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Thromboxane A_2 causes retarded clearance of aggregated protein in glomeruli of nephritic mice

Toshiyuki Nagao, Jun-ichi Koseki, Yoshio Suzuki, Tadashi Nagamatsu *

Department of Pharmacology, Faculty of Pharmacy, Meijo University, 150 Yagotoyama Tenpaku-Ku, Nagoya 468-8503, Japan

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Abstract

Recently, it has been demonstrated that the production of prostaglandins and thromboxane is increased in patients with chronic glomerulonephritis and lupus nephritis. We recently demonstrated that thromboxane A_2 delayed the clearance of heat-aggregated bovine serum albumin deposited in glomeruli. In the present study, we investigated the effect of thromboxane A_2 on the clearance of macromolecules in nephritic glomeruli. First, we attempted to clarify the conditions for the clearance of heat-aggregated bovine serum albumin in nephritic glomeruli, using glomeruli isolated from control and anti-glomerular basement membrane nephritic mice. Heat-aggregated bovine serum albumin was injected twice into each mouse. The glomeruli were then isolated and incubated in culture medium. The heat-aggregated bovine serum albumin content of control glomeruli gradually diminished with incubation time up to 24 h. The heat-aggregated bovine serum albumin content of nephritic glomeruli was 69% higher than that of control glomeruli at 24 h incubation. The production of thromboxane B_2 (the stable metabolite of thromboxane A_2) in nephritic glomeruli showed about a sevenfold increase compared with control. DP-1904 [6-(1-imidazolylmethyl)-5,6,7,8-tetrahydro-naphthalene-2-carboxylic acid hydrochloride], a thromboxane A_2 synthase inhibitor, and KT2-962 [sodium 3-(4-(4-chlorophenyl-butylsulfonamido) butyl)-6-isopropylazulene-1-sulfonate], a selective thromboxane A_2 receptor antagonist, significantly reduced the heat-aggregated bovine serum albumin content in nephritic glomeruli. Normal glomeruli treated with U-46619 [15*S*-hydroxy-11a,9a-(epoxymethano)prosta-5Z,13E-dienoic acid], a stable analogue of thromboxane A_2, had significantly more heat-aggregated bovine serum albumin than control glomeruli. We next investigated whether thromboxane A_2 could affect the uptake/disposal of heat-aggregated bovine serum albumin by cultured rat mesangial cells. U-46619 significantly enhanced the uptake and inhibited the disposal of heat-aggregated bovine serum albumin by mesangial cells. Finally, we performed experiments to elucidate the role of the thromboxane A_2 receptor (TP receptor) in the clearance of heat-aggregated bovine serum albumin using TP-deficient mice. The glomerular heat-aggregated bovine serum albumin content of TP-receptor knockout [TP(−/−)] mice was lower than that of wild-type [WT(+/+)] mice. U-46619 dose dependently increased the uptake of heat-aggregated bovine serum albumin by mesangial cells in WT(+/+) mice, but not in the TP(−/−) mice. These findings suggest that thromboxane A_2 retards the clearance of aggregated protein in nephritic glomeruli and may contribute to the pathophysiology of glomerulonephritis.

Keywords: Thromboxane A_2; Clearance; Aggregated protein; Glomeruli; Nephritic mouse

1. Introduction

A large amount of protein is deposited in the glomeruli of patients with chronic glomerulonephritis or glomerulosclerosis (Alpers et al., 1987; Okada et al., 1994). Akai et al. (1994) reported that repeated administration of bovine serum albumin caused glomerular injury via an increase in the intraglomerular expression of mRNA for platelet derived growth factor-B and interleukin-1β in mice. The long-term deposition of macromolecules in glomeruli is considered to cause inflammation and glomerular injury, which can lead to dysfunction of the kidney (Klahr et al., 1988). However, few reports are available on how glomeruli regulate the removal of macromolecules in glomerulonephritis. Therefore, we investigated the clearance of macromolecules in nephritic glomeruli.

Recently, it has been demonstrated the production of prostaglandins and thromboxane A_2 is increased in patients with chronic glomerulonephritis and lupus nephritis

* Corresponding author. Tel.: +81-52-832-1781; fax: +81-52-834-8090.

E-mail address: nagamats@meijo-u.ac.jp (T. Nagamatsu).

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(Niwa et al., 1987, 1988; Woo, 1996; Patrono and Pierucci, 1986; Yoshida et al., 1996). It is believed that thromboxane A_2 mediates the deterioration of renal function through vasoconstrictive, platelet pro-aggregator and chemotactic actions (Lianos et al., 1983). Based on these findings, we have already investigated the antinephritic effect of DP-1904, a thromboxane A_2 synthase inhibitor, on crescentic type anti-glomerular basement membrane nephritis in rats, which resembles rapidly progressive glomerulonephritis in humans (Nagao et al., 1994, 1995). DP-1904-treated nephritic rats exhibited less deposition of exogenously administered rabbit immunoglobulin G in glomeruli than did control rats (Nagao et al., 1994). Additionally, we recently reported that thromboxane A_2 is involved in the disposal of aggregated protein in vivo (Nagamatsu et al., 1997). Singhal et al. (1991) also reported that a thromboxane A_2 analogue increased the endocytosis of immunoglobulin G complexes by cultured mesangial cells. Such findings led us to hypothesize that thromboxane A_2 interferes with the clearance of macromolecules, such as immune complexes, in nephritic glomeruli. To test this possibility, we investigated the effect of thromboxane A_2 on the clearance of aggregated protein in isolated anti-glomerular basement membrane nephritic glomeruli and cultured mesangial cells.

