Age and hormonal dependence of tonin levels in rat submandibular gland as determined by a new direct radioimmunoassay

Hsien-Cheng SHIH,*† Lee CHAO† and Julie CHAO*†

Departments of †Biochemistry and *Pharmacology, Medical University of South Carolina, Charleston, SC 29425, U.S.A.

A simple and sensitive direct radioimmunoassay for tonin (EC.3.4.99.–) has been developed. This assay incorporates a modified and convenient poly(ethylene glycol) technique for separation of free from bound tonin. A rabbit antiserum in a final dilution of 1:160000 was used and the purified tonin was labelled with ^{125}I by using a lactoperoxidase method. It detects 20 pg of immunoreactive tonin per tube. Serial dilutions of rat submandibular gland extracts showed complete parallelism with tonin standard curves. No cross-reactivity with rat tissue kallikrein was seen. Intra- and inter-assay errors were 3.2 and 5.6%, respectively. Using this assay, immunoreactive tonin was detected in the rat submandibular gland as early as 3 weeks after birth (body wt. ~ 50–60 g). Tonin levels are shown to be dependent on age and sex with significantly higher levels in male than in female rats. Castration results in decrease of tonin levels, and 17 α -methyltestosterone replacement reversed the level to higher than the sham-operated control rats. Cortisol treatment increased, but thyroxine or oestradiol had no effect, on tonin levels in the submandibular gland of castrated rats. This newly developed radioimmunoassay can now be used to measure low levels of tonin in various tissues and body fluids to address questions about its regulation and functional significance.

INTRODUCTION

Tonin (EC 3.4.99.–), a newly identified esteroproteinase of the serine proteinase family, is capable of producing the potent vasoactive peptide angiotensin II from angiotensin I or from renin substrates such as tetradecapeptide or angiotensinogen (Schriffin & Genest, 1983; Boucher *et al.*, 1974). Despite the substrate specificity, tonin is unrelated to angiotensin I converting enzyme and is different from renin (Boucher *et al.*, 1977). The serine proteinase, similar to tissue kallikrein, is rich in the adult rat submandibular gland (Boucher *et al.*, 1972).

Previously, it has been reported that tonin in the rat submandibular glands cannot be detected until the rat has reached a weight of 250 g, that it then increases with age and that the submandibular gland tonin is lower in female than in male rats (Boucher *et al.*, 1972). We have developed a simple and sensitive radioimmunoassay for tonin using a modified form of the poly(ethylene glycol) separation technique of Shimamoto *et al.* (1979). Using this newly developed assay, we are able to demonstrate changes of tonin levels with age, sex or hormonal manipulation in rat submandibular glands.

EXPERIMENTAL

Enzyme isolation and antiserum production

Rat tonin from submandibular gland was purified as described previously (Demassieux *et al.*, 1976). The homogeneity of rat tonin was determined by SDS/polyacrylamide-gel electrophoresis according to the previously described procedure (Chao & Chao, 1981). Antiserum against purified rat tonin was raised in rabbits in a

similar way to that described for rat urinary kallikrein (Chao & Margolius, 1979) or rat urinary esterase A (Chao, 1983). Rat urinary kallikrein was purified and the antiserum was prepared as described previously (Chao & Margolius, 1979).

Iodination and purification of ^{125}I -labelled tonin

Purified rat tonin was labelled with ^{125}I by using a lactoperoxidase method according to the procedure for preparing ^{125}I -labelled human urinary kallikrein (Shimamoto *et al.*, 1980). Briefly, 30 μl (5 μg) of purified rat tonin, 5 μl (0.5 mCi) of Na^{125}I in 0.1 M-NaOH and 10 μl (1.5 μg) of lactoperoxidase in 0.1 M-sodium acetate buffer, pH 5.6, were added to 50 μl of 0.4 M-sodium acetate buffer, pH 5.6. The reaction was initiated by the addition of 10 μl of H_2O_2 (20 $\mu\text{g}/\text{ml}$). After a 30 min incubation at 37 °C, 0.2 ml of 0.1% bovine serum albumin in 0.05 M-phosphate buffer, pH 7.4 (elution buffer) was added to the reaction mixture which was then immediately applied to a column (1 cm $\times$ 30 cm) of Sephadex G-100 equilibrated with the elution buffer. The first five fractions of 1 ml each contained no radioactivity and were discarded. Subsequent 1 ml fractions were collected and radioactivity therein was measured with a Beckman well-type γ spectrometer. To identify labelled rat tonin, samples (0.2–7 μl) containing 10000 c.p.m. were removed from each tube, incubated with an excess of the rabbit anti-tonin serum (1:3200 dilution in a total incubation volume of 400 μl) for 24 h at 20 °C and assayed as described below.

