Antinephritic Effect of Y-19018, a Thromboxane A Synthetase Inhibitor, on Crescentic-Type Anti-GBM Nephritis in Rats

Yoshio SUZUKI, Yoshihito TSUKUSHI, Mikio ITO and Tadashi NAGAMATSU
Department of Pharmacology, Faculty of Pharmacy, Meijo University, Tenpaku-cho, Tenpaku-ku, Nagoya 468, Japan
Accepted June 29, 1987

Abstract—In order to investigate the antinephritic effect of Y-19018, a thromboxane A synthetase inhibitor, on crescentic-type anti-glomerular basement membrane (anti-GBM) nephritis in rats, the present study was undertaken. Male Sprague Dawley rats were immunized with rabbit γ-globulin in Freund's complete adjuvant following i.v. injection of anti-GBM serum. Y-19018 at a dose of 0.3 and 3.0 mg/kg was given orally to rats from the day after the injection of anti-GBM serum (day 1) to day 39. Y-19018, 3.0 mg/kg, significantly inhibited both urinary protein excretion (30.6%) on day 19 and plasma cholesterol (39.8%) on day 15. Moreover, light microscopy demonstrated that this drug at both doses remarkably prevented histological involvement of the glomeruli on day 40 in a dose-dependent manner. In the blood obtained from nephritic rats, platelet aggregation was increased. Y-19018 suppressed (48.7%) the hyperaggregability of platelets on day 40 at a high dose, although the suppression of platelet aggregation was not in a dose-dependent manner. It is concluded from these data that Y-19018 shows beneficial effects on crescentic-type anti-GBM nephritis and may exert its action partly through inhibition of glomerular TXA₂.

Recent studies have indicated that platelet aggregability was promoted (1) and platelet aggregates were increased in the circulation in patients with various forms of glomerulonephritis (2). Additionally, anti-platelet drugs could diminish protein excretion into urine and improve the kidney function in such cases. It is well-known that platelets are aggregated by a very small amount of thromboxane A₂ (TXA₂) as compared with other stimulants. Lianos et al. (3) demonstrated that thromboxane B₂ (TXB₂), a stable metabolite of TXA₂, remarkably increased in the glomeruli 2 hr after the injection of nephrotoxic serum into rats and that the increase was sustained for 14 days. They also reported that there was a positive relationship between the amount of glomerular TXB₂ and the extent of proteinuria in such an experimental model. Therefore, it is expected that the decrease of TXA₂ in glomerulonephritis could lead to an inhibition of the progress of glomerulonephritis. In the present studies, we have found that Y-19018, a TXA synthetase inhibitor, could suppress the development of crescentic-type anti-GBM nephritis in rats.

Materials and Methods

1. Animals: Male Sprague-Dawley rats (Sizuoka Jikken Dobutsu Kyodo Kumiai) weighing 160–170 g were used in the experiments.

2. Drugs (4): Y-19018 (2,4,6,2'-tetramethyl-5'-(1-imidazoly)-diphenyl hydroxy methan) (Yoshitomi Seiyaku) was used. Y-19018 was suspended in 0.5% gum arabic solution. Animals orally received the drug in a volume of 1 ml/100 g of body weight.

3. Experimental nephritis and drug treatment: Thirty-five rats were intravenously injected with 0.56 ml/animal of anti-GBM serum which was produced in rabbits as reported (5). On the next day, 24 hr urinary
protein was determined; and 3 groups were formed, each consisting of 8 rats with the same level of average proteinuria. Then, the rats of each group were subcutaneously immunized with 6.5 mg of rabbit r-globulin in 0.25 ml of Freund’s complete adjuvant into the hind foot pads to induce crescentic-type anti-GBM nephritis. Two of the 3 groups were administered daily with 0.3 or 3.0 mg/kg of Y-19018 (p.o.), from day 2 (the injection of rabbit r-globulin) to day 39 in all experiments. Mikashima et al. (4) demonstrated that Y-19018 at 3.0 and 0.3 mg/kg decreased serum TXB2 by about half of the normal level in rabbits. The remaining group, given 0.5% gum arabic solution instead of the drugs (p.o.), served as the control. In addition, a nontreated (normal) group of 8 rats was used in the experiment.