Recently, Namba et al. (1992) cloned a cDNA for a mouse thromboxane A_2 receptor (TP receptor) and demonstrated that TP receptor mRNA is expressed in the mouse kidney. Narumiya et al. disrupted the gene encoding TP receptor in mice by homologous recombination and created TP-deficient mice (Sugimoto et al., 1997; Ushikubi et al., 1998). We performed experiments to elucidate the role of the TP receptor in the clearance of heat-aggregated bovine serum albumin using TP-deficient mice.

2. Materials and methods

2.1. Animals

Male ICR mice, 4 weeks old and weighing 30–35 g, were obtained from Nihon Crea (Tokyo, Japan). TP-deficient mice were obtained from Dr. S. Narumiya of Kyoto University, and generated basically as described previously (Sugimoto et al., 1997; Ushikubi et al., 1998). In brief, the mouse genes encoding the TP receptor were disrupted, and chimeric mice were generated. The animals were then back-crossed with C57BL/6 mice, and the resulting heterozygous litter mates [TP(+/−)] were bred to produce homozygous TP(−/−) mice. Homozygous mice were born at the predicted Mendelian frequency, grew normally, lived longer than 1 year, and were fertile. We detected no abnormalities in general body appearance. The distribution of the TP receptor gene was verified by Northern blot hybridization, which failed to detect messenger RNA encoding the receptor in TP(−/−) mice. The animals were housed in an air-conditioned room at 22 ± 2°C with a 12-h light–dark cycle until use. They had access to water and standard mouse chow ad libitum. Male Sprague–Dawley rats weighing 100–200 g (Nihon Crea) were used to obtain mesangial cells.

2.2. Drugs

The thromboxane A_2 synthase inhibitor DP-1904 [6-(1-imidazolylmethyl)-5,6,7,8-tetrahydro-naphthalene-2-carboxylic acid hydrochloride] was purchased from Daiichi Pharmaceutical (Tokyo, Japan). The thromboxane A_2 receptor antagonist KT2-962 [sodium 3-(4-(4-chlorophenylbutylsulfonamido) butyl)-6-isopropylazulene-1-sulfonate] was provided by Kotobuki Pharmaceutical (Nagano, Japan). U-46619 [15*S*-hydroxy-11a,9a-(epoxymethano)prosta-5Z,13E-dienoic acid] was purchased from Sigma (St. Louis, MO, USA). U-46619 was prepared as a stock solution in ethanol and used after an appropriate dilution with RPMI 1640 (Nissui Pharmaceutical, Tokyo, Japan).

2.3. Protocol for the experiment on nephritic glomeruli

2.3.1. Preparation of heat-aggregated bovine serum albumin

Heat-aggregated bovine serum albumin was prepared as reported previously (Ford and Kosatka, 1981). Briefly, crystallized bovine albumin (Bayer, Kankakee, IL, USA) was dissolved in saline at 30 mg/ml. The solution was alkalinized (pH 10) with 0.2 N NaOH, then heated at 70°C for 20 min and at 79°C for 15 min. After it had cooled at room temperature, aggregation of bovine serum albumin was confirmed by measuring absorbance at 525 nm. The solution was then neutralized with 0.2 N HCl and centrifuged at 3500 rpm for 30 min. The supernatant containing heat-aggregated bovine serum albumin was stored in a freezer until use. Heat-aggregated bovine serum albumin consisted of molecules with molecular weights of 5.0×10^5, 1.0×10^6 and 2.8×10^6 Da, as determined by gel filtration chromatography using Cellulofine GCL-2000-st (Seikagaku Kogyo, Tokyo, Japan).

2.3.2. Protocol for the experiment on the clearance of nephritic glomeruli

Anti-glomerular basement membrane nephritis was induced in mice by injecting 0.1 ml of rabbit anti-mouse glomerular basement membrane serum into the tail vein, in accordance with the modified method reported previously (Ito et al., 1983). On the 15th day after the injection, urine samples were collected for 6 h. We used only animals with a urinary protein excretion > 0.5 mg/6 h as nephritic mice. In addition, a control group that received normal rabbit serum was used for comparison with the nephritic group. Both control and nephritic mice were injected twice with 0.8 mg/g body weight of heat-aggregated bovine serum albumin in the tail vein with an interval of 3 h. The

glomeruli were isolated 6 h after the first injection and then incubated. Glomerular heat-aggregated bovine serum albumin content was measured 6 and 24 h after the first injection.

To test the effect of thromboxane A_2 on the clearance of heat-aggregated bovine serum albumin in the nephritic glomeruli, glomeruli were isolated 6 h after the first injection of heat-aggregated bovine serum albumin. The isolated glomeruli were then incubated in the presence of DP-1904, a thromboxane A_2 synthase inhibitor, or KT 2-962, a selective thromboxane A_2 receptor antagonist, at 10^{-7}, 10^{-6} and 10^{-5} M for 3 h at 37°C. Furthermore, control glomeruli with heat-aggregated bovine serum albumin were isolated and incubated with U-46619, a thromboxane A_2 analogue, at 10^{-7}, 10^{-6} and 10^{-5} M for 3 h at 37°C.