Rat urinary kallikrein labelled with ^{125}I was prepared either by the chloramine T method as described previously (Shimamoto *et al.*, 1979) or by the lactoperoxidase method (Shimamoto *et al.*, 1980).

Abbreviation used: Tos-Arg-OMe, tosylarginine methyl ester.

† To whom correspondence and reprint requests should be addressed.

Radioimmunoassay procedures

NaCl/P_i (0.14 M-NaCl in 0.01 M-NaH₂PO₄, pH 7.0) including 1% bovine serum albumin was used as the assay buffer. The volume of assay buffer including purified tonin (0.020–10 ng) or tissue samples totalled 200 μ l. Tonin antiserum was diluted 1:40000 with assay buffer, and 100 μ l was added to each tube. ¹²⁵I-tonin (8000–10000 c.p.m.) in 100 μ l of assay buffer was added, making the final incubation volume 400 μ l, and the final antiserum dilution 1:160000. All samples or standards were assayed in duplicate. Tubes were incubated at room temperature (~20 °C) for 18 h. Antibody-bound tonin was separated from free enzyme by using a modification of the poly(ethylene glycol) separation technique of Shimamoto *et al.* (1979). Bovine γ -globulin (1%) in NaCl/P_i (30 μ l) and 25% poly(ethylene glycol) in NaCl/P_i (250 μ l) were added to the tubes and mixed. Tubes were centrifuged at 2500 *g* for 30 min at 4 °C. The supernatant was carefully aspirated, and the radioactivity in the unwashed precipitates was counted in the automatic well-type γ spectrometer. Control tubes (*B*₀) contained no standard or sample. Precipitated radioactivity was expressed as a percentage of the control tube radioactivity.

Animal studies

Groups of five or six Sprague–Dawley castrated or sham-operated male, normal female (200–250 g body wt.) or Wistar Kyoto rats (50–310 g body wt.) were obtained from Sprague–Dawley, Madison, WI, U.S.A. Animals were housed in groups of three or four in a light-controlled (12 h light/12 h darkness) constant temperature (25 °C) room. All rats were fed a normal lab chow (0.18 mequiv. of Na⁺/g; 0.2 mequiv. of K⁺/g; Wayne Pet Food Division, Chicago, IL, U.S.A.) and allowed free access to tap water. At 3 weeks after surgery by the supplier, rats began to receive subcutaneous injections of 17 α -methyltestosterone (40 mg/kg), cortisol (20 mg/kg), or 17 β -oestradiol (0.1 mg/kg) suspended in sesame oil, or thyroxine (0.3 mg/kg) suspended in saline, or vehicle, every other morning for 2 weeks.

Preparation of submandibular gland extracts

After the treatment periods, rats were anaesthetized with pentobarbital (50 mg/kg). The vena cava was cut and the circulation perfused via cardiac puncture with at least 30 ml of normal saline until tissue appeared blood-free. The submandibular glands were either frozen at –20 °C or immediately minced and homogenized in phosphate-buffered saline, pH 7.3, at 4 °C. Homogenates were centrifuged at 600 *g* for 10 min, and the supernatant was treated with deoxycholate (0.5% w/v) for 30 min at room temperature. The extracts were centrifuged at 20000 *g* for 30 min and supernatants collected for assays of tonin and total protein. Protein concentration was measured according to Lowry *et al.* (1951) using bovine serum albumin as the standard.

