000 glomeruli (n = 6). Results are expressed as means ± S.E.M. **P*<0.05 and ***P*<0.01, compared with the vehicle control.

Table 1. Comparison of a-BSA in glomeruli and culture medium after incubation with PGE_2

	Glomeruli (a-BSA ng/3000 glomeruli)		Culture medium (a-BSA ng/ml)	
	0 h	3 h	0 h	3 h
Vehicle	32.2 ± 2.7	16.0 ± 1.1	9.1 ± 0.8	40.5 ± 3.5
PGE_2 10^{-7} M	—	13.9 ± 1.6	—	34.0 ± 2.3
10^{-6} M	—	12.9 ± 0.8	—	34.5 ± 2.6

Glomeruli were incubated with vehicle or PGE_2 for 3 h. A-BSA was determined in glomeruli and culture medium by ELISA. Numbers at 0 h show a-BSA before the incubation. Results are expressed as means ± S.E.M. n = 4.

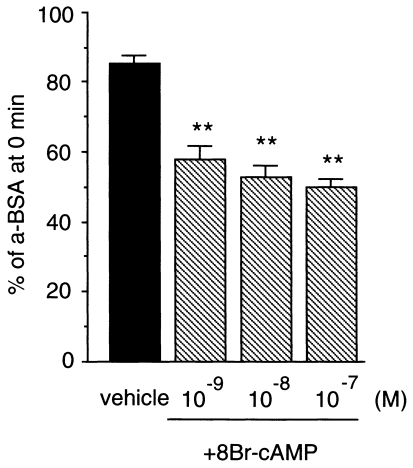

Fig. 4. Effect of 8-bromo-cyclic AMP on clearance of a-BSA in isolated glomeruli. A-BSA-loaded glomeruli were isolated and incubated with vehicle or 8-bromo-cyclic AMP. Vehicle-treated glomeruli showed 16.7 ± 2.0 ng/3000 glomeruli (n = 8), and 10^{-7} M 8-bromo-cyclic AMP treated glomeruli showed 9.5 ± 1.5 ng/3000 glomeruli (n = 6). Results are expressed as means ± S.E.M. ***P*<0.01, compared with the vehicle control.

lated glomeruli according to incubation time. This decrease in a-BSA-loaded glomeruli was accelerated by PGE_2 and 8-bromo-cyclic AMP. In contrast, a specific protein kinase A inhibitor (KT 5720) suppressed the decrease of a-BSA in the presence or the absence of 8-bromo-cyclic AMP. We demonstrated also that PGE_2 and 8-bromo-cyclic AMP exerted the accelerated effect on decrease of a-BSA in cultured mesangial cells.

Glomeruli were isolated 3 h after the last injection of a-BSA and then incubated in the culture medium in the current investigation. Although it was noted in the previous report that glomerular a-BSA did not decrease until 6 h after the last injection of a-BSA into mice (11), in isolated glomeruli, a-BSA began to decrease immediately after the incubation. Because serum levels of a-BSA were highest at 3 h after the injection (11), it is likely that glomeruli are still taking up a-BSA from the circulation. Therefore, studies with isolated glomeruli and cultured mesangial cells are required to clarify the disposal process of aggregated protein. We observed a 30% decrease of a-BSA in isolated glo-