2.3.3. Measurement of heat-aggregated bovine serum albumin in glomeruli

Glomeruli were isolated using sieves. Briefly, the kidneys were removed, decapsulated and minced. Renal tissue was passed through a 90-μm pore sieve. Glomeruli and tubules retained on a 38-μm pore sieve were washed with phosphate-buffered saline (pH 7.2) and then collected into a centrifuge tube. The purity of isolated glomeruli was more than 60% when assessed by light microscopy after washing with phosphate-buffered saline. The isolated glomeruli were then resuspended in RPMI 1640 at 3000-glomeruli/ml. One milliliter of glomerular suspension was moved to each well of a 48-well tissue culture plate (Corning Inc., Corning, NY, USA) to be incubated for 30 min in a CO_2 incubator. After the incubation, glomeruli were transferred to a sonication tube, washed with medium, and frozen in fresh medium at −20°C. On the day of measurement, the frozen glomeruli were thawed and disrupted by sonication. The lysate was used as a sample of heat-aggregated bovine serum albumin in glomeruli. Heat-aggregated bovine serum albumin content was determined by enzyme-linked immunosorbent assay with affinity purified rabbit anti-bovine serum albumin antibody (Yagai, Yamagata, Japan), peroxidase-conjugated rabbit anti-bovine serum albumin antibody (Cappel, Durham, NC, USA) and *o*-phenylenediamine (Sigma). The amount of glomerular heat-aggregated bovine serum albumin measured by this method is given as ng/3000 glomeruli. The result is expressed as a percentage of the vehicle control group.

2.3.4. Measurement of thromboxane B_2 in isolated glomeruli of nephritic mice

The glomeruli were isolated using a differential sieving technique as mentioned in Section 2.3.3. The isolated glomeruli were incubated in Krebs–Ringer phosphate-buffered saline (pH 7.2) at 37°C for 60 min. The incubation mixture was then centrifuged and the supernatant was frozen at −70°C for the determination of thromboxane B_2 (the stable metabolite of thromboxane A_2). The amount of thromboxane B_2 in glomeruli was determined by enzyme immunoassay (Amersham International, Buckinghamshire, England). The result is expressed as pg/10^4 glomeruli.

2.4. Protocol for experiments with cultured mesangial cells

2.4.1. Mesangial cell culture

Mesangial cells were obtained by culturing glomeruli isolated from kidneys of rats by conventional sieving methods (Kreisberg and Karnovsky, 1983). Cells, which grew in RPMI 1640 containing 20% fetal calf serum, were identified by phase-contrast, immunofluorescence and electron microscopy. They were characterized as intrinsically mesangial by using previously reported criteria (Lovett et al., 1983). These criteria are the presence of a spindle-shaped morphologic structure and the absence of polygonal-shaped cells; bright immunofluorescence staining for myosin, actin, desmin and Thy-1; and negative staining for common leukocyte antigen, cytokeratin and factor VIII. All experiments were performed using cells between the 5th and 10th passage.

2.4.2. Protocol for experiments with cultured mesangial cells

Mesangial cells were transferred to 48-well plates at 1×10^4 cells/well and cultured until subconfluent. Mesangial cells were incubated in serum-free medium for 12 h. Heat-aggregated bovine serum albumin was biotinylated by using a biotinylation kit (American Qualex, San Clemente, CA, USA). After treatment of mesangial cells with U-46619 at 10^{-8}, 10^{-7} and 10^{-6} M for 10 min, biotinylated heat-aggregated bovine serum albumin was added to the culture medium at 500 μg/200 μl of serum-free RPMI 1640 in the uptake experiment. In the disposal experiment, 500 μg of biotinylated heat-aggregated bovine serum albumin was added to the culture medium. Mesangial cells were incubated for 60 min to allow the uptake of biotinylated heat-aggregated bovine serum albumin. After being washed with phosphate-buffered saline, mesangial cells were treated with U-46619 at 10^{-8}, 10^{-7} and 10^{-6} M for 30 min. Mesangial cells were washed with phosphate-buffered saline and permeabilized with 120 μl of 0.5% NP-40. The cytosol fraction was collected by centrifugation. Mesangial heat-aggregated bovine serum albumin content was determined by enzyme-linked immunosorbent assay with peroxidase-conjugated streptoavidine and *o*-phenylendiamine. The result is expressed as a percentage of vehicle control.

