Effects of Tonin on the Adrenal Secretion in the Rat

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SUMMARY Tonin infused intravenously at a rate of 12 \( \mu g/kg/min \) for 2 hours stimulated aldosterone and corticosterone secretion in conscious rats. In pentobarbital-anesthetized rats treated with dexamethasone and morphine, tonin stimulated aldosterone secretion, but corticosterone was unaffected. Tonin did not stimulate steroidogenesis in isolated rat adrenal glomerulosa cells unless substrate, in the form of "stripped" rat plasma, was added, in which case large amounts of immunoreactive angiotensin II were generated. These results suggest that tonin may generate an angiotensin peptide in vivo, which in turn stimulates aldosterone secretion. ACTH is secreted in response to tonin infusion, and contributes to the adrenal response.

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KEY WORDS • tonin • angiotensin II • ACTH • aldosterone • adrenal

TONIN is an enzyme of the serine protease family\(^1\) found in large amounts in free form in the rat submaxillary gland from which it has been purified to homogeneity\(^2\) and crystallized.\(^3\) It is a polypeptide containing 272 amino acids, with molecular weight (MW) of 28,700.\(^4\) N-terminal sequence analysis has permitted the identification of 34 of the first 40 residues and revealed extensive homology with the sequence of many serine proteases of the trypsin-chymotrypsin family. Tonin acts on angiotensinogen, the Skeggs tetradecapeptide, or angiotensin I (AI) to generate angiotensin II (AII) directly\(^1\) and forms AII from des-Asp\(^{1}\)-AI.\(^5\) It cleaves substance P,\(^6\) and produces peptide fragments from \( \beta \)-lipotropin (including an opioid-like material), adrenocorticotropic, and proopiomedanocortin.\(^7\) Tonin is secreted into the venous effluent of the submaxillary gland and is bound in plasma to a powerful circulating inhibitor,\(^8\) at present identified as \( \alpha_{1} \)-macroglobulin.\(^9\)

Since tonin produces AII, which is a potent stimulant of aldosterone secretion,\(^10\) we infused it into conscious and anesthetized rats and studied its action on isolated rat adrenal cells to determine whether it would affect adrenal secretion.

Materials and Methods

In Vivo Experiments

Male Sprague-Dawley rats weighing about 300 g were kept in individual cages and exposed to light from 6 am to 6 pm at a temperature of 22°C and relative humidity of 60%. They were fed normal rat chow containing 152 mmoles/kg of sodium (Purina, Ralston, Indiana) and had access to water ad libitum. Under pentobarbital anesthesia (Nembutal, Abbott) (60 mg/kg intraperitoneally), a PE-20 polyethylene catheter filled with 0.9% NaCl containing 100 U/ml of heparin (Sodium Heparin, Abbott) was placed in the femoral vein, passed under the skin, and brought out at the scruff of the neck. After 48 hours, the conscious rats were studied between 0900 and 1200 hours. Special care was taken to avoid stress in handling the animals. Rats weighing less than before surgery were not used. 0.9% NaCl or tonin purified according to Demassieux et al.\(^1\) and dissolved in 0.9% NaCl were infused in a total volume of 0.75 ml over 2 hours at a rate of 12 \( \mu g \) of tonin/kg/min with a Harvard pump model 600-950 (Harvard Apparatus Company,
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Blood was immediately centrifuged at 4°C and plasma was separated and stored at -20°C until assayed.

To block the secretion of ACTH, anesthetized rats treated with dexamethasone and morphine were studied. A group of intact rats was anesthetized with pentobarbital (40 mg/kg intraperitoneally) and 10 minutes later they were injected with morphine sulphate (12.5 mg/kg intramuscularly). A PE-20 catheter was inserted into the femoral vein. The rats were infused with 0.9% NaCl or tonin (12 μg/kg/min) in 0.9% NaCl for 2 hours. At the end of the experiment, blood was withdrawn from the abdominal aorta for measurement of aldosterone, corticosterone, PRA, and AII. A similar group of rats was injected with dexamethasone (Decadron) 1 mg/kg intramuscularly. Ninety minutes later the rats were anesthetized, treated with morphine and cannulated as the previous group.