4. General condition, body weight and urine volume: The general condition of the rats was observed, and body weight and urine volume were determined during the experiment.

5. Urine, blood and kidney: Twenty-four hr urine samples were obtained on days 0, 5, 9, 29 and 39 after the injection of anti-GBM serum. The animals were loaded with 8 ml of tap water and placed in separate metabolic cages for 24 hr without feeding and water. Urine was then centrifuged at 3,000 rpm for 20 min at 4°C, and the supernatant was used for urinalysis. Blood samples were obtained on days 7, 15, 25 and 36 after the injection of anti-GBM serum. From the tail vein of conscious rats, 0.6 ml of blood was drawn with a disposable microsyringe and put into a tube with indomethacin at 2 µg/ml of blood and EDTA-2Na-2H2O at 4.5 µmol. The blood sample was centrifuged at 3,000 rpm for 20 min at 4°C, and the supernatant was used for urinalysis. Blood samples were obtained on days 0, 5, 9, 29 and 39 after the injection of anti-GBM serum. From the tail vein of conscious rats, 0.6 ml of blood was drawn with a disposable microsyringe and put into a tube with indomethacin at 2 µg/ml of blood and EDTA-2Na-2H2O at 4.5 µmol. The blood sample was centrifuged at 3,000 rpm for 20 min at 4°C, and the supernatant was used for urinalysis. Blood samples were obtained on days 7, 15, 25 and 36 after the injection of anti-GBM serum. From the tail vein of conscious rats, 0.6 ml of blood was drawn with a disposable microsyringe and put into a tube with indomethacin at 2 µg/ml of blood and EDTA-2Na-2H2O at 4.5 µmol. The blood sample was centrifuged at 3,000 rpm for 20 min at 4°C, and the supernatant was used for urinalysis. Blood samples were obtained on days 7, 15, 25 and 36 after the injection of anti-GBM serum. From the tail vein of conscious rats, 0.6 ml of blood was drawn with a disposable microsyringe and put into a tube with indomethacin at 2 µg/ml of blood and EDTA-2Na-2H2O at 4.5 µmol. The blood sample was centrifuged at 3,000 rpm for 20 min at 4°C, and the supernatant was used for urinalysis. Blood samples were obtained on days 7, 15, 25 and 36 after the injection of anti-GBM serum. From the tail vein of conscious rats, 0.6 ml of blood was drawn with a disposable microsyringe and put into a tube with indomethacin at 2 µg/ml of blood and EDTA-2Na-2H2O at 4.5 µmol. The blood sample was centrifuged at 3,000 rpm for 20 min at 4°C, and the supernatant was used for urinalysis.

6. Determination of biochemical parameters in urine and plasma: The protein content in urine was determined by the method of Kingsbury et al. (6) and expressed as mg per 24 hr urine. N-acetyl-β-glucosaminidase (NAG) activity in urine was determined in accordance with the method of Hasebe (7) using 4-nitrophenyl-N-acetyl-β-glucosaminide (Sigma) as substrate and expressed as mU per 24 hr urine. One unit refers to the ability to produce 1 µM of p-nitrophenol from substrate for 1 hr incubation in 1 l of urine. Urea nitrogen content in plasma was determined by the urease-indophenol spectrophotometric assay (8). Cholesterol content in the plasma was determined with a commercial assay kit (Determina TC-5, Kyouwa Medix Co., Ltd.) (9). Both urea nitrogen and cholesterol were expressed as mg per 1 dl of plasma.

7. Determination of platelet aggregability in whole blood: Platelet aggregability was measured with a whole blood aggregometer C-560 (Crono-log Co.). On day 40, 500 µl of blood was added to 495 µl of 0.9% saline in a small aggregometer cuvette. This cuvette was maintained at 37°C by a heated block and the contents were stirred with a “flea” magnet. An electrode was inserted into the sample. Five µg of collagen (Collagen reagent Horm, Hormon-Chen) was then added to a sample in the cuvette, and measurements were taken for 5 min. Results were represented as the conductance (ohm) between the two electrodes 5 min after the addition of aggregating agents.