2.5. Protocol for the experiment with TP-deficient mice

The experiments were performed in the following two groups: wild-type [WT(+/+)] mice ($n = 5$) and TP receptor knockout [TP(−/−)] mice ($n = 10$). Each animal

was injected twice with 0.8 mg/g body weight of heat-aggregated bovine serum albumin into the tail vein with an interval of 3 h. The glomeruli were isolated 24 h after the first injection of heat-aggregated bovine serum albumin, and the glomerular heat-aggregated bovine serum albumin content was then measured as mentioned in Section 2.3.3. Mesangial cells were obtained by culturing glomeruli isolated from kidneys of TP-deficient mice by conventional sieving methods (Kreisberg and Karnovsky, 1983). Cells which grew in RPMI 1640 containing 20% fetal calf serum were identified as mesangial cells by phase-contrast microscopy according to the morphological criteria, that is, flat polygonal or spindle-shaped cells with multiple processes. All experiments were performed using cells between the 4th and 13th passage. Mesangial cells were preincubated with serum-free medium containing vehicle or U-46619 for 10 min at 37°C and were then incubated with serum-free medium containing heat-aggregated bovine serum albumin for 60 min at 37°C. Mesangial heat-aggregated bovine serum albumin content was measured as mentioned in Section 2.4.2.

2.6. Statistical analysis

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

3. Results

3.1. Induction of anti-glomerular basement membrane nephritis in mice

Nephritic glomeruli were obtained from anti-glomerular basement membrane nephritic mice on the 15th day after the injection of anti-glomerular basement membrane serum. On that day, the urinary protein excretion and plasma cholesterol content increased significantly compared with control. In the sections from control mice, no diaminobenzidine tetrahydrochloride staining for rabbit immunoglobulin G was detected in glomeruli. However, deposition of rabbit immunoglobulin G was observed along the glomerular basement membrane in the kidney sections from anti-glomerular basement membrane serum-treated nephritic mice (Table 1, Fig. 1).

Table 1
Urinary protein excretion and plasma cholesterol content of mice with anti-glomerular basement membrane serum-induced nephritis

Group	*n*	Urinary protein excretion content (mg/6 hr)	Plasma cholesterol content (mg/dL)
Control-mice	6	0.24 ± 0.04	87.5 ± 16.0
Nephritic-mice	6	3.79 ± 0.50[a]	124.7 ± 8.0[b]

Urine and plasma were taken on the 15th day after injection of anti-GBM. Values indicate the means ± S.D. and *n* indicates the number of mice used.

[a] Indicates significant differences from the control mice at $P < 0.05$.

[b] Indicates significant differences from the control mice at $P < 0.01$.

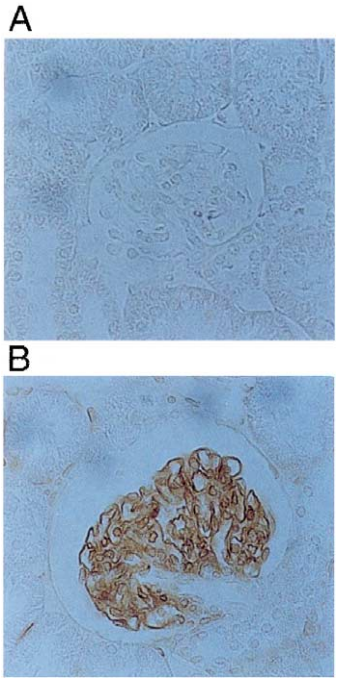

Fig. 1. Light micrographs of glomeruli from control (A) and anti-glomerular basement membrane nephritic (B) mice. Kidneys were taken on the 15th day after injection of anti-glomerular basement membrane serum for light microscopic study. In the sections from control mice, no diaminobenzidine tetrahydrochloride staining for rabbit immunoglobulin G was detected in glomeruli. However, deposition of rabbit immunoglobulin G was observed along the glomerular basement membrane in the sections from anti-glomerular basement membrane nephritic mice.

3.2. Time course of heat-aggregated bovine serum albumin level in control and nephritic glomeruli

Both control and nephritic mice were injected twice with 0.8 mg/g body weight of heat-aggregated bovine serum albumin in the tail vein with an interval of 3 h. The glomeruli were isolated 6 h after the first injection and then incubated. The amount of heat-aggregated bovine serum albumin in the control and nephritic glomeruli was 4.53 and 5.07 ng/3000 glomeruli just after glomeruli isolation, respectively. Thereafter, the glomerular heat-aggregated bovine serum albumin content in control mice gradually decreased, reaching 30% within 24 h, while in nephritic mice the heat-aggregated bovine serum albumin content decreased to 50%. At 24 h after the first injection of heat-aggregated bovine serum albumin, the glomerular heat-aggregated bovine serum albumin content of nephritic mice was higher than that of control mice, suggesting

nephritic glomeruli have a retarded clearance of aggregated protein (Fig. 2).

3.3. Glomerular thromboxane A_2 production of isolated glomeruli of nephritic mice

The glomerular thromboxane B_2 production of the nephritic mice showed an about sevenfold increase compared with that of the control mice after a 60-min incubation. Since we have reported that thromboxane A_2 interferes with macromolecule disposal in normal glomeruli (Nagamatsu et al., 1997), this increase in thromboxane A_2 levels may cause the retarded clearance of aggregated protein in nephritic glomeruli (Fig. 3).

3.4. Effect of thromboxane A_2 synthase inhibitor and thromboxane A_2 receptor antagonist on the clearance of heat-aggregated bovine serum albumin in nephritic glomeruli

Then we isolated glomeruli from nephritic mice that had been injected with heat-aggregated bovine serum albumin. The isolated nephritic glomeruli were incubated in the presence of DP-1904 or KT 2-962 for 3 h. DP-1904 significantly reduced the glomerular heat-aggregated bovine serum albumin content at 10^{-5} M compared with that of the vehicle control. KT 2-962, a selective thromboxane receptor antagonist, also significantly diminished the glomerular heat-aggregated bovine serum albumin content at 10^{-5} M (Fig. 4).

