Lack of Evidence for the Participation of Tonin in the Pathogenesis of One-Kidney, One-Clip Renovascular Hypertension

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SUMMARY. It has been reported that immunization against tonin normalizes blood pressure, and that sialoadenectomy, during which the tonin-rich salivary glands are removed, decreases blood pressure in one-kidney, one-clip hypertension. To investigate the role of tonin on this form of hypertension further, we actively immunized one-kidney, one-clip hypertensive rabbits with tonin and measured both the blood pressure response and the titer of antibodies raised against tonin. In addition, because sialoadenectomy may alter food intake, we assessed the effect of sialoadenectomy on the blood pressure of one-kidney, one-clip hypertensive rats fed a liquid diet to facilitate eating. After immunization, all rabbits developed antitonin-antibody titers ranging from 1:300 to 1:56,000. However, in none of the rabbits did the blood pressure decrease significantly (114 ± 3 mm Hg before immunization; 129 ± 6 mm Hg at 16 weeks after immunization). In one-kidney, one-clip hypertensive rats, sialoadenectomy did not lower blood pressure (179 ± 5 mm Hg before sialoadenectomy; 202 ± 9 mm Hg 3 weeks after sialoadenectomy). Neither blood pressure nor body weight differed between sialoadenectomy and sham-sialoadenectomy one-kidney, one-clip hypertensive rats (n = 6). In conclusion, neither active immunization against tonin in one-kidney, one-clip hypertensive rabbits nor sialoadenectomy in one-kidney, one-clip hypertensive rats significantly reduced established hypertension. These results do not support the hypothesis that tonin is involved in the pathogenesis of one-kidney, one-clip hypertension in these animal models. (Circ Res 55: 580–584, 1984)

TONIN is a serine protease that releases angiotensin II directly from natural renin substrate (angiotensinogen), from the synthetic tetradecapeptide renin substrate, and from angiotensin I. Although it is found in various rat tissues, it exists in particularly high concentrations in the submandibular glands (Boucher et al., 1972, 1974). Recently, several investigators have suggested that tonin may be involved in the pathogenesis of one-kidney, one-clip renovascular hypertension (lk,lc hypertension) (Garcia et al., 1978, 1979; Cheng et al., 1982). Garcia et al. (1978) reported that a single intravenous administration of tonin antiserum restored blood pressure (BP) to normal in seven of 10 rats with lk,lc hypertension, although decreases in BP were transient. In rabbits with lk,lc hypertension, active immunization against tonin normalized BP in those rabbits which developed antitonin antibodies, whereas no BP change occurred in rabbits that did not produce antibodies (Garcia et al., 1979). In this study, however, the decreases in BP were observed before the antibody titer was detected, and no correlation was found between decreases in BP and elevations of antitonin-antibody titer. In addition, Cheng et al. (1982) reported that sialoadenectomy (Sx) in lk,lc hypertensive rats caused a substantial lowering of BP, which they attributed to the elimination of the tonin-rich submandibular gland. However, they did not report the rats’ body weight, which, secondary to Sx, would be affected by a decrease in food intake (Plagge, 1938), and which, in turn, could affect BP. Nevertheless, these studies suggested an important role of tonin in the pathogenesis of lk,lc hypertension.

Therefore, to investigate further the mechanism by which active immunization with tonin normalized BP in lk,lc hypertensive rabbits, we attempted to duplicate those studies (Garcia et al., 1979). However, even though our rabbits produced higher antitonin-antibody titers than were reported by Garcia et al. (1979), we observed no significant changes in BP. Further, we investigated whether Sx would affect BP in lk,lc hypertensive rats when the steady increase in body weight after Sx was maintained by feeding the rats a liquid diet. We also observed no significant antihypertensive effect.

Methods

Active Immunization with Tonin in lk,lc Hypertensive Rabbits

Male, New Zealand white rabbits (2–2.5 kg) were housed in individual cages in a temperature-controlled room at 22°C with a 12-hour light/dark cycle. The rabbits
were maintained on standard rabbit chow (Ralston Purina Co.) and allowed free access to tap water.

After a control period of 3–4 weeks, the right kidney was removed through a flank incision. Two weeks later, the left renal artery was constricted with a round-shaped, silver clip having an internal diameter of 1.2 mm. Body weight of the rabbits at the time of clipping ranged from 2.2 to 3.1 kg. All surgical procedures were performed under ketamine hydrochloride (50 mg/kg, im) and halothane inhalation anesthesia.