8. Determination of blood pressure: Systolic blood pressure was measured before and 6, 12, 24, 30 and 37 days after the injection of anti-GBM serum by tail plethysmography (KN-209, Natsume). Before the measurement, animals were warmed at 60°C for 3 min in the preheating box.

9. Light microscopic studies: The kidney was stepwise dehydrated and fixed by immersing the tissue into low to high concentrations of alcohol. Thereafter, the tissue was embedded in paraffin and cut into 3 µm thick sections. The sections were stained by hematoxylin and eosin, periodic acid Schiff and Masson’s trichrome.

Fifty glomeruli per section were observed under a light microscope for evaluating
crescentic formation, adhesion of capillary wall to Bowman's capsule and fibrinoid degeneration. Each histopathological parameter was graded normal (0 point), mild (1 point), moderate (2 point) and severe (3 point) according to the extent of alteration. The number of glomeruli corresponding to each score was represented as \( n_0, n_1, n_2 \) and \( n_3 \). Indexes for crescent formation (CI), for adhesion of capillary wall to Bowman's capsule (AI) and for fibrinoid degeneration (FI) were calculated as follows: \( CI, AI \) or \( FI = (1 \times n_1) + (2 \times n_2) + (3 \times n_3) \). Moreover, the index for glomerular lesion (IGL) was calculated as follows:

\[
IGL = \frac{(3 \times CI) + (2 \times AI) + (1 \times FI)}{(3+2+1) \times 50}
\]

We gave 3, 2 and 1 points to CI, AI and FI, respectively, because we consider that CI, AI and FI are associated with the glomerular lesion in this order; and the "50" shows the number of glomeruli.

10. Statistical analysis: All data were presented as the mean±S.D., and significance of difference was determined by analysis of variance, Student's \( t \)-test and Mann-Whitney's \( U \)-test. Inhibitory percentage was calculated as follows:

\[
inhibitory\ percentage\ (%) = \frac{\text{Control}-\text{Test\ drug}}{\text{Control}-\text{Normal}} \times 100
\]

Results

1. General condition

No rats died with Y-19018 during the experiment. A similar amount of rat chow was consumed in both the control and Y-19018 groups. In addition, with Y-19018, we did not observe any rats that showed abnormal behavior and diarrhea.

2. Body weight and urine volume (Table 1)

On day 6, the control and Y-19018 groups already showed lighter body weight than that in the normal group. At the end of the experiment, the Y-19018 group gained slightly less body weight than that of the control group (normal group: 95 g, control group: 92 g, 0.3 mg/kg Y-19018: 89 g, 3.0 mg/kg Y-19018: 84 g). Both control and Y-19018 groups had a similar urine volume throughout the experiment.

3. Effect of Y-19018 on biochemical parameters in urine and plasma

Urinary protein (Fig. 1): Normal rats excreted about 10 mg/day of protein into the urine throughout the experimental period.

![Fig. 1. Effect of Y-19018 on urinary protein content and N-acetyl-β-glucosaminidase activity in crescentic-type anti-GBM nephritis in rats. Y-19018 was given daily p.o. for 39 days. Each plot denotes the mean±S.D. of 8 rats on days 0, 5, 9, 29 and 39, respectively. * indicates a significant difference from each control at \( P<0.05 \).]
Table 1. Changes in body weight and urine volume during administration of Y-19018 in crescentic anti-GBM nephritis in rats