Preparation and Incubation of Cell Suspension

The effect of tonin on isolated rat adrenal glomerulosa or fasciculata cells was studied on cells prepared according to a technique previously described. Briefly, female Fisher rats weighing approximately 250 g and kept on a normal-sodium diet were killed by decapitation. Adrenal glands were immediately removed and dissected free of fat and capsules were separated from fasciculata-reticulans by manual compression. The minced capsules were incubated for 40 minutes at 37°C in phosphate-buffered saline containing Dispase (Boehringer Mannheim GmbH, lot 1369304) (8 μg/ml), deoxyribonuclease (DNase I from bovine pancreas, Sigma Chemical Company, St. Louis, Missouri) (3 μg/ml), collagenase (C2139, Sigma Chemical Co., St. Louis, Missouri) (1.7 mg/ml) and 3% chicken serum (Gibco, New York). The cells were dispersed mechanically, filtered through a 100 μm nitex nylon filter (Tetko Inc., Elmsford, New York) and centrifuged at 200 × g for 2 minutes. The cells were resuspended in F12 nutrient medium (Gibco, New York) containing 0.35% hepes (Calbiochem., La Jolla, California), 0.12% sodium bicarbonate, 0.002% gentamicin (Schering, Kenilworth, New Jersey) and 0.5% bovine serum albumin (Sigma Chemical Company, St. Louis, Missouri).

Cells were first incubated for 2 hours at 37°C in a Dubnoff metabolic shaker. After centrifugation they were resuspended in the same medium. The cell suspension, 0.8 ml, was pipetted into 1.5-ml Eppendorf tubes containing 0.1 ml of stimulating agent and/or 0.1 ml of antagonist dissolved in F12 medium. The volume was made up to 1 ml with F12 medium as appropriate. The tubes were stoppered and incubated for 90 minutes at 37°C in a specially designed rack to keep them in an oblique position to facilitate adequate agitation of the cell suspension inside the tube. At the end of the incubation, tubes were centrifuged in an Eppendorf 3200 microcentrifuge (Eppendorf, Hamburg, Germany) for 2 minutes. The supernatant was decanted into glass tubes and frozen at -20°C until assayed for aldosterone and corticosterone by radioimmunoassay. Response of this preparation to AII, K+, and adrenocorticotropic (ACTH) has been previously demonstrated.

Rat α1-macroglobulin used in these studies was purified by the method of Gauthier et al. by J. Tremblay as previously described. "Stripped" rat plasma was prepared by treating rat plasma with 1% activated charcoal (Fisher Scientific Company, Fair Lawn, New Jersey) and 0.1% dextran T 70 (Pharmacia, Uppsala, Sweden) at 4°C overnight. Plasma was then centrifuged, and the supernatant was again treated similarly for 1 hour. After recentrifugation, plasma was stored at -70°C until use.

Biochemical Determinations

Aldosterone in plasma was measured by radioimmunoassay by a modification of the method of Underwood and Williams. Plasma corticosterone was determined by radioimmunoassay after extraction of plasma with dichloromethane. Antibodies for both methods were kindly provided by Dr. P. Vecsei (University of Heidelberg). Aldosterone and corticosterone in the incubation media of isolated cells were measured by direct radioimmunoassay without extraction or chromatography. These results were previously validated by comparison with radioimmunoassay after chromatography for aldosterone or after extraction for corticosterone. Plasma renin activity (PRA) was determined by radioimmunoassay of AI generated during a 2-hour incubation at 37°C and pH 6.5. Plasma AI levels were measured by a modification of the direct radioimmunoassay of Oster et al. Cross-reactivity of AII and the angiotensin C-terminal pentapeptide with the AII antibody was 70%, and that of the angiotensin C-terminal hexapeptide was 97%. Cross-reactivity of AI was 0.27%. A blank value of 39 ± 4.7 pg/ml was not subtracted, as previously described. DNA was measured by the method of Schneider.

Statistical Analysis

Statistical analysis used the unpaired Student t test. The null hypothesis was rejected when p < 0.05. Results are means ± SEM.

Results

In Vivo Experiments

Normal conscious rats infused intravenously with tonin for 2 hours had a significantly higher plasma aldosterone and corticosterone concentration at the end of the experiment (fig. 1, left panel). PRA was
similar in both groups of rats. We found no difference in plasma AII concentration between the tonin-infused and saline-infused rats (49 ± 8 pg/ml in the former vs 36 ± 1 pg/ml in controls, $p < 0.1$).

Pentobarbital-anesthetized rats treated with morphine had very high plasma aldosterone concentrations, and there were no significant differences between saline-infused and tonin-infused rats (fig. 1, middle panel). Basal plasma corticosterone was significantly lower in the anesthetized morphine-treated rats (2.8 ± 0.6 ng\% ) than in conscious ones (26.1 ± 3.4 ng\% ), indicating the effectiveness of morphine in suppressing basal ACTH release. Plasma corticosterone in the anesthetized morphine-treated rats infused with tonin was significantly higher than in controls (fig. 1, middle panel), indicating an ACTH secretory response. PRA was elevated in both groups, secondary to anesthesia, and this increase appeared to be responsible for the high plasma aldosterone concentration. Plasma AII concentration was 88 ± 16 pg/ml in controls, and 217 ± 42 pg/ml in tonin-infused rats ($p < 0.01$).