**Measurement of BP**

The rabbits were placed in an incubator (37°C), and xylene was applied at the tip of the ear to dilate left arteries. Blood pressure was measured indirectly by the left central ear artery while the rabbits were in a conscious resting state, using a modification of the Grant-Roschild capsule, as described by Fujii and Yazaki (1972). The average of five readings was recorded. The animals were considered hypertensive when BP was consistently at least 25 mm Hg higher than during the control period.

**Active Immunization with Tonin in Rabbits**

Rat tonin was purified from the rat submandibular gland by ionic exchange and gel filtration chromatography, as previously described (Hayashi et al., 1981). Specific activity of purified rat tonin was 1700 nmol/His-Leu/mg per min.

A single dose of 50 μg of purified rat tonin emulsified in Freund's complete adjuvant was injected subcutaneously at several sites in rabbits with hypertension of 3–5 weeks duration, as described by Garcia et al. (1979). The rabbits that did not raise sufficient antibodies by a single injection of tonin received booster injections of 50 μg of tonin with Freund's incomplete adjuvant 2 or 3 months later. Arterial blood samples were taken every 2 weeks to determine plasma renin activity (PRA), hematocrit, and serum antitonin titer.

**Effect of Sx on BP of Rats with lk,lc Hypertension**

Experiments were performed on male Wistar rats (175–200 g) placed in individual cages. To avoid the effect of Sx on the amount of food intake, since it could affect body weight and, subsequently, BP, the rats were maintained on a liquid diet (AIN-76 Semipurified, ICN Nutritional Biochemicals) and on tap water ad libitum throughout the experiment.

After an adaptation period of 5 days, the rats underwent a right nephrectomy, and the left renal artery was clipped with a U-shaped silver clip of 0.23 mm internal gap. Ether anesthesia was used during the surgery.

A right nephrectomy, and the left renal artery was constricted with a round-shaped, silver clip of 0.23 mm internal gap. Ether anesthesia was used during the surgery.

Under ketamine hydrochloride (50 mg/kg, im) and halothane inhalation anesthesia.

Four weeks after surgery, hypertensive rats were divided into two groups: group 1 (n = 9), Sx, and group 2 (n = 6), sham operation. Sialoadenectomy was performed under ether anesthesia, and both submandibular glands were removed from the rats, each with its attached sublingual glands. Blood pressure and body weight were measured every week. Blood pressure was measured by the tail cuff method, and animals were considered hypertensive when their systolic BP exceeded 140 mm Hg.

**Analytical Procedures**

PRA was measured by a radioimmunoassay (RIA) using a modification of the RIA of Haber et al. (1969), as previously described (Carretero et al., 1973). Hematocrit was determined in a Readacrit centrifuge (Clay-Adams), according to the manufacturer's recommendations. Serum antitonin was measured by RIA using a modification of the RIA of Gutkowska et al. (1978). The assay was performed in polyethylene tubes with 0.1 M Tris-HCl, pH 7.4, containing 0.1% BSA as a buffer. One hundred microliters of 125I-tonin with approximately 5000 counts/min and 100 μl of antiserum at four different dilutions with the assay buffer (1:500, 1:1000; 1:5000; 1:10,000 dilutions, respectively) were transferred into tubes. The final volume was adjusted to 500 μl with the assay buffer. After 18 hours of incubation at 4°C, 100 μl of a 1:50 dilution of sheep anti-rabbit γ-globulin were added to each tube to separate free from antibody-bound 125I-tonin. After the tubes were incubated for another 24 hours at 4°C, they were centrifuged at 1700 g for 45 minutes, and the precipitate was counted in a γ-counter (Micromedic 4/600, Micromedic Systems). Unspecific binding was simultaneously determined using normal rabbit serum and subtracted from the binding obtained with immunized rabbits. Under this condition, tonin antiserum titers were expressed as the dilution at which 50% binding of the labeled tonin was observed.

All results were expressed as mean ± SEM. Statistical evaluations were performed by Student's paired and unpaired t-tests. A P value of less than 0.05 was considered statistically significant.

