Antithrombin III Prevents Blood Pressure Elevation and Proteinuria Induced by High Salt Intake in Pregnant Stroke-Prone Spontaneously Hypertensive Rats

Hiroshi Shinyama, Katsumi Yamanaga, Toshiaki Akira, Takeshi Uchida, Masafumi Yaguchi, Masahiro Watanabe, and Yoshio Kagitani


Received November 15, 1995; accepted March 5, 1996

In pregnant stroke-prone spontaneously hypertensive rats, salt-loading causes symptoms similar to those of human preeclampsia, such as hypertension and proteinuria. To seek evidence of the therapeutic potential in preeclampsia of antithrombin III (AT III), which is a serine protease inhibitor active on various enzymes of the coagulation cascade, we examined the effect of consecutive treatment with AT III on hypertension and proteinuria in this animal model. Salt-loading (2% NaCl diet) caused a significant elevation of systolic blood pressure on day 15—17 and of urinary protein excretion on day 17—19 of gestation, as compared with animals fed a normal diet. AT III, administered i.v. at a dose of 60 or 300 U/kg/d for 10 d from day 9—11 to 18—20, attenuated these pathological changes in a dose-dependent manner. Histological examination of the kidney revealed that AT III prevented the occurrence of arteriosclerosis and thickening of the capillary basement membrane. However, the pathological changes induced by salt-loading were not attributable to activation of the blood coagulation system. These results demonstrate that AT III has preventive action against salt-induced hypertension and proteinuria in pregnancy through a mechanism largely independent of its anticoagulant action. AT III may thus be beneficial for the treatment of clinical symptoms of preeclampsia.

Key words antithrombin III; preeclampsia; SHRSP; hypertension; proteinuria; pregnancy

Preeclampsia, still a major cause of maternal and fetal morbidity and mortality in pregnancy, is associated with clinical symptoms such as hypertension, proteinuria and edema. Since its etiology is unclear and apparently heterogeneous, there is no treatment regarded as comprehensively effective. So far, most research into the pathophysiology of preeclampsia has focused on injury to and functional alteration of endothelial cells, and an imbalance between prostacyclin and thromboxane A2 in favor of the latter. Several clinical studies have indicated that preeclampsia is associated with enhanced intravascular coagulation and suppression of the fibrinolytic system. Compared to a normal pregnancy, the level of antithrombin III (AT III), a serine protease inhibitor which inactivates various enzymes of the coagulation cascade, is reduced. The extent of hematological abnormality, especially the decrease in plasma AT III activity, correlates with the severity of maternal morbidity. Accordingly, supplementation of AT III concentrates in preeclamptic patients, especially those with decreased plasma AT III level, may be of benefit, and Terao et al. have indeed shown the effectiveness of such treatment in a small pilot study.

It is, however, difficult to establish a theoretical basis for this treatment, since there is no established animal model of preeclampsia accompanied by or resulting from coagulation abnormality. Recently, Shibukawa et al. reported that pregnant stroke-prone spontaneously hypertensive rats (SHRSP) given water containing 1% NaCl displayed symptoms similar to those of preeclampsia, such as proteinuria, blood pressure elevation and fetal growth retardation. We therefore investigated the present study whether treatment with AT III concentrates would alleviate proteinuria and hypertension induced by high dietary salt intake in pregnant SHRSP. Further, in order to investigate the possible involvement of coagulation abnormality in the pathological alterations produced by a high-salt regime, and to clarify whether the efficacy of AT III can be explained in terms of the alleviation of such changes, a number of biochemical parameters which serve as indices for the state of the coagulation cascade were also monitored.

MATERIALS AND METHODS

Reagents Human AT III was provided by our factory (The Green Cross Corp., Osaka, Japan) at a concentration of 99.9 U/ml, and was diluted with saline when necessary.

Animals 24–28 week-old female SHRSP (Seiwa Experimental Animals Ltd., Fukuoka, Japan) were housed in the same room, with free access to tap water and food (CE-2, Crea, Tokyo, Japan). Animals were housed with fertile males for 1–3 nights, and the first day on which spermatozoa were detected in a vaginal smear was designated day 0 of gestation.