Findings in rats injected with dexamethasone prior to anesthesia and morphine are depicted in the right panel of figure 1. Plasma aldosterone was much lower than in the previous groups of rats. After tonin infusion, plasma aldosterone was twofold higher than in controls. Plasma corticosterone was extremely low in controls (0.85 ± 0.09 ng\% ) and did not rise after tonin infusion (0.75 ± 0.03 ng\% ). PRA in these rats was much lower than in those not receiving dexamethasone (fig. 1, lower part of right panel).

In Vitro Experiments

The actions of tonin on an isolated adrenal glomerulosa cell preparation are shown in table 1. Angiotensin II produced a significant increase in aldosterone output by the isolated glomerulosa cells at a concentration of $10^{-8}$ moles/liter which was abolished by Sar'-Ile'-AII or des-Asp'-Ile'-AII at a concentration of $10^{-10}$ moles/liter. Neither tonin ($1.6 \times 10^{-7}$ moles/liter) nor the tonin-a-macro-globulin complex modified aldosterone output. When "stripped" rat plasma was added to the incubation medium, aldosterone output was lower than in control vessels. When tonin was added at a concentration of $16. \times 10^{-7}$ moles/liter in the presence of "stripped" rat plasma, aldosterone output increased threefold. At this concentration, immunoreactive AII in the incubation medium at the end of 90 minutes was 823 ± 60 pg/ml vs 28 ± 1 pg/ml (blank value of the method, and therefore equivalent to 0 pg/ml) in samples containing lower concentrations of tonin. Higher concentrations of tonin produced a similar increase in aldosterone output, whereas lower concentrations were ineffective. The increase in aldosterone output in the presence of rat plasma was abolished by adding Sar'-Ile'-AII or des-Asp'-Ile'-AII. The generation of AII immunoreactive material during the incubation in the presence of plasma and the blockade by angiotensin antagonists, suggested that the increased aldosterone output found was caused by the action of AII formed by tonin acting on substrate present in plasma.
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<th>Table 1. Effect of Angiotensin II, Tonin, and Angiotensin Antagonists on Aldosterone Output by Isolated Rat Adrenal Glomerulosa</th>
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<tr>
<td><strong>Aldosterone output (ng/mg DNA)</strong></td>
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<tr>
<td>Control</td>
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<tr>
<td>Angiotensin II (10^-6 moles/liter)</td>
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<tr>
<td>Angiotensin II (10^-3 moles/liter) + Sar^-1-Ile^-1-angiotensin II (10^-6 moles/liter)</td>
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<tr>
<td>Angiotensin II (10^-6 moles/liter) + des-Asp^-1-Ile^-6-angiotensin II (10^-6 moles/liter)</td>
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<tr>
<td>Tonin (1.6 X 10^-7 moles/liter)*</td>
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<tr>
<td>Tonin-α-macroglobulin complex (1.6 X 10^-7 moles/liter)</td>
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<tr>
<td>Control + 2% &quot;stripped&quot; rat plasma</td>
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<tr>
<td>Tonin (1.6 X 10^-7 moles/liter) + 2% &quot;stripped&quot; rat plasma</td>
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<tr>
<td>Tonin (1.6 X 10^-7 moles/liter) + 2% &quot;stripped&quot; rat plasma + Sar^-1-Ile^-6-angiotensin II (10^-6 moles/liter)</td>
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<tr>
<td>Tonin (1.6 X 10^-7 moles/liter) + 2% &quot;stripped&quot; rat plasma + des-Asp^-1-Ile^-6-angiotensin II (10^-6 moles/liter)</td>
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*5 μg/ml.

†Lower concentrations of tonin were ineffective, and higher concentrations produced a similar increase in aldosterone output. Results are means of close duplicates of a representative preparation. Similar results were obtained in at least two different experiments.

 Neither tonin, with or without added rat plasma, nor the tonin-inhibitor complex, stimulated corticosterone output by isolated rat adrenal fasciculata cells. Corticosterone secretion by these cells increased 30-fold in the presence of ACTH (3.4 X 10^-4 moles/liter).