**Results**

**Study I: Active Immunization with Tonin in Rabbits**

Twelve rabbits developed low renin lk,lc hypertension. Blood pressure increased from 72 ± 2 to 114 ± 3 mm Hg (Δ42 ± 3 mm Hg; P < 0.001), and PRA decreased from 5.8 ± 0.5 to 1.2 ± 0.3 ng/ml per hr (Δ4.6 ± 0.5 ng/ml per hr; P < 0.001) by 4–10 weeks after clipping (before immunization). Hematocrit did not change significantly (41 ± 1 and 42 ± 1%).

Subsequent active immunization against tonin produced antitonin-antibody titers ranging from 1:300 to 1:56,000. However, none of the rabbits showed a significant decrease in BP. Individual values of BP and antitonin-antibody titers are shown in Table 1. Regardless of the levels of antibody titers, the rabbits still remained hypertensive at 16 weeks after immunization (129 ± 6 mm Hg). There was no relationship between changes in BP and the titer of antibodies achieved. Figure 1 shows the BP and antitonin-antibody titer in rabbit no. 1, which developed the highest titer. The antitonin-antibody titer started to rise 6 weeks after the tonin injections and increased to a titer of 1:56,000. However, BP still remained unchanged or increased further at 20 weeks after tonin injections.

**Study II: Sx in Rats**

Figure 2 depicts the courses of BP in both group 1 (lk,lc hypertension and Sx) and group 2 (lk,lc hypertension and sham operations). Sialoadenectomy did not lower BP in lk,lc hypertensive rats (179
TABLE 1

<table>
<thead>
<tr>
<th>Rabbit no.</th>
<th>BP before clipping</th>
<th>BP after clipping</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>80</td>
<td>124</td>
</tr>
<tr>
<td>2</td>
<td>72</td>
<td>126</td>
</tr>
<tr>
<td>3</td>
<td>85</td>
<td>126</td>
</tr>
<tr>
<td>4</td>
<td>68</td>
<td>96</td>
</tr>
<tr>
<td>5</td>
<td>74</td>
<td>120</td>
</tr>
<tr>
<td>6</td>
<td>70</td>
<td>112</td>
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<tr>
<td>7</td>
<td>72</td>
<td>119</td>
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<td>8</td>
<td>69</td>
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</tr>
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<td>9</td>
<td>70</td>
<td>106</td>
</tr>
<tr>
<td>10</td>
<td>71</td>
<td>102</td>
</tr>
<tr>
<td>11</td>
<td>63</td>
<td>102</td>
</tr>
<tr>
<td>12</td>
<td>68</td>
<td>132</td>
</tr>
</tbody>
</table>

Blood Pressure (mm Hg) and Antibody Titers in Rabbits before and after Active Immunization against Tonin

<table>
<thead>
<tr>
<th>Rabbit no.</th>
<th>4 Weeks</th>
<th>8 Weeks</th>
<th>12 Weeks</th>
<th>16 Weeks</th>
<th>20 Weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>119 (&lt;1:1)</td>
<td>120 (1:1,500)</td>
<td>120 (1:32,000)</td>
<td>132 (1:52,000)</td>
<td>138 (1:56,000)</td>
</tr>
<tr>
<td>2</td>
<td>137 (&lt;1:1)</td>
<td>150* (&lt;1:1)</td>
<td>162 (1:290)</td>
<td>162 (1:300)</td>
<td>Died</td>
</tr>
<tr>
<td>3</td>
<td>132 (&lt;1:460)</td>
<td>127* (1:2,250)</td>
<td>124 (1:4,600)</td>
<td>134 (1:8,200)</td>
<td>128 (1:4,500)</td>
</tr>
<tr>
<td>4</td>
<td>102 (&lt;1:1)</td>
<td>105* (&lt;1:1)</td>
<td>107 (1:3,400)</td>
<td>116 (1:1,750)</td>
<td>114 (1:2,100)</td>
</tr>
<tr>
<td>5</td>
<td>121 (&lt;1:1)</td>
<td>117* (1:240)</td>
<td>118 (1:500)</td>
<td>125 (1:900)</td>
<td>128 (1:1,600)</td>
</tr>
<tr>
<td>6</td>
<td>114 (&lt;1:1)</td>
<td>121* (&lt;1:1)</td>
<td>130 (1:620)</td>
<td>131 (1:890)</td>
<td>132 (1:2,100)</td>
</tr>
<tr>
<td>7</td>
<td>132 (&lt;1:1)</td>
<td>138 (1:320)</td>
<td>135* (1:760)</td>
<td>135 (1:4,000)</td>
<td>Died</td>
</tr>
<tr>
<td>8</td>
<td>110 (1:60)</td>
<td>116 (1:600)</td>
<td>115* (1:3,000)</td>
<td>123 (1:3,800)</td>
<td>126 (1:3,600)</td>
</tr>
<tr>
<td>9</td>
<td>104 (1:1)</td>
<td>104 (1:70)</td>
<td>114* (1:1,000)</td>
<td>116 (1:1,100)</td>
<td>116 (1:900)</td>
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<tr>
<td>10</td>
<td>104 (&lt;1:1)</td>
<td>105* (1:1,400)</td>
<td>105 (1:2,100)</td>
<td>104 (1:2,200)</td>
<td>104 (1:2,000)</td>
</tr>
<tr>
<td>11</td>
<td>100 (&lt;1:1)</td>
<td>104 (1:940)</td>
<td>102* (1:3,700)</td>
<td>104 (1:4,100)</td>
<td>104 (1:4,000)</td>
</tr>
<tr>
<td>12</td>
<td>140 (&lt;1:1)</td>
<td>148 (&lt;1:1)</td>
<td>164* (1:400)</td>
<td>169 (1:400)</td>
<td>176 (1:300)</td>
</tr>
</tbody>
</table>