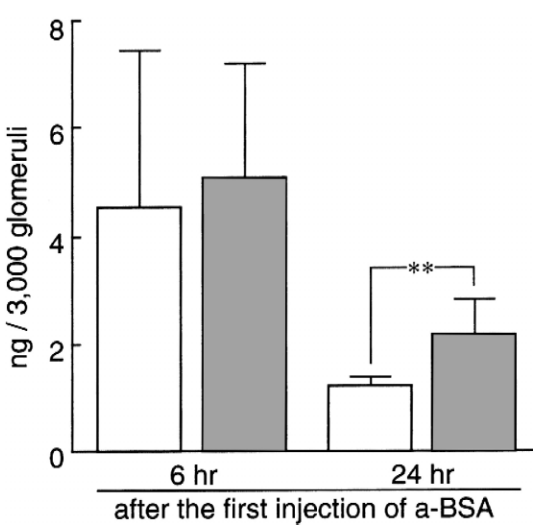

Fig. 2. Time course of heat-aggregated bovine serum albumin level in control and nephritic glomeruli. On the 15th day after injection of anti-glomerular basement membrane serum, heat-aggregated bovine serum albumin was injected into control and nephritic mice twice with a 3-h interval. Glomeruli were isolated 6 h after the first injection of heat-aggregated bovine serum albumin and incubated in serum-free RPMI-1409 medium for 24 h at 37°C. □: control; ■: anti-GBM. Each column denotes the mean with S.E.M. for 5–10 mice. $^{**}P < 0.01$ versus control mice.

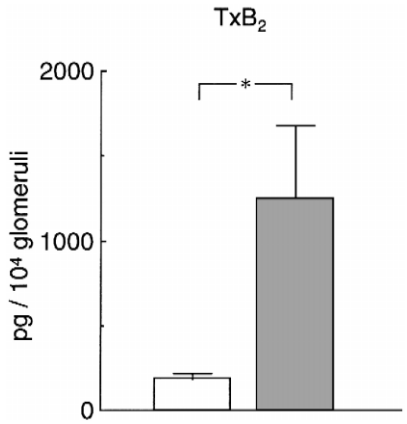

Fig. 3. Glomerular thromboxane A_2 production in isolated glomeruli of control and nephritic mice. On the 15th day after injection of anti-glomerular basement membrane serum, heat-aggregated bovine serum albumin was injected into control and nephritic mice twice with a 3-h interval. Glomeruli were isolated 6 h after the first injection for measurement of thromboxane A_2 production. □: control; ■: anti-GBM. Each column denotes the mean with S.E.M. for 18–19 mice. $^{*}P < 0.05$ versus control mice.

3.5. Effect of thromboxane A_2 analogue on the clearance of heat-aggregated bovine serum albumin in normal glomeruli

Therefore, to elucidate the effect of thromboxane A_2 on the clearance of heat-aggregated bovine serum albumin in

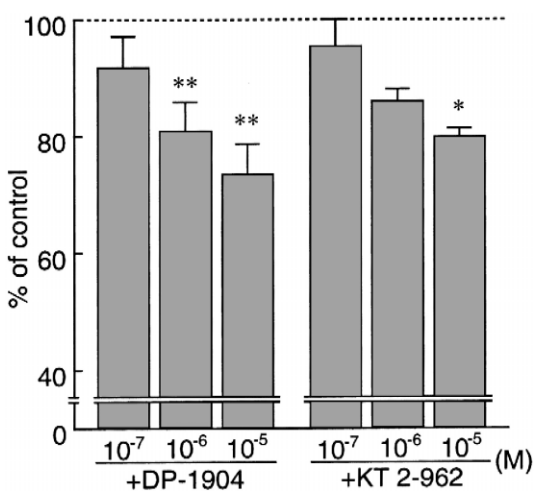

Fig. 4. Effect of thromboxane A_2 synthase inhibitor and thromboxane A_2 receptor antagonist on the clearance of heat-aggregated bovine serum albumin in nephritic glomeruli. On the 15th day after injection of anti-glomerular basement membrane serum, heat-aggregated bovine serum albumin was injected into nephritic mice twice with a 3-h interval. Isolated nephritic glomeruli with heat-aggregated bovine serum albumin were incubated in the presence of DP-1904, a thromboxane A_2 synthase inhibitor, or KT 2-962, a selective thromboxane A_2 receptor antagonist, for 3 h at 37°C. Broken line indicates the glomerular heat-aggregated bovine serum albumin content of the vehicle control. Results are expressed as means ± S.E.M. from four series of experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ versus vehicle control.