<table>
<thead>
<tr>
<th>Groups</th>
<th>B.W. (g)</th>
<th>U.V. (ml)</th>
<th>1</th>
<th>5</th>
<th>9</th>
<th>19</th>
<th>29</th>
<th>39 Days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>158.9±2.5</td>
<td>11.6±2.1</td>
<td>179.1±3.8</td>
<td>12.2±1.3</td>
<td>186.6±5.0</td>
<td>10.3±1.5</td>
<td>211.8±8.5</td>
<td>12.5±1.6</td>
</tr>
<tr>
<td>Control</td>
<td>158.9±2.5</td>
<td>12.2±2.3*</td>
<td>141.0±6.3###</td>
<td>15.6±4.1</td>
<td>146.7±6.0##</td>
<td>11.8±2.8</td>
<td>174.9±23.6###</td>
<td>13.2±2.2</td>
</tr>
<tr>
<td>Y-19018</td>
<td>158.9±2.5</td>
<td>11.1±2.2</td>
<td>140.1±9.1</td>
<td>15.1±2.4</td>
<td>143.8±11.3</td>
<td>10.8±2.0</td>
<td>166.8±20.4</td>
<td>12.9±2.2</td>
</tr>
<tr>
<td>0.3 mg/kg</td>
<td>158.9±2.5</td>
<td>12.9±2.2</td>
<td>141.8±3.3</td>
<td>13.1±3.9</td>
<td>139.6±10.5</td>
<td>11.9±2.1</td>
<td>165.0±23.9</td>
<td>10.8±1.1*</td>
</tr>
</tbody>
</table>

Numbers indicate the mean±S.D. obtained from 8 rats. * and ### show significant difference from the normal at P<0.05 and P<0.001. * shows a significant difference from the control at P<0.05. B.W.: body weight, U.V.: urine volume.

Table 2. Effect of Y-19018 on blood pressure in crescentic anti-GBM nephritis in rats

<table>
<thead>
<tr>
<th>Groups</th>
<th>6</th>
<th>12</th>
<th>24</th>
<th>30</th>
<th>37</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>127.4±7.4</td>
<td>119.3±4.9</td>
<td>122.3±8.4</td>
<td>116.6±6.3</td>
<td>123.0±8.6</td>
</tr>
<tr>
<td>Control</td>
<td>140.0±13.8*</td>
<td>133.3±7.6*</td>
<td>138.7±3.2###</td>
<td>148.5±9.8###</td>
<td>143.5±12.1##</td>
</tr>
<tr>
<td>Y-19018 0.3mg/kg</td>
<td>139.8±10.2</td>
<td>133.9±5.7</td>
<td>135.4±11.4 (20.1)</td>
<td>137.3±8.9 (35.1)</td>
<td>143.6±7.3</td>
</tr>
<tr>
<td>Y-19018 3.0mg/kg</td>
<td>128.6±14.0 (90.5)</td>
<td>124.7±11.9 (61.4)</td>
<td>130.4±14.8 (50.6)</td>
<td>136.4±11.2 (37.9)</td>
<td>138.6±12.0 (23.9)</td>
</tr>
</tbody>
</table>

Numbers indicate the mean±S.D. obtained from 8 rats. *, ## and ### show significant difference from the normal at P<0.05, P<0.01 and P<0.001. Numbers in parentheses indicate inhibitory percentage: \( C-T \times 100 \) (C: Control, T: Test drug, N: Normal).
Control rats showed severe proteinuria as high as 250 mg/day on day 1 after the i.v. injection of anti-GBM serum. On day 5, proteinuria once diminished to 85.8 mg/day, and then the largest amount of proteinuria was observed on day 19. On the other hand, 3.0 mg/kg Y-19018 resulted in about 30% less proteinuria than that of the control rats on day 19, which was a significant difference. In 0.3 mg/kg Y-19018, the rats showed the same level of proteinuria as that of the control rats.

NAG activity (Fig. 1): In the control group, the NAG activity in the urine was transiently increased on day 5. Y-19018 failed to suppress the increase in the activity at both doses.

Plasma cholesterol (Fig. 2): The content of plasma cholesterol in control rats rapidly increased after the injection of anti-GBM serum, reaching a peak of 247.4 mg/dl on day 15. The administration of Y-19018 at 3 mg/kg significantly decreased plasma cholesterol to about 60% of that of the control group by day 15, and at 0.3 mg/kg, the drug had the tendency to diminish it from day 25.