Ab titers are shown in parentheses and expressed as the dilution at which 50% binding of labeled tonin was observed.

* Indicates the booster injections of tonin.

± 5 mm Hg before Sx; 202 ± 9 mm Hg 3 weeks after Sx). Throughout the entire experiment, the BP in group 1 did not differ from that in group 2.

Body weight between groups 1 and 2 did not differ from each other (Fig. 3). Although the increase in body weight was transiently blunted after Sx or sham operation, the rats steadily gained weight in both groups.

Discussion

In the present experiments, neither active immunization against tonin in lk,lc hypertensive rabbits nor Sx in lk,lc hypertensive rats was able to decrease BP significantly. Consequently, these results do not confirm the results of previous studies in other laboratories (Garcia et al., 1978; Cheng et al., 1982).

Garcia et al. (1978, 1979) showed that both passive and active immunization against tonin decreased BP to normal in lk,lc hypertensive animals. They also reported that tonin potentiated the vasoconstrictor response to norepinephrine, especially in lk,lc hypertension (Garcia et al., 1981a), and suggested that tonin could be involved in the pathogenesis of lk,lc hypertension through the mechanisms that might partially be explained by the vascular action of tonin. However, our results do not support the hypothesis that tonin plays a role in the pathogenesis of lk,lc hypertension.

Our hypertensive rabbits have lk,lc hypertension with low renin. Control BP which was indirectly measured on a central ear artery, was slightly higher than indirect control BP reported by Romero et al. (1973) and as slightly lower than the values of Fujii and Yazaki (1972). On the other hand, control BP, which was also measured indirectly on the central ear artery by Garcia et al., showed extremely low values compared to our values and to direct BP (Johnson et al., 1975). It has been suggested that ear vessels of rabbits are strongly innervated by sympathetic nerves and that values of BP are changed easily by the tone of the sympathetic nerve (Kawaguchi, 1931). To minimize the effects of sympathetic nerves on ear vessels, we dilated ear vessels by warming the rabbit in an incubator and by applying xylenes at the tip of the ear. Because Garcia et al. measured indirect BP at room temperature, their BP may have been influenced by sympathetic tone and may not reflect the true level of arterial pressure. However, this point could not explain the discrepancy, because the BP of their rabbits normalized only when antitonin-antibodies developed.

![Figure 1](http://circres.ahajournals.org/)

**Figure 1.** Time course of blood pressure and antitonin-antibody titers in alk,lc hypertensive rabbit (No. 1). Nx = right nephrectomy, Clip = left renal artery clipping.
We expressed antitonin-antibody titer as the dilution at which 50% binding of labeled tonin was observed, whereas Garcia et al. expressed it as the percentage of bound labeled tonin with serum at 1:1,000 dilution. Their titers ranged from 5% to 57%.