Experimental Protocol The experimental protocol is shown in Fig. 1. Animals confirmed to have mated were individually housed with free access to water and fed either a normal diet (0.3% NaCl) or a high-salt diet (2% NaCl; Funabashi Farm, Chiba, Japan) from day 0 of gestation. Urine was collected three times, for 24 h each time, before mating, on day 8—10, and on day 17—19 of gestation, to measure urine volume and the urinary concentration of protein. Systolic blood pressure (SBP) was measured three times, on day 0, day 6—8 and day

© 1996 Pharmaceutical Society of Japan

* To whom correspondence should be addressed.
15—17 of gestation, with an automatic device (BP-98, Softtron, Tokyo, Japan) using the tail-cuff method. Animals on the high-salt diet were assigned to one of three study groups on day 9—11 of gestation (the day after the second urine collection): a control group receiving saline (3 ml/kg/d) and two groups receiving AT III (60 or 300 U/kg/d). AT III and saline were administered intravenously once daily through the tail vein for 10 d from day 9—11 to day 18—20 (the day after final urine collection) of gestation. On day 18—20, 1 h after the final administration, animals were anesthetized with an intraperitoneal injection of pentobarbital sodium (40 mg/kg). An abdominal midline incision was made and arterial blood was withdrawn into a syringe containing sodium citrate (final concentration, 3.8 mg/ml). Animals found not to be pregnant were excluded from the final data. After measuring platelet counts, blood was centrifuged at 5000 rpm for 10 min at 4°C, and plasma was separated and stored at −40°C until the assay of biochemical parameters described below. The kidneys were then removed and fixed for at least 5 d in 10% formalin in phosphate-buffered saline (pH 7.3). Transversal slices of the kidneys were embedded in paraffin, and 3 μm-thick sections were stained with hematoxylin and eosin (HE), periodic acid-Schiff (PAS) or phosphotungstic acid hematoxylin (PTAH). The histological sections were evaluated microscopically for lesions in a blind manner by an observer, and the severity of the lesions was graded from 0 to 4 according to abnormalities, with a numerical value (in parenthesis) allocated for statistical analysis, as follows: no change (0); very slight (1); slight (2); moderate (3); marked (4).

Biochemical Parameters Urinary protein concentration was measured using the Bio-Rad Protein Assay (Bio-Rad, Hercules, CA, U.S.A.). AT III activity was measured by its amidolytic activity using the chromogenic substrate S-2238 and was expressed as a percentage of human control plasma (Daiichi Kagaku, Tokyo, Japan). Thrombin-AT III complex (Teijin, Tokyo, Japan) and D-dimer fibrin degradation products (D-dimer FDP, Boehringer Mannheim-Yamanouchi, Tokyo, Japan) were assayed by EIA using a test kit. Platelet count was calculated with a microcellcounter (Sysmex F-800, Toa Medical Electronics, Kobe, Japan).

Statistical Analysis Student's t-test was used for the analysis of values between the high-salt groups and the normal diet group. Analysis of variance (ANOVA) followed by Dunnett's method for multiple comparison was used for statistical analysis among the high-salt groups. The Kruskal–Wallis rank test, followed by Mann–Whitney's U test, was used for statistical analysis for histological lesions. The significant difference was set at p levels of less than 5%. All data are expressed as the mean ± S.E.M.

RESULTS

Changes in Blood Pressure Changes in SBP are shown in Fig. 2. There were no significant differences in SBP between the 3 high-salt groups on either day 0 or day 6—8 of gestation. In the saline-treated animals fed a high-salt diet, SBP was significantly higher (by 22 mmHg, p < 0.01) on day 15—17 of gestation than in animals fed a normal diet. AT III tended to attenuate (60 U/kg/d) or significantly attenuate (300 U/kg/d; p < 0.05) this elevation of blood pressure.

Changes in Urine Volume and Urinary Protein Concentration Changes in urine volume (UV) and urinary protein excretion (Uprotein V) over the days of gestation are shown in Fig. 3. UV as measured before conception and on days 8—10 and 17—19 of gestation revealed no significant differences between any of the groups. However, compared with animals fed a normal diet, Uprotein V was significantly elevated (p < 0.05) in the salt-loaded groups, proportionately to the number of
days of treatment. AT III tended to suppress (60 U/kg/d) or significantly suppressed (300 U/kg/d) the increase in $U_{\text{protein}}$.