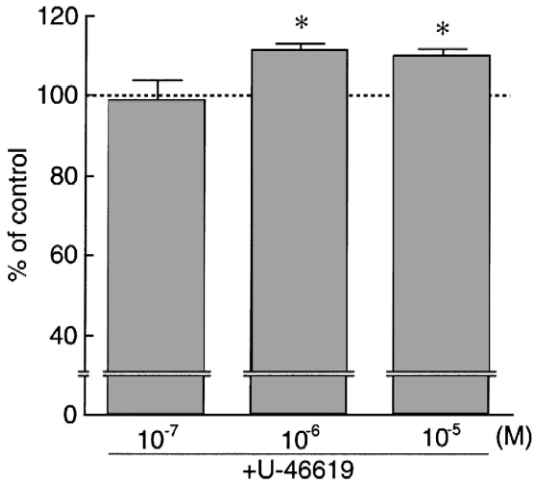

Fig. 5. Effect of thromboxane A_2 analogue on the clearance of heat-aggregated bovine serum albumin in normal glomeruli. Heat-aggregated bovine serum albumin was injected into control mice twice with a 3-h interval. Isolated normal glomeruli with heat-aggregated bovine serum albumin were incubated in the presence of U-46619, a thromboxane A_2 analogue, for 3 h at 37°C. Broken line indicates the glomerular heat-aggregated bovine serum albumin content of the vehicle control. Results are expressed as means ± S.E.M. from three series of experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ versus vehicle control.

glomeruli, normal glomeruli were treated with U-46619, a stable thromboxane A_2 analogue. Glomeruli obtained from normal mice injected with heat-aggregated bovine serum albumin were incubated in the presence of U-46619 for 3 h. U-46619 at 10^{-6} and 10^{-5} M significantly delayed the clearance of glomerular heat-aggregated bovine serum albumin compared with that of the vehicle control (Fig. 5).

3.6. Effects of thromboxane A_2 analogue on uptake of heat-aggregated bovine serum albumin by cultured rat mesangial cells

Since Singhal et al. (1991) reported that thromboxane A_2 increased the uptake of aggregated immunoglobulin G by mesangial cells, we examined whether thromboxane A_2 increased the uptake of heat-aggregated bovine serum albumin by mesangial cells. Mesangial cells were preincubated with serum-free medium containing vehicle or U-46619, a thromboxane A_2 analogue, for 10 min and were then incubated with serum-free medium containing heat-aggregated bovine serum albumin for 60 min. U-46619 significantly increased the uptake of heat-aggregated bovine serum albumin at 10^{-7} and 10^{-6} M compared with vehicle alone. These results are consistent those reported by Singhal et al. (1991) (Table 2).

3.7. Effect of thromboxane A_2 analogue on disposal of heat-aggregated bovine serum albumin by cultured rat mesangial cells

Next, we examined the effect of thromboxane A_2 on the disposal of heat-aggregated bovine serum albumin in mesangial cells. The mesangial cells were incubated first with serum-free medium containing heat-aggregated bovine serum albumin for 60 min and then with serum-free medium containing vehicle or U-46619 for 2 h. U-46619 significantly inhibited the disposal of heat-aggregated bovine serum albumin at 10^{-6} and 10^{-5} M indicating that the disposal process in mesangial cells is delayed by thromboxane A_2 (Table 2).

Table 2
Effects of TxA_2 analogue on the uptake or disposal of a-BSA in cultured rat mesangial cells

Treatment	dose (M)	*n*	Uptake (% of control)	Disposal (% of control)
U-46619	10^{-8}	4	113.7 ± 5.9	–
	10^{-7}	4	160.7 ± 22.2[a]	84.7 ± 5.3
	10^{-6}	4	234.7 ± 77.8[a]	126.9 ± 8.2[a]
	10^{-5}	4	–	121.9 ± 8.4[a]

In the uptake experiment, cultured rat mesangial cells were preincubated with serum-free medium containing vehicle or U-46619 for 10 min at 37°C and were then incubated with serum-free medium containing heat aggregated bovine serum albumin a-BSA for 60 min at 37°C. In the disposal experiment, cultured rat mesangial cells were incubated with serum-free medium containing a-BSA for 60 min at 37°C and were then incubated with serum-free medium containing vehicle or U-46619 for 2 h. Results are expressed as the means ± S.E.M. from four series of experiments.

[a] Indicates a significant difference from the vehicle control at $P < 0.05$.

3.8. The clearance of heat-aggregated bovine serum albumin in isolated glomeruli of TP-deficient mice

To further elucidate the role of the TP receptor in the clearance of aggregated protein in glomeruli, we used

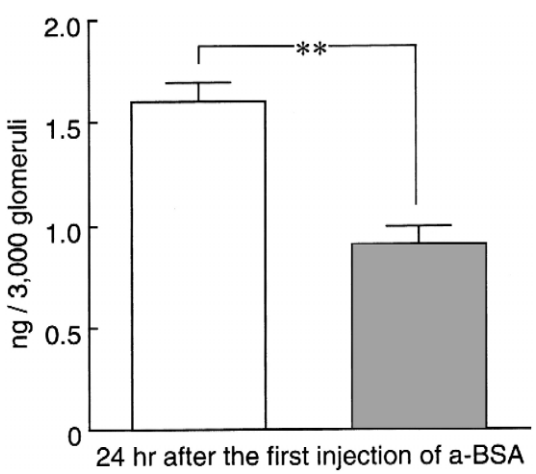

Fig. 6. The clearance of heat-aggregated bovine serum albumin in isolated glomeruli of TP-deficient mice. The experiments were performed in the following two groups: wild-type [WT(+/+)] mice, TP receptor knockout [TP(−/−)] mice. Heat-aggregated bovine serum albumin (a-BSA) was injected into [TP(−/−)] and [WT(+/+)] mice twice with a 3-h interval. The glomeruli were isolated at 24 h after the first injection of heat-aggregated bovine serum albumin. □: WT(+/+) mice; ■: TP(−/−) mice. Each column denotes the mean with S.E.M. for 5–10 mice. $^{*}P < 0.05$, $^{**}P < 0.01$ versus WT(+/+) mice.