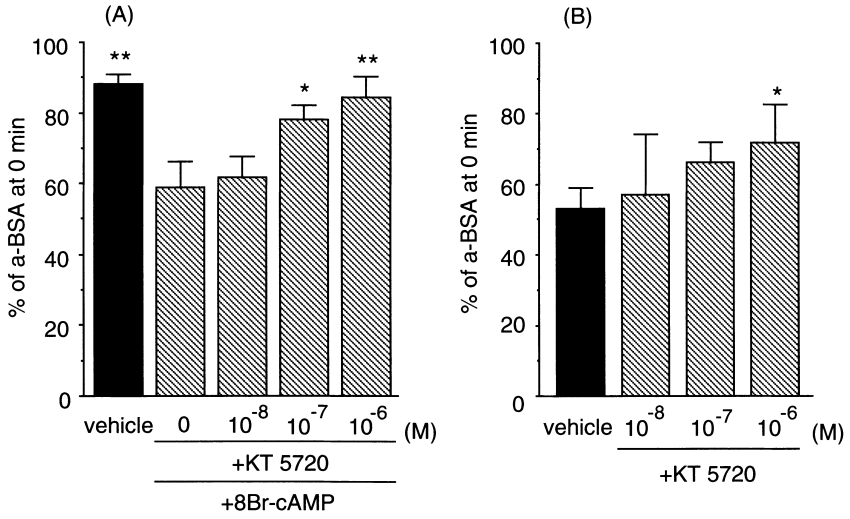

Fig. 5. Involvement of protein kinase A in disposal of a-BSA in isolated glomeruli. A-BSA-loaded glomeruli were isolated and incubated with vehicle, 8-bromo-cyclic AMP alone or 8-bromo-cyclic AMP with KT 5720 (A) and with vehicle or KT 5720 (B). In experiment A, vehicle-treated glomeruli showed 13.2 ± 1.1 ng/3000 glomeruli (n = 7); 10^{-8} M 8-bromo-cyclic, 9.8 ± 1.1 ng/3000 glomeruli (n = 9); and 10^{-8} M 8-bromo-cyclic AMP plus 10^{-6} M KT 5720, 12.4 ± 0.7 ng/3000 glomeruli (n = 9). Results are expressed as means ± S.E.M. **P*<0.05 and ***P*<0.01, compared with the group that was exposed to 8-bromo-cyclic AMP alone. In experiment B, vehicle-treated glomeruli showed 6.4 ± 0.3 ng/3000 glomeruli (n = 6), and 10^{-6} M KT 5720 treated glomeruli showed 8.4 ± 0.5 ng/3000 glomeruli (n = 6). Results are expressed as means ± S.E.M. **P*<0.05, compared with vehicle control.

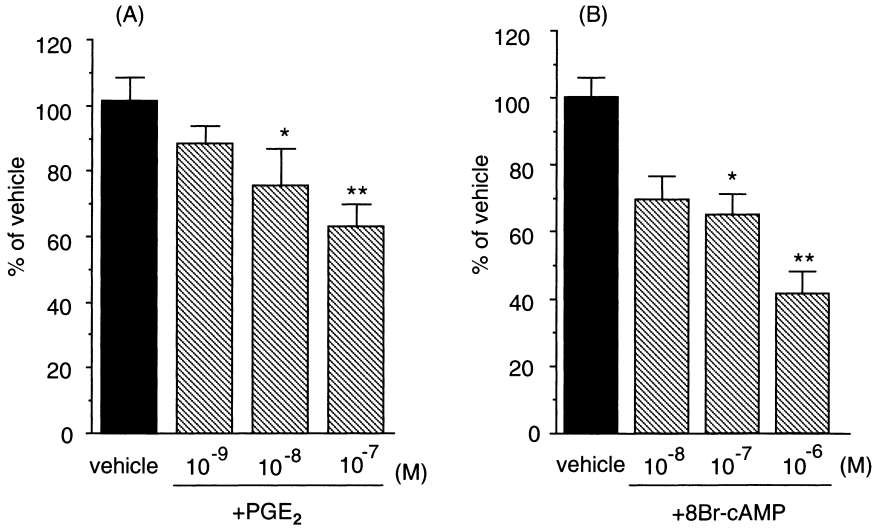

Fig. 6. Effect of PGE_2 (A) and 8-bromo-cyclic AMP (B) on clearance of a-BSA in cultured mesangial cells. Mesangial cells were incubated with biotin-labeled a-BSA, and then the medium was changed to fresh medium containing vehicle, PGE_2 or 8-bromo-cyclic AMP. In experiment A, the vehicle control group showed 0.171 ± 0.024 O.D. (n = 12), and the 10^{-7} M PGE_2 group showed 0.111 ± 0.024 O.D. (n = 11). In experiment B, the vehicle control group showed 0.169 ± 0.020 O.D. (n = 10), and the 10^{-6} M 8-bromo-cyclic AMP group showed 0.073 ± 0.020 O.D. (n = 9). Results are expressed as means ± S.E.M. **P*<0.05 and ***P*<0.01, compared with vehicle control.

meruli after 3 h-incubation. Dean studied the kinetics of degradation of intracellular protein in cultured mouse macrophages (17). They observed 15% degradation of intracellular protein within 4 h. The difference in the rate of decrease of intracellular protein may be explained by the amount of each protein in glomeruli and macrophages; the amount of protein they used is equivalent to the physiological amount, and on the other hand, the amount of protein we used is a non-physiological amount. In the present study, more a-BSA was observed in the culture medium of a-BSA-loaded glomeruli after the incubation than that before the incubation. This increase of a-BSA in the medium indicates the

possibility that more a-BSA releases from glomeruli during the incubation than a-BSA, which is decomposed in glomeruli. It is also speculated that a fraction of a-BSA is released from glomeruli into the medium, and polyclonal antibody could trap these molecules. Further investigation is needed to clarify the cause for this increase of a-BSA in culture medium.