**Histopathological Analysis** Figure 4 shows typical histological views of the kidney of saline-treated and AT III- (300 U/kg/d) treated animals fed the high-salt diet; Table 1 records the severity of pathological changes in individual animals. In the saline-treated animals (Fig. 4A), proliferative arteritis and typical features of arteriosclerosis were observed in small calibration arteries, especially in arterioles in which the appearance of glomeruli was relatively normal, although such lesions were also found

![Histological Views of Kidneys](image)

**Table 1.** Histological Determination of Kidneys of Pregnant Stroke-Prone Spontaneously Hypertensive Rats (SHRSP) Fed High-Salt or Normal Diet

<table>
<thead>
<tr>
<th>Items</th>
<th>Normal diet</th>
<th>Saline</th>
<th>Saline</th>
<th>AT III 60 U/kg/d</th>
<th>AT III 300 U/kg/d</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grade</td>
<td>0 1 2 3 4</td>
<td>0 1 2 3 4</td>
<td>0 1 2 3 4</td>
<td>0 1 2 3 4</td>
<td>0 1 2 3 4</td>
</tr>
<tr>
<td>Arteriosclerosis</td>
<td>4*</td>
<td>0 1 2 1 1</td>
<td>0 1 3 4 1</td>
<td>3 1 2 0 2</td>
<td>8 1 2 0 0</td>
</tr>
<tr>
<td>Fibrin deposition in vessels</td>
<td>5 1 1 1 0</td>
<td>8 0 0 1 0</td>
<td>8 0 0 0 0</td>
<td>9 2 0 0 0</td>
<td>9 2 0 0 0</td>
</tr>
<tr>
<td>Glomerulosclerosis</td>
<td>6 0 0 2 0</td>
<td>4 1 1 1 2</td>
<td>5 0 1 2 0</td>
<td>5 2 4 0 0</td>
<td>5 2 4 0 0</td>
</tr>
<tr>
<td>Thickening of glomerular</td>
<td>7 0 1 0 0</td>
<td>3 0 5 1 0</td>
<td>7 0 1 0 0</td>
<td>8 1 2 0 0</td>
<td>8 1 2 0 0</td>
</tr>
<tr>
<td>basement membrane</td>
<td></td>
<td></td>
<td></td>
<td>$p = 0.027^{*}$</td>
<td>$p = 0.027^{*}$</td>
</tr>
<tr>
<td>Fibrin deposition in glomeruli</td>
<td>7 0 0 1 0</td>
<td>8 0 0 1 0</td>
<td>5 2 0 1 0</td>
<td>9 2 0 0 0</td>
<td>9 2 0 0 0</td>
</tr>
</tbody>
</table>

0, no change; 1, very slight; 2, slight; 3, moderate; 4, marked; $p$: values calculated by $H$-test followed by $U$-test. a) Number of animals. b) Comparison with saline-treated animals fed normal diet ($U$-test). c) Comparison with saline-treated animals fed high-salt diet.
### Table 2. Effect of Consecutive Treatment with Antithrombin III (AT III) on Plasma AT III Activity, Thrombin-ACT III (TAT) Complex, D-Dimer FDP and Platelet Counts in Pregnant SHRSP on Day 18–20 of Gestation

<table>
<thead>
<tr>
<th>Treatment dose (U/kg/d)</th>
<th>n</th>
<th>AT III activity (%)</th>
<th>TAT complex (ng/ml)</th>
<th>D-Dimer FDP (ng/ml)</th>
<th>Platelet counts (× 10⁹/µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal diet</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline</td>
<td>8</td>
<td>106 ± 1</td>
<td>2.8 ± 0.6</td>
<td>9.0 ± 0.2</td>
<td>82.6 ± 11.1</td>
</tr>
<tr>
<td>High salt diet</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline</td>
<td>9</td>
<td>113 ± 2*</td>
<td>1.3 ± 0.2*</td>
<td>7.8 ± 0.4**</td>
<td>74.6 ± 10.0</td>
</tr>
<tr>
<td>AT III 60</td>
<td>8</td>
<td>196 ± 4¹</td>
<td>4.0 ± 0.7</td>
<td>6.7 ± 0.3</td>
<td>96.9 ± 14.2</td>
</tr>
<tr>
<td>AT III 300</td>
<td>11</td>
<td>492 ± 11¹</td>
<td>13.2 ± 2.4¹</td>
<td>7.5 ± 0.4</td>
<td>80.8 ± 6.8</td>
</tr>
</tbody>
</table>

Values represent means ± S.E.M.; *p < 0.05; **p < 0.01 vs. saline-treated pregnant rats fed normal diet (Student’s t-test); ¹p < 0.01 vs. saline-treated pregnant rats fed high salt diet (Dunnett’s test); n: number of animals; arterial blood obtained 1 h after final drug or saline administration.