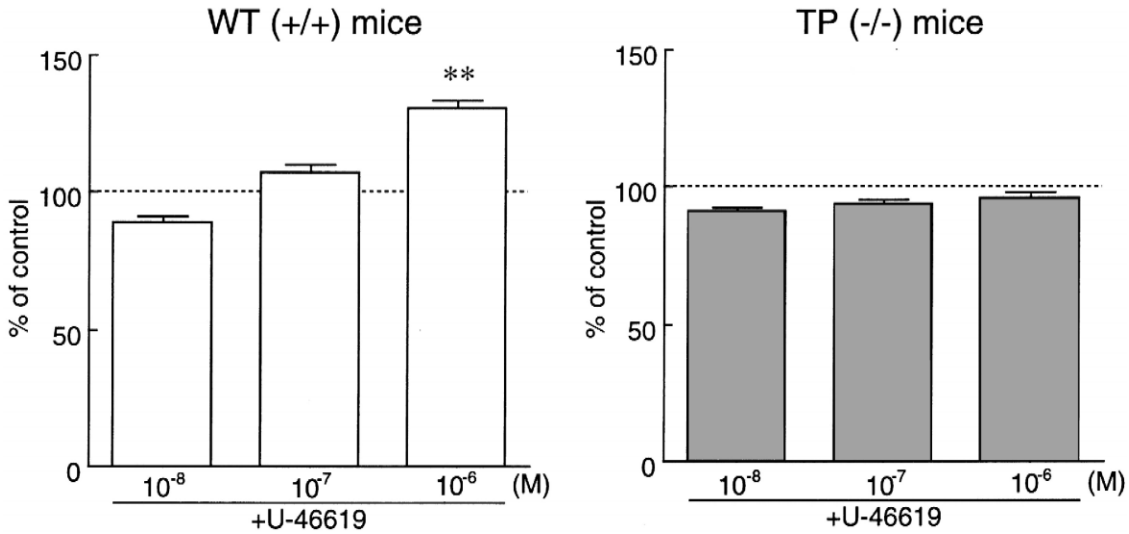

Fig. 7. Effects of thromboxane A_2 analogue on the uptake of heat-aggregated bovine serum albumin by mesangial cells obtained from TP-deficient mice. Mesangial cells were preincubated with serum-free medium containing vehicle or U-46619, a thromboxane A_2 analogue, for 10 min at 37°C and were then incubated with serum-free medium containing heat-aggregated bovine serum albumin for 60 min at 37°C. □: WT(+/+) mice; ■: TP(−/−) mice. Each column denotes the mean with S.E.M. from four series of experiments. Broken line indicates the mesangial heat-aggregated bovine serum albumin content of vehicle control in each experiment. $^{*}P < 0.05$, $^{**}P < 0.01$ versus vehicle control.

TP-deficient mice. WT(+/+) mice and TP(−/−) mice were injected twice with 0.8 mg/g body weight of heat-aggregated bovine serum albumin in the tail vein with an interval of 3 h. The glomeruli were isolated 24 h after the first injection of heat-aggregated bovine serum albumin. The glomerular heat-aggregated bovine serum albumin content of TP(−/−) mice was 38% lower than that of WT(+/+) mice. Glomerular thromboxane A_2 production in TP(−/−) mice was the same as that of WT(+/+) mice (data not shown) (Fig. 6).

3.9. Effects of thromboxane A_2 analogue on uptake of heat-aggregated bovine serum albumin by mesangial cells obtained from TP-deficient mice

Mesangial cells were preincubated with serum-free medium containing vehicle or U-46619 for 10 min and then incubated with serum-free medium containing heat-aggregated bovine serum albumin for 60 min. U-46619 dose dependently increased the uptake of heat-aggregated bovine serum albumin by the mesangial cells obtained from WT(+/+) mice but not TP(−/−) mice. This result indicates that the TP receptor is involved in the uptake of aggregated protein by mesangial cells (Fig. 7).