The treatment with 8-bromo-cyclic AMP accelerated the decrease of a-BSA in isolated glomeruli as well as the treatment with PGE_2. We have demonstrated that PGE_2 is generated by glomeruli that have taken up a-BSA and that an increase of PGE_2 is accompanied by a decrease of a-BSA in glomeruli (12). Schlondorff et al. reported that rat glomeruli produce cyclic AMP in response to PGE_2 (18). PGE_2 actually stimulated isolated mouse glomeruli to generate cyclic AMP. There are pharmacologically four kinds of receptor subtypes of PGE_2, namely EP_1 to EP_4, and that cDNA is cloned for each receptor subtype (19, 20). All the receptor subtypes were demonstrated to be expressed in the kidney (21, 22). Expression of EP_2- and EP_4-receptor mRNA in mouse glomeruli was demonstrated by RT-PCR assay. Their positions on the agarose gel were consistent with those reported by Arakawa et al. (16). These receptors have a 7-transmembrane structure and are coupled with G_s protein that stimulates adenylate cyclase. EP_4-receptor subtypes are demonstrated to be predominantly expressed in glomeruli by in situ hybridization in mice (23). Therefore, it is reasonable to consider that glomeruli engulf a-BSA and generate PGE_2, and then PGE_2 binds to the EP_2/EP_4 receptor. PGE_2 stimulates glomeruli to generate intracellular cyclic AMP in an autocrine manner thereafter.

Macrophages have an ability to ingest macromolecules, and this function is reported to be subject to downregulation by PGE_2 or cyclic AMP (24). Mesangial cells actively scavenge the denatured protein to maintain the environment in glomeruli as do macrophages (5). Therefore, we further examined whether PGE_2 and cyclic AMP accelerate disposal of aggregated protein using cultured mesangial cells and a-BSA. The results were similar to those obtained with isolated glomeruli, namely, PGE_2 and cyclic AMP accelerated the disposal of a-BSA in cultured mesangial cells. We did not observed any floating cells after the incubation. Additionally, PGE_2 and 8-bromo-cyclic AMP did not have any effect on the uptake of a-BSA into mesangial cells (data not shown). The present data suggest that PGE_2 accelerates the disposal process of aggregated protein in mesangial cells via cyclic AMP as a second messenger and that mesangial cells contribute to this disposal process in glomeruli.

We also studied the involvement of protein kinase A in the disposal of aggregated protein in glomeruli. In isolated glomeruli, we observed that a selective inhibitor of protein kinase A (KT 5720) abolished the accelerating effect of 8-bromo-cyclic AMP. In the additional experiment, a-BSA-loaded glomeruli were incubated with KT 5720 for 12 h. We considered that 30-min incubation without pharmacological manipulation was insufficient to investigate the role of protein kinase A in the disposal process of aggregated protein in isolated glomeruli, because the rate of decrease of glomerular a-BSA was only 20% as compared with that at 0 min. The incubation with KT 5720 delayed clearance of glomerular a-BSA in the absence of indomethacin as compared with the vehicle control. In the previous report, glomeruli increased the production of PGE_2 after the uptake of a-BSA (12). These results indicate that protein kinase A mediates a signal of PGE_2 to accelerate the disposal of aggregated protein in glomeruli. Further studies are needed to clarify which PGE_2 receptors, EP_2 or EP_4, are associated with accelerating this process, and which lysosomal enzyme or proteasome is involved in it.

Expansion of the mesangial area and much protein deposition are observed in glomeruli from patients with chronic glomerulonephritis (25), glomeruloscrelosis (26) or diabetic nephropathy (27). Long lasting deposition of such protein including excessive extracellular matrix in glomeruli is considered to cause the persistent inflammation, cell death, and expansion of mesangial area that lead to dysfunction of the kidney (28 – 32). Therefore, acceleration of the removal of such protein from glomeruli with certain agents may prevent the development of glomerulonephritis and glomerulosclerosis. It is of interest what alterations would be induced in this removal process in glomerulonephritis and diabetic nephropathy. In conclusion, PGE_2 accelerates the clearance of aggregated protein in glomeruli via cyclic AMP and protein kinase A.

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