Plasma urea nitrogen (Fig. 2): The control rats had a higher level of plasma urea nitrogen than that of the normal rats from day 15 after the i.v. injection of anti-GBM serum, and they exhibited a value of 22.2 mg/dl on day 36. However, the rats treated with Y-19018 revealed significantly less content of urea nitrogen on days 25 and 36 at 0.3 mg/kg and on day 36 at 3.0 mg/kg in comparison with those in the control group. The inhibitory percentages varied from 50 to 70%.

4. Effect of Y-19018 on platelet aggregability (Fig. 3)

While platelet aggregability was 7.8±3.1 Ω in the normal group, the control rats showed 12.9±2.6 Ω on day 40, which was about 1.5-fold that of the normal rats. The inhibitory percentage of 3.0 mg/kg Y-19018 was 60.8% (9.8±1.6 Ω).

5. Effect of Y-19018 on blood pressure (Table 2)

In the control group, the blood pressure rose with the passage of time after the induction of nephritis, and it reached 144 mmHg on day 37. The 3.0 mg/kg of Y-19018 group showed 24% inhibition as compared with the control group on day 37, although it was statistically not significant.

6. Effect of Y-19018 on histological alteration of glomeruli (Fig. 4, Photo 1)

In the control group, a crescent was formed in 58.9% of the glomeruli (CI 48.9), adhesion of capillary wall to Bowman's
Fig. 3. Effect of Y-19018 on collagen-induced platelet aggregation in crescentic-type anti-GBM nephritis in rats. Y-19018 was given daily p.o. for 39 days. Each column denotes the mean±S.D. of 8 rats on day 40. * indicates a significant difference from the control at P<0.05. ** shows significant difference from the normal at P<0.05.

capsule in 40.0% (Al 29.0), and fibrinoid necrosis in 19.4% (Fl 13.7). Y-19018 at 0.3 mg/kg significantly suppressed CI by 78.1% and Al by 49.7% as compared with that of the control. However, no effect was observed on Fl. Y-19018 at 3.0 mg/kg markedly protected the glomeruli from these alterations, that is, with an inhibitory percentage of 96.7% in CI, 87.2% in Al and 64.9% in Fl. When these indexes for alterations were transformed into values of the IGL, the control group had an IGL of 0.727; 0.3 mg/kg Y-19018, an IGL of 0.274; and 3 mg/kg Y-19018, an IGL of 0.056. The inhibitory percentage was dose-dependent: 66.0% for 0.3 mg/kg and 92.3% for 3 mg/kg.

Discussion

In the present study, 3.0 mg/kg of Y-19018 significantly suppressed proteinuria on day 19 and notably inhibited glomerular

Fig. 4. Effect of Y-19018 on glomerular histological parameters in crescentic-type anti-GBM nephritis in rats. Y-19018 was given daily p.o. for 39 days. Each column denotes the mean±S.D. of 8 rats on day 40. The number in parenthesis indicates inhibitory percentage which is derived from the following: C-T x 100 (C: Control, T: Test drug, N: Normal). * and *** indicate a significant difference from the control at P<0.05 and 0.001, respectively.

Photo 1. Kidney sections from the control rat (A), Y-19018, 0.3 mg/kg/day, p.o.-treated rat (B) and Y-19018, 3.0 mg/kg/day, p.o.-treated rat (C) on day 40 after injection of anti-GBM serum in crescentic-type anti-GBM nephritis in rats. Y-19018 was given daily p.o. for 39 days. Masson’s trichrome stain (x400). A: the glomerulus is swollen and has a crescent (●) in Bowman’s space, adhesion (▼) of capillary wall and Bowman’s capsule, proliferated mesangium, thickness of capillary wall and hypercellularity. B: Small crescent and adhesion are still present in the glomerulus. C: There are proliferated mesangium and thickness of capillary wall, but no crescent and adhesion in the glomerulus.
alteration on day 40. The rats with Y-19018 showed no abnormality in general aspects including behavior during the experiment, but slightly less body weight than that of the control on day 40.