RESULTS

Isolation of tonin and antiserum characterization

Rat tonin was purified to apparent homogeneity as shown by polyacrylamide slab gel electrophoresis under reducing conditions. Fig. 1 shows the single polypeptide chain of rat tonin with *M*_r ~ 32000 (lane 2) and rat

Fig. 1. SDS/polyacrylamide-gel electrophoresis analysis of purified rat tonin and kallikrein

Purified rat tonin (10 μ g) and rat urinary kallikrein (4 μ g) were analysed in a 7.5–17.5% gradient gel under reducing conditions. The protein bands were stained with Coomassie Blue R250. Lane 1, *M*_r markers (bovine serum albumin, 67000; ovalbumin, 43000; aldolase, 40000; chymotrypsinogen, 25000; ribonuclease, 13700); lane 2, rat tonin; lane 3, rat urinary kallikrein.

urinary kallikrein with *M*_r ~ 38000 (lane 3). Purified tonin has angiotensin II converting as well as esterolytic and kininogenase activities. In contrast with rat urinary kallikrein, tonin esterolytic activity is strongly inhibited by soya-bean trypsin inhibitor but not by aprotinin at the concentrations described previously (Chao, 1978). The specific activity of purified tonin is 18 Tos-Arg-OMe esterase units/mg and that of kallikrein is 125 Tos-Arg-OMe esterase units/mg (Chao, 1978). Antiserum curves showed that the maximum specific binding (58%) of added rat tonin labelled with ¹²⁵I occurred with a final antiserum dilution of 1:12800; 30% specific binding occurred using a final antiserum dilution of 1:200000, a dilution greater than that used in the assay.

Radioiodination of tonin

Fig. 2 shows the distribution of total radioactivity and the radioactivity precipitable with excess antiserum in fractions collected from a Sephadex G-100 column. Two peaks of radioactivity were present. The iodinated enzyme was precipitable by the antiserum in the first peak (tubes 12–14). The second peak was free iodide (tubes 20–21). The radioactivity precipitated in blank tubes (tubes without antiserum) using the optimal concentration of poly(ethylene glycol) and bovine

Fig. 2. Purification of iodinated rat tonin

Tonin (5 µg) was iodinated with 0.5 mCi of Na¹²⁵I by the lactoperoxidase method, as described in the Experimental section. The distribution of total radioactivity (●) and radioactivity bound to excess antiserum (bars) in the eluate fractions (1.0 ml each) from the Sephadex G-100 column is shown. To determine radioactivity bound to antiserum, tubes containing 200 µl of assay buffer, 100 µl of antiserum (1:800 in assay buffer), and samples (0.2–7.0 µl) from each tube containing 10000 c.p.m. in 100 µl of assay buffer were incubated and antiserum-bound enzyme was separated as described in the Experimental section.

Fig. 3. Log-logit transformations of a representative radioimmunoassay standard curve for tonin, rat urinary kallikrein and rat submandibular gland extract

The abscissa shows the log scale of quantities in weight. The final dilution of tonin antiserum in the tube was 1:160000. No cross-reactivity was seen with rat urinary kallikrein (1.25–80 ng).

γ-globulin as described in the Experimental section was < 7% of the total added counts. The specific radioactivity of tonin labelled with ¹²⁵I was determined by measuring the fractional reduction in binding of labelled enzyme to antiserum after the addition of unlabelled rat tonin (0.02–10 ng). The labelled enzyme was stable when stored at 4 °C for 1 month.

Radioimmunoassay characterization

Standard curves with purified rat tonin and the cross-reactivity of rat urinary kallikrein with the tonin antiserum are shown in Fig. 3. The displacement curve

Table 1. Recovery of rat tonin from rat submandibular gland extract

Each value represents the mean ± S.E.M. for n = 3.