**Discussion**

Our results suggest that tonin may stimulate aldosterone and corticosterone secretion in vivo after intravenous infusion into conscious rats. Since tonin is strongly inhibited in the circulation by α1-macroglobulin, whether an angiotensin peptide apparently formed was generated in the circulation or tissues is unclear. The difference between plasma levels of immunoreactive AI1 of controls and of tonin-infused rats was at the limit of statistical significance (p > 0.05 < 0.1). Moreover, the AI1/PRA ratio was increased by tonin infusion in another study. We have previously demonstrated that tonin may generate small but significant amounts of AI1 in plasma in vitro at a rate which follows first order kinetics and achieves Vmax at high substrate concentration, such as found in nephrectomized rats. This raises the possibility that such a peptide is generated but escapes detection because of the high blank value of the method we have used for radioimmunoassay of AI1 immunoreactive material. The site of action of the putative angiotensin peptide remains to be determined, but it is likely that it acts directly on the adrenal glomerulosa cell.

Pentobarbital-anesthetized rats treated with morphine had a very high plasma aldosterone level, which was not modified by the tonin infusion. Since PRA was extremely elevated in these rats, the aldosterone response was presumably caused by the action of the renin-angiotensin system. The overriding influence of the renin system probably masked the effects of tonin as was previously the case in the investigation of the steroidogenic action of AI1. Plasma corticosterone in control rats was extremely low, indicating an adequate inhibition of ACTH secretion. After tonin infusion, however, corticosterone rose significantly. Thus morphine and pentobarbital were unable to block the stimulation of secretion of ACTH by tonin. A direct effect of tonin on the adrenal cannot explain this increase in plasma corticosterone since tonin does not stimulate isolated rat adrenal fasciculata cells. Plasma AI1 was much higher in these tonin-infused rats than in their controls. This may be caused by the AI1-converting enzyme activity of tonin acting on the increased concentration of AI1 generated by the elevated PRA of these rats. These high levels of AI1 may have contributed to the ACTH secretory response resulting in elevated plasma corticosterone.

In pentobarbital-anesthetized, morphine-injected rats pretreated with dexamethasone, plasma renin was much lower than in rats that had not received dexamethasone. This may be due to effects on sodium excretion. However, previous evidence has suggested that salt and mineralocorticoid treatment will not prevent renin-release produced by anesthesia. Dexamethasone may also reduce renin-release by the suppression of ACTH secretion, which has been reported to stimulate renin secretion. Corticosterone was suppressed even further than in rats not receiving dexamethasone, and there was no difference between controls and tonin-infused rats, demonstrating a complete inhibition of ACTH secretion. In these rats plasma aldosterone was very low. Dexamethasone may inhibit steroidogenesis directly by feedback inhibition. Plasma aldosterone was increased twofold by tonin infusion. In similarly treated rats, a dose of AI1 of 20 ng/kg/min, which elevates plasma aldosterone in conscious rats from 11.2 ± 2.4 ng% to 37.2 ± 6.8 ng%, increased plasma aldosterone from 1.9 ± 0.9 ng% to 3.4 ± 0.6 ng% (unpublished results). This dose of AI1 produces a plasma concentration of AI1 of 115 ± 19 pg/ml. Thus, although the response to tonin found in anesthetized rats treated with dexamethasone and morphine is a weak one, so is the response to a dose of AI1 which produces an important elevation of plasma aldosterone in conscious rats.

Since corticosterone rises in pentobarbital-anesthetized morphine-treated rats after tonin infusion, tonin appears to stimulate ACTH release. The mechanism for this effect remains to be determined. It is interesting in this respect that tonin produces an opioid peptide and other fragments from pro-
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opiomelanocortin. This raises the possibility of a number of peptides, from the pituitary or elsewhere, which may contribute to the in vivo steroidogenic effect of tonin. The high plasma concentration of All in pentobarbital-morphine-treated rats, in which corticosterone in plasma rose markedly after tonin infusion, suggests that the ACTH release produced by tonin may be at least partially mediated by All. The study on isolated adrenal glomerulosa cells showed that neither tonin nor the tonin-inhibitor (tonin-α, macroglobulin) complex stimulate adrenal cells directly. When tonin substrate (angiotensinogen) was added in the form of 2% “stripped” cells directly. When tonin substrate (angiotensinogen) for tonin to act upon. Although unlikely, the possibility that tonin may be acting in vitro on Al cannot be excluded in our experiments. It is possible that substrate is available in the adrenal in vivo, but is absent from isolated cells, and that some of the in vivo findings are caused by local generation of All.

In conclusion, tonin appears to stimulate aldosterone secretion in part by generation of an All peptide and in part by stimulating the secretion of ACTH. The physiological significance of these findings remains to be determined.

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