All rabbits in the present study produced detectable antibodies titer. Although the levels of antibodies titer are affected partly by the specific activity of labeled tonin, 10 out of 12 rabbits appeared to have higher antitonin-antibody titers than those reported by Garcia et al. All rabbits in the present study produced detectable antibodies titer. Although the levels of antibodies titer are affected partly by the specific activity of labeled tonin, 10 out of 12 rabbits appeared to have higher antitonin-antibody titers than those reported by Garcia et al. (1979). These authors reported that all rabbits that developed antibodies against tonin normalized their BP. In contrast, in the present study, all rabbits remained hypertensive, and no relationship was observed between BP and titer of antibodies. A difference between our protocol and the one used by Garcia et al. is that we used booster injections of tonin to increase antibody titers. However, it is unlikely that discrepant results could be explained by a qualitative difference in the antibodies related to the number of immunizations used, since the rabbits that developed significant antibody titer before the booster injections (numbers 1, 3, 7, 8, and 11) had no change in BP.

In contrast to our results, Garcia et al. (1979) showed that production of antitonin-antibodies as low as 5% binding by serum at 1:1,000 dilution normalized BP of lkglc hypertensive rabbits. However, they found no correlation between antibody titer and decreased BP, and there was a discrepancy in time between reduction of BP and detection of antibodies. Thus, it is possible that the observed decrease in BP was not directly related to the presence of antitonin antibodies.

Cheng et al. (1982) showed that removal of the submandibular gland (Sx), which contains tonin at very high concentrations, reduced BP in over half of their lkglc hypertensive rats, and they suggested a partial role of the submandibular gland, including tonin, in the pathogenesis of lkglc hypertension. However, we could not find decreases in BP of lkglc hypertensive rats after Sx was performed.

Sialoadenectomy has been reported to impair growth, especially in young rats (Plagge, 1938; Bixler et al., 1955; Shaw and Wolman, 1958; Haldi and Wynn, 1963). Plagge (1938) and Bixler et al. (1955) suggested that this impairment might be attributed to a low food intake associated with some mechanical factors in the feeding process caused by Sx. However, Haldi and Wynn (1963) showed that, even when animals were fed an identical amount of food by a stomach tube, sialoadenectomized rats gained less weight than their controls. Sialoadenectomized rats also showed various changes in their adrenals, testes, and uteri (Bixler et al.) and widespread changes in oxidative metabolism of different tissues (Plaza et al., 1979).

In older rats, impairment of growth by Sx was less pronounced when observed over long-term periods (Plaza et al., 1979). However, Sx could impair the amount of food intake because of surgical stress and mechanical factors, such as pain, physical effects of sutures, and reduced salivary excretion, even in older rats, until they recover fully from surgical procedures and until compensatory excretion of saliva by other salivary glands has occurred. This effect could alter body weight and, subsequently, BP.

In fact, Cheng et al. (1982) reported that all hypertensive rats that underwent Sx showed initial decreases in BP halfway to normal pressure. Thereafter, BP returned to presialoadenectomized levels in about 40% of the animals within 15 days, whereas it remained at that low level in the rest of the rats. Unfortunately, these investigators did not report the changes in the body weight of those rats.

In our experiment, we maintained the rats on a liquid diet throughout the experiment to facilitate eating. We could keep the rats healthy and maintain steady increases in body weight, although the increase in body weight was slightly blunted after Sx.

![Figure 2: Blood pressure of rats throughout the experiment. Abbreviations are the same as in Figure 2.](http://circres.ahajournals.org/)

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Under this condition, Sx did not lower the BP in lk,lc hypertensive rats.

In conclusion, neither active immunization against tonin in lk,lc hypertensive rabbits nor Sx in lk,lc hypertensive rats resulted in a decrease in BP. These results do not support the hypothesis that tonin is involved in the pathogenesis of lk,lc hypertension in these animal models.

This study was supported by National Institutes of Health Grants HL 21092 and HL 28982.

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Received March 7, 1984; accepted for publication August 6, 1984.

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INDEX TERMS: Tonin • Renal vascular hypertension • One-kidney, one-clip hypertension • Pathogenesis of hypertension • Plasma renin activity • Sialoadenectomy
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Circ Res. 1984;55:580-584
doi: 10.1161/01.RES.55.5.580

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