Tonin added (ng/tube)	Mean measured value (ng/tube)	Recovered (ng/tube)
0	1.45 ± 0.02	—
0.32	1.84 ± 0.07	0.39 ± 0.07
0.64	2.18 ± 0.18	0.73 ± 0.18
1.25	2.68 ± 0.06	1.23 ± 0.06
2.5	3.96 ± 0.26	2.51 ± 0.26
5.0	6.24 ± 0.06	4.79 ± 0.06

for submandibular gland homogenates was parallel with the standard curve of tonin. However, there was no cross-reactivity between purified rat urinary kallikrein (1.25–80 ng per tube) and the tonin antiserum in the assay. Using a B/B₀ of 93% as the limit of detectability, the assay sensitivity was 20 pg of tonin. The intra-assay coefficient of variation was 3.2% as determined by using seven submandibular gland samples. The between-assay error was evaluated on the same sample in seven separate assays and the coefficient of variation was 5.6%. The recovery of various amounts of rat tonin added to six rat submandibular gland extracts is shown in Table 1.

Age dependence of tonin and kallikrein content in rat submandibular gland

Table 2 shows the immunoreactive tonin and kallikrein levels in the submandibular gland of male Wistar Kyoto rats from different age groups. Using this sensitive direct radioimmunoassay, tonin was detected in the rat submandibular gland of 3-week-old rats (body wt. ~ 50–60 g). The quantity of tonin increased approx. 10000-fold from 0.28 ± 0.05 ng/mg of protein in 3-week-old rats to 2.07 ± 0.65 µg/mg of protein in 7–8-week-old rats. Thereafter, tonin content in the submandibular gland increased gradually from 10.46 ± 1.22 µg/mg of protein at the 10th week to 29.76 ± 3.16 µg/mg of protein at the 16th week. In comparison with the kallikrein level in the same submandibular gland extract of the 3-week-old rats, the levels of tonin (0.28 ± 0.05 ng/mg) were more than 10000-fold lower than that of kallikrein (7.3 ± 1.52 µg/mg). From the young rat (wt. 50–60 g) to the adult rat (wt. 280–310 g), the kallikrein level increased 18.6-fold while tonin levels increased 100000-fold (Table 2).

Sex and hormonal dependence of submandibular gland tonin

Table 3 shows hormonal effects on tonin levels in the rat submandibular gland of castrated male and normal female Sprague–Dawley rats. Castration of males resulted in a 44% decrease in immunoreactive tonin in the adult male submandibular gland (93.5 ± 6.6 versus 52.5 ± 4.4 ng/mg of protein, P < 0.005). Testosterone or cortisol treatment in castrated males showed 114% and 46% increase, respectively, in submandibular gland tonin level over the castrated males receiving vehicle while thyroxine and estradiol had no effect. Normal female rats contained 11.5-fold less tonin in the submandibular

Table 2. Age dependence of tonin and kallikrein levels in rat submandibular gland

Values shown are the mean $\pm$ S.E.M. for five to six submandibular glands of male Wistar Kyoto rats in each group.

Age (weeks)	Body wt. (g)	Tonin ($\mu\text{g}/\text{mg}$ of protein)	Kallikrein ($\mu\text{g}/\text{mg}$ of protein)
3	50–60	$(2.8 \pm 0.05) \times 10^{-4}$	7.31 ± 1.52
7–8	100–150	2.07 ± 0.65	59.31 ± 3.26
8–10	180–250	10.46 ± 1.22	108.9 ± 9.96
14–16	280–310	29.72 ± 3.16	130.0 ± 6.17

Table 3. Effects of hormones on tonin in rat submandibular gland

Values shown are the means $\pm$ S.E.M. for five or six submandibular glands of Sprague–Dawley rats in each group. ^a $P < 0.005$ versus castrated control rat; ^b $P < 0.005$ versus castrated control rat; ^c $P < 0.025$ versus castrated control rat; ^d $P < 0.005$ versus normal female rats; ^e $P < 0.025$ versus normal female rats.

Sex	Surgical treatment	Hormone treatment	Tonin ($\mu\text{g}/\text{ml}$ of protein)
Male	Sham-operated	–	93.5 ± 6.6^a
	Castration	–	52.5 ± 4.4
	Castration	17α -Methyltestosterone	112.9 ± 12.5^b
	Castration	Thyroxine	54.2 ± 6.0
	Castration	Cortisol	76.4 ± 9.9^c
	Castration	Oestradiol	52.3 ± 3.4
Female	–	–	7.8 ± 1.2
	–	17α -Methyltestosterone	72.1 ± 12.8^d
	–	Thyroxine	26.7 ± 3.4^e

gland when compared with that in the sham-operated male rats (Table 3). After administration of testosterone or thyroxine to normal female rats, submandibular gland tonin levels increased 8.2- and 2.4-fold, respectively (Table 3). These results indicate that tonin levels are strongly androgen-dependent and that thyroxine and cortisol might also play a role in the regulation of tonin synthesis.