4. Discussion

Macromolecules and aggregated protein injected into experimental nephritic animals are deposited in the mesangial area in glomeruli. However, the regulatory mechanism for the clearance of aggregated protein in glomeruli is still unclear. These foreign substances are considered to be eliminated by two pathways: the mesangial channel that runs from the peripheral mesangial area to the lacis area at the vascular pole of the glomerulus via the mesangial axis (Elema et al., 1976) and endocytosis by mesangial cells (Sterzel et al., 1983). Regarding the clearance of macromolecules via the mesangial channel, Lee and Vernier (1980) observed aggregated human albumin in mesangial matrix channels and in the endosomes of mesangial cells by immunoelectron microscopy. They emphasized that the mesangial channel is a pathway for the clearance of aggregated protein from glomeruli. We previously demonstrated in in vivo experiments that injected heat-aggregated bovine serum albumin accumulated in glomeruli and was cleared within 48 h, and that injected carbon particles taken up into the mesangial area drained out over 2–7 weeks (Nagamatsu and Suzuki, 1987, 1988). This difference was possibly due to the properties of the injected substances, namely their ability to be degraded by mesangial cells. We considered that the glomerular clearance of protein composed-macromolecules such as aggregated albumin and immune complex was related to endocytosis by mesangial cells in glomeruli and not to drainage through mesangial matrix channels. In the present study, we showed the clearance of heat-aggregated bovine serum albumin to be significantly delayed in nephritic glomeruli compared with normal glomeruli. Long-term deposition of macromolecules in glomeruli is considered to cause the inflammation and glomerular injury that lead to dysfunction of the kidney (Klahr et al., 1988). Akai et al. (1994) reported that repeated administration of bovine serum albumin caused immune complex-mediated glomerulonephritis with glomerular injury, such as mesangial proliferation, in mice. Disruption of the glomerular clearance of macromolecules

in nephritic glomeruli may be related to the progression of glomerulonephritis.

Recently, it has been demonstrated that factors such as angiotensin II and eicosanoids (Singhal et al., 1991), atrial natriuretic peptide, dopamine and adenosine 3′,5′-cyclic monophosphate (Singhal et al., 1990) and repetitive mechanical strain (Mattana et al., 1995) are associated with the clearance of macromolecules in glomeruli and mesangial cells. Previously, we reported that the long-term administration of thromboxane A_2 synthase inhibitor reduced glomerular immune deposits in anti-glomerular basement membrane serum-induced crescentic-type nephritis in rats (Nagao et al., 1995), and that the clearance of aggregated protein in mice glomeruli was delayed by the action of thromboxane A_2 (Nagamatsu et al., 1997). However, the mechanism of glomerular clearance by thromboxane A_2 remains unclear. Therefore, we performed experiments to elucidate the effect of thromboxane A_2 on macromolecule clearance using isolated glomeruli and cultured mesangial cells.

In the present study, we demonstrated that the glomerular clearance of heat-aggregated bovine serum albumin was significantly delayed in nephritic compared with normal glomeruli. The production of thromboxane A_2 was increased significantly in nephritic glomeruli compared with normal glomeruli. In addition, thromboxane A_2 synthase inhibitor and thromboxane A_2 receptor antagonist each improved the delay in glomerular clearance in nephritic glomeruli. In contrast, thromboxane A_2 analogue delayed glomerular clearance in normal glomeruli. These findings suggest that the glomerular clearance of heat-aggregated bovine serum albumin is disrupted by increasing glomerular thromboxane A_2 production in glomerulonephritis. In the present study, it was found that thromboxane A_2 analogue not only promoted the uptake of heat-aggregated bovine serum albumin but also delayed the disposal of heat-aggregated bovine serum albumin by mesangial cells. The delaying action of U-46619 on heat-aggregated bovine serum albumin disposal occurred at a concentration of U-46619 10 times higher than that needed to accelerate heat-aggregated bovine serum albumin uptake. This result suggested that thromboxane A_2 had a greater effect on heat-aggregated bovine serum albumin uptake than on heat-aggregated bovine serum albumin disposal. Schlondorff and Mori (1990) demonstrated that the uptake of immunoglobulin G complexes by cultured mesangial cells was acutely increased by angiotensin II and inhibited by c-AMP, and that these effects were independent of the number of specific Fc receptors, but were mediated by changes in the cytoskeleton. Thromboxane A_2 and U-46619, as well as angiotensin II, evoke activation of phospholipase C with generation of inositol triphosphate and an increase in intracellular calcium concentration in cultured mesangial cells (Mene et al., 1987, 1988). It seems reasonable to consider that the rise in intracellular calcium concentration induced by thromboxane A_2 retards the clearance of macromolecules by influencing the microfilaments.

Finally, we performed experiments to elucidate the role of the thromboxane A_2 receptor (TP receptor) in the clearance of heat-aggregated bovine serum albumin using TP-deficient mice. The glomerular heat-aggregated bovine serum albumin content was lower in TP($-/-$) mice than in WT($+/+$) mice. Furthermore, heat-aggregated bovine serum albumin uptake by U-46619 was not accelerated in cultured mesangial cells obtained from TP($-/-$) mice, but was in cells from WT($+/+$) mice. These results for the TP($-/-$) mice support our findings concerning thromboxane A_2 in the pharmacological experiments. In Fig. 2, heat-aggregated bovine serum albumin-loaded glomeruli were isolated 6 h after the first injection and then incubated up to 24 h, whereas glomeruli of the WT($+/+$) mice were isolated 24 h after the first injection of heat-aggregated bovine serum albumin. Despite the difference in experimental protocol, glomerular heat-aggregated bovine serum albumin levels were quite similar. This result suggests that incubated glomeruli preserve their ability to clear aggregated protein. The deposition of macromolecules, such as immune complexes, in glomeruli is characteristic of immunologically mediated glomerulonephritis. The retarding effect of thromboxane A_2 on the clearance of macromolecules contributes to the pathophysiology of glomerulonephritis with an increase in glomerular thromboxane A_2 synthesis.

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