Mikashima et al. (4) reported that Y-19018 reduced TXB2 generation and increased 6-keto-PGF1α in the serum, and this effect was present until 24 hr after the administration of Y-19018. They showed that this inhibitory effect on TXA synthetase is dose-dependent. Y-19018 is a specific TXA synthetase inhibitor, since Y-19018 inhibits TXA synthetase with an IC50 of 8.2×10⁻⁷ M (4) and does not inhibit cyclooxygenase and PGI synthetase up to 10⁻⁴ M (H. Mikashima, personal communication).

Crescentic-type anti-GBM nephritis was used in this experiment to evaluate the anti-nephritic effect of Y-19018. This model of nephritis is induced by the injection of anti-GBM serum and rabbit r-globulin, and it shows more proteinuria and more severe glomerular alteration than those in the original-type of anti-GBM nephritis (10).

In the present study, hyperaggregability of platelets, was demonstrated in the blood obtained from the nephritic rats on day 40. It is considered that in the heterologous phase, immune complexes are formed on the GBM after the injection of anti-GBM antibody, and then they activate the platelet (11, 12). Furthermore, the exfoliation of endothelial cytoplasm from GBM is induced in this phase of anti-GBM nephritis and the collagen that is one of components in GBM is exposed to the circulation (13). This exposed GBM also stimulates the platelets.

In the autologous phase, nephritic rats produce antibody against anti-GBM antibody and a lot of immune complexes are formed on the GBM (14). In the present nephritic model, the animals were immunized with rabbit r-globulin to enhance the immunity against rabbit anti-GBM antibody. Therefore, it is conceivable that more platelets could be activated in the autologous phase than in the heterologous phase, and activated platelets might cause increasing proteinuria and fibrinoid degeneration and crescent formation in the glomeruli.

Recently, many authors have supported the concept that arachidonic acid-metabolites, especially TXA2, were predominantly associated with irreversible platelet aggregation with the release reaction. Evidence indicates that stimulating platelets could implicate the development of glomerulonephritis (1, 2). Therefore, it seems reasonable to consider that the synthesis of TXA2 in platelets is inhibited by Y-19018 and that if the platelets are stimulated by immune complexes and collagen on GBM during the treatment with Y-19018, no platelets are activated and aggregated. However, Y-19018 dose-dependently inhibited only histological parameters in this experiment. On the other hand, platelet aggregability was suppressed to the same degree at both doses of Y-19018. By the light microscopy, we found no platelet aggregates in the glomerular capillary in the control group on day 40. As mentioned above, Y-19018 decreased TXB2 generation dose-dependently. Therefore, we do not consider that Y-19018 exerts its effect on glomerular alteration through only the inhibition of platelet aggregation.

Lianos and his colleagues (3) demonstrated that TXB2 remarkably increased in the glomeruli 2 hr after the i.v. injection of rabbit nephrotoxic serum into rats, and then this increase was sustained for 14 days. In their experiments, glomerular TXA2 could reduce the glomerular filtration rate and renal plasma flow after the injection of nephrotoxic serum. Therefore, Y-19018 may inhibit synthesis of the glomerular TXA2, and it may improve the micro circulation in the glomerulus and then inhibits the development of glomerulonephritis. Additionally, reduced GFR and RPF were restored by an increase of PGI2 and PGE2 (3). It was demonstrated that PGI2 and PGE2 dilate renal blood vessels (15) and prominently increase renal blood flow (16). PGI2 also more strongly suppressed platelet aggregation than other prostanoids (17). Since Y-19018 increases prostanoids including PGI2 and PGE2 by inhibiting TXA2 synthetase (4), it is likely that Y-19018 may exert its action through not only inhibition of the glomerular TXA2 but also by effects on PGI2 and PGE2.

As TXA2 synthetase inhibitor seems to be
Antinephritic Effect of Y-19018

a very promising antinephritic medicine from these results, experiments to evaluate the antinephritic effect of other TXA₂ synthetase inhibitor are underway.

References


17 Grygowski, R.J., Bunting, S., Moncada, S., Flower, R.J. and Vane, J.R.: Arterial walls are protected against deposition of platelet thrombin by a substance (prostaglandin X) which they make from prostaglandins. Prostaglandins 12, 685–713 (1976)