DISCUSSION

We have developed a simple and sensitive radioimmunoassay of tonin (minimum detectable amount, 20 pg/tube) using a modified poly(ethylene glycol) separation technique. The application of an optimal combination of poly(ethylene glycol) and γ -globulin to the assay mixture effectively separates the free from antibody-bound tonin with the blank tube (omission of antibody) giving $< 7\%$ of the total added tracer. The radioimmunoassay system reported here eliminates the need for a second antibody (Gutkowska *et al.*, 1978) which involves additional time and expense. We have compared the results obtained by both the poly(ethylene glycol) separation and double-antibody methods without observing any significant difference between the two methods (results not shown). In the assay reported here, rat urinary kallikrein up to 80 ng/tube showed no competition for ^{125}I -tonin binding to anti-tonin serum. Therefore, the assay is specific since no cross-reactivity exists between kallikrein and anti-tonin serum in this assay system. The assay can detect as little as 20 pg of immunoreactive tonin in the tissue extracts or body fluid.

Therefore, this sensitive and specific assay may be used as a powerful tool for studying the regulation and functional significance of tonin in various glandular tissues such as kidney, pancreas, prostate or testis.

Two direct radioimmunoassays have been described previously for measuring immunoreactive tonin. One is the double-antibody method (Gutkowska *et al.*, 1978) and the other is the solid-phase radioimmunoassay (Gutkowska *et al.*, 1982). Both methods require more than 48 h of total incubation time and the latter method requires a large quantity of antiserum for coating the assay tubes. The newly developed tonin assay described here not only provides higher sensitivity, but is simple and less expensive. Most significantly, the assay can be completed within 16–18 h as compared with the double-antibody method which requires 48 h.

It has been reported that free tonin in the rat submandibular gland could not be demonstrated until the rat had reached a weight of 250 g; then it increased progressively with age (Boucher *et al.*, 1972). By using this radioimmunoassay, we can quantify submandibular gland tonin from different age rats. In 3-week-old rats (body wt. 50–60 g), tonin content in the submandibular gland was very low, at 0.28 ± 0.05 ng/mg of protein and increased dramatically (~ 10000 -fold) up to 2.07 ± 0.65 $\mu\text{g}/\text{mg}$ of protein in the 7–8-week-old rats with body wt. ranging from 100 to 150 g. From 7–8 weeks, tonin levels increase progressively with age (Table 2). The possible significant relationship between age and submandibular gland tonin levels is a subject of much interest.

Tonin content in the submandibular gland is not only

age- but is also sex-dependent. The submandibular tonin content of male rats is approx. 11.5-fold higher than that of the female rats (Table 3). Sex difference of tonin (Boucher *et al.*, 1972), renin (Gecse *et al.*, 1976) and kallikrein (Chao & Margolius, 1983) has been shown previously. Similar to our previous studies on hormonal regulation of tissue kallikrein in the rat submandibular gland (Chao & Margolius, 1983), the present study demonstrates androgen-dependency of tonin levels. Castration results in reduction of submandibular gland tonin in male rats. Testosterone replacement increased the submandibular gland tonin levels higher than that of castrated rats receiving vehicle. Cortisol treatment also increases tonin levels but thyroxine or oestradiol has no effect on tonin content in the submandibular gland of castrated rats. In normal female rats both testosterone and thyroxine administration caused an increase in submandibular gland tonin levels, but testosterone was approx. three times more potent than thyroxine. A sharp drop in tonin activity has been shown in hypophysectomized rats, whereas testosterone alone did not produce a significant effect to restore tonin activity (Lis *et al.*, 1977). The present study shows sex dimorphism of tonin in the submandibular gland, and the level of this enzyme is clearly androgen-dependent.

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