Although its precise mechanism is not fully defined, it is well documented that high salt intake causes blood pressure elevation with an increase in total peripheral resistance in humans and experimental hypertensive animals. The ability to excrete sodium is reduced in SHRSP, compared with normotensive Wistar Kyoto rats, so that salt-loading accelerates the development of hypertension, resulting in functional and structural deterioration of the kidney. Substantial data has been gathered as to the mechanism of such salt-induced pathological changes which involve structural and functional changes of the vascular bed, altered regulation of the baroreflex and renal dopaminergic systems, and altered ionic homeostasis across the cell membrane of vascular smooth muscle cells, all leading to a rise in systemic vascular resistance. Since the clinical symptoms of preeclampsia are peculiar to pregnancy and disappear within a few weeks after labor, the pathogenesis of hypertension and renal dysfunction in this condition appears to be different from that in salt-induced alterations. However, the mechanisms involved may have common features, since preeclampsia is also marked by abnormal sodium retention.

Changes in Blood Coagulation Parameters

Changes in plasma AT III activity, thrombin-AT III complex, D-dimer FDP, and platelet counts are shown in Table 2. Salt-loading caused a slight but significant increase in plasma AT III activity (p < 0.01), and decreases in levels of D-dimer FDP (p < 0.01) and thrombin-AT III complexes (p < 0.05). AT III treatment at doses of 60 and 300 U/kg/d caused respective elevations of AT III activity to 1.7-fold (p < 0.01) and 4.4-fold (p < 0.01) that of saline-treated animals, while thrombin-AT III complex levels reached relative elevations of 3.1-fold and 10.1-fold, respectively (p < 0.01). Platelet counts were not influenced significantly by either salt-loading or treatment with AT III.

DISCUSSION

Several clinical studies have indicated that preeclampsia is associated with chronic intravascular coagulation. Since the severity of clinical symptoms is correlated with the reduction in plasma AT III activity, some researchers have tried to restore activity with AT III concentrate and have obtained good clinical outcomes. However, there have been few basic studies offering a theoretical basis for AT III supplementation. Moreover, although its physiological role in the hemostatic system is well documented, little data is available to the physiological action of AT III on the cardiovascular system. In the present study, we describe how dietary salt-loading causes blood pressure elevation and proteinuria in pregnant SHRSP, as also reported by Shibukawa et al., and we demonstrate that AT III has preventative effects against salt-induced rises in blood pressure and renal injury in pregnancy.
In SHRSP, high salt intake causes severe histopathological changes such as fibrinoid necrosis and proliferative arteritis of the renal arterioles, as well as fibrin thrombi and sclerotic changes in the glomeruli.\(^3,\(^4\)\) Histological analysis in the present study indicated that the ability of AT III treatment to protect against the pathological changes could be related primarily to a vascular protective effect against arteriosclerosis, since AT III at 300 U/kg/d markedly reduced the incidence and severity of arteriosclerosis. This finding may imply that some direct action of AT III on the renal vasculature contributes largely to the protective effect. It has been reported that the pathogenesis of arteriosclerosis involves increased arteriolar endothelial permeability and leakage of plasma protein into the vascular wall.\(^3,\(^7\)\) This leads to the local conversion of fibrinogen to fibrin, resulting in necrosis of the vessel wall.\(^3\) AT III might therefore be thought to inhibit the conversion of fibrinogen to fibrin in the vascular wall, thus preventing necrosis of the renal arterioles. However, such a theory is not supported by the varying incidence, in salt-loaded animals, of arteriosclerosis and fibrin deposition in the vessels, both of which are salt-induced phenomena. It therefore appears that, in the present study, the protective effects of AT III against functional and structural impairment induced by salt-loading cannot be accounted for in terms of fibrin-related vascular changes in renal arterioles. To clarify the mechanism of AT III’s preventive effect against salt-induced renal injury, further studies are required.

In conclusion, we demonstrated in this study that AT III mitigates the development of salt-induced hypertension and proteinuria as well as the histological deterioration of the kidneys. Although the extent of the pathological similarity between human preeclampsia and our experimental model is unclear, our present data give evidence of the efficacy of AT III against hypertension and its complications, including renal dysfunction, both also major clinical symptoms of human preeclampsia, and suggest that this may be attributable in part to some mechanism independent of its anticoagulant action.

REFERENCES