

Lower Levels of Thyrotropin-Releasing Hormone-Degrading Activity in Human Cord and in Maternal Sera Than in the Serum of Euthyroid, Nonpregnant Adults

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ABSTRACT Thyrotropin-releasing hormone (TRH)-degrading activity was investigated in human cord, maternal, and euthyroid adult sera by measuring (a) the rate of disappearance of TRH and (b) the rate of formation of degradation products. The rate of TRH degradation in cord and maternal sera was 25–33% of that in euthyroid adult serum. Concomitantly, in cord and maternal sera, the rate of formation of proline, a major TRH degradation product in serum, was one-quarter to one-third that in euthyroid adult sera. The differences were highly significant ($P < 0.001$). The decreased levels of TRH-degrading activity in cord and maternal sera cannot be explained by (a) the presence of a dialyzable inhibitor, (b) the absence of an activator of TRH degradation, or (c) a reversal of the degradation process. There was no difference in the types of radioactive degradation products formed by cord, maternal, and euthyroid adult sera.

The low level of TRH-degrading activity and its possible relationship to high thyrotropin-stimulating hormone levels in cord serum suggest that TRH-degrading activity may be a factor to consider in investigations of the perinatal pituitary-thyroid axis, but further studies are needed to determine the role of serum degradation of TRH in regulating physiological levels of TRH.

INTRODUCTION

Recent studies have focused on differences in thyroid physiology in infants and adults. In human cord serum,

Received for publication 21 July 1977 and in revised form 8 December 1977.

triiodothyronine (T_3)¹ is below adult standards (1), thyroxine (T_4) is at the upper level of adult standards (2), and thyrotropin-stimulating hormone (TSH) (3), reverse T_3 (4), and thyroxine-binding globulin are above adult standards (5). In the newborn infant, T_3 and T_4 levels rise to greater than adult values and then decline to normal adult values during development (2).

An important question concerning the regulation of perinatal thyroid function is the physiologic role of thyrotropin-releasing hormone (TRH) and the factors affecting TRH activity. Serum degradation is one way in which TRH activity may be regulated. Adult serum contains a potent TRH inactivation system (6–8), but we have recently observed that the plasma of neonatal rats does not degrade TRH extensively (9). Hence, it was of interest to measure the level of TRH-degrading activity in humans at birth.

METHODS

Serum collection, storage, and stability of TRH-degrading activity

Mixed arterial-venous cord blood was collected from normal newborn infants at delivery. Maternal samples were taken on admission to the labor floor or at the time of delivery. All were normal term pregnancies of patients at the Boston Hospital for Women, Boston, Mass. Serum was collected from adults judged to be euthyroid by T_3 , T_4 , and TSH. For some assays, the following serum pools were used: nonpregnant euthyroid adult,

¹ *Abbreviations used in this paper:* T_3 , triiodothyronine; T_4 , thyroxine; TRH, thyrotropin-releasing hormone; TSH, thyrotropin stimulating hormone; [³H-Pro]TRH, [³H]-L-Pro-2,3-[³H]-L-pGlu-L-His-L-Pro-NH₂.

10 males plus 10 females; cord, 5 males plus 5 females; maternal, 10 females.

Serum was stored frozen at -12°C . Serum TRH-degrading activity was stable for at least 2 mo at -12°C . Assays of sera before and after freezing showed that no loss in TRH-degrading activity occurred after the first freeze/thaw cycle, but five freeze/thaw cycles over a 2-mo period resulted in a 10% loss of activity.

Measurement of TRH-degrading activity

Radioimmunoassay for TRH disappearance. Serum (200 μl) was added to 800 μl of a buffer solution (0.25% bovine serum albumin, 150 mM NaCl, 10 mM Na phosphate, pH 7.5) containing 2 μg of TRH. Duplicate samples were incubated at 37°C ; 100 μl aliquots were withdrawn at 15, 30, 60, 90 and 120 min, diluted to 1 ml with the bovine serum albumin, NaCl, phosphate buffer, and the tubes were immediately placed into a boiling water bath for 3 min. The samples were then diluted further for TRH radioimmunoassay, which was conducted as previously described (9).

Separation and quantitation of TRH and degradation products by paper electrophoresis. Incubation conditions were: 5 μM TRH (containing 500–600 dpm [^3H -Pro]TRH/pmol TRH), 100 mM Na phosphate buffer, pH 7.4, and 5 μl of euthyroid adult or 10 μl of maternal or cord serum in a final volume of 50 μl . [^3H -Pro]TRH (New England Nuclear, Boston, Mass.; 20 Ci/mmol) was purified according to the method of McKelvy (10) with minor modifications.² After 90 min at 37°C , 150 μl of 10 mM Na phosphate buffer, pH 5.1, were added to the tubes, and the tubes were immediately transferred to a boiling water bath for 3 min. 15- μl aliquots were applied to paper electrophoresis strips, and electrophoresis was conducted for 80 min (10 mA, constant current) in a Beckman Durham (Beckman Instruments, Inc., Fullerton, Calif.) type cell in citrate-phosphate buffer, pH 2.8 (11). Strips were cut into 0.5-cm sections and transferred to counting vials. The sections were covered with 1 ml of 50 mM NaCl; after gentle shaking for 1 h, 10 ml of liquid scintillation solution (12) was added. Vials were counted for 10 min. TRH degraded was calculated by adding the radioactivity in the TRH sections and comparing it with that obtained from a control incubation with heat-inactivated serum (30 min, 55°C) (6–8). 90% of the applied radioactivity was recovered on the strips. The sum of the strip sections for TRH (4–5.5 cm from the origin), proline (0.5–2.0 cm), dipeptide-containing proline (6–7 cm), deamido-TRH (2.5–3.5 cm), and prolineamide (8–11 cm) accounted for 95% of the recovered radioactivity. The remainder of the radioactivity was located at the origin; this corresponds to an impurity in the [^3H -Pro]TRH which is not completely removed upon purification (10). No other peaks of radioactivity were observed. The positions of TRH and the degradation products were identified by comparison with standards stained with Pauly reagents or with ninhydrin. Standards of ^3H -Pro (New England Nuclear) and [^3H -Pro]TRH were also used.

Measurement of the major degradation product, proline, by a rapid Dowex 50 separation procedure. Incubation conditions were identical to those described for the paper electrophoresis procedure, except that the incubation solution contained 250–300 dpm [^3H -Pro]TRH/pmol TRH. All assays were conducted in duplicate and at 2 different vol of serum. Both volumes were within the linear portion of the velocity vs. enzyme concentration graph.² After incubation, proline was

separated from undegraded TRH by a Dowex 50 (Dow Chemical Co., Midland, Mich.) batchwise procedure at pH 5.2 (10 mM sodium phosphate).² Analysis of the unbound fraction by thin-layer chromatography and paper electrophoresis² showed that proline is the only radioactive product in the unbound fraction. Enzyme units are expressed as picomoles proline per hour per microliter at the standard incubation conditions. Results obtained by this procedure and the amount of proline as measured in the paper electrophoresis procedure agreed within $\pm 5\%$. Duplicate samples in the same Dowex assay varied by 3%; interassay variation was 5%.

TSH, T_4 , and T_3 were determined by radioimmunoassay (13, 14).

RESULTS

TRH (2 $\mu\text{g}/\text{ml}$) was incubated with pooled sera (20% by volume), and TRH disappearance was measured by radioimmunoassay. The rates of TRH degradation were: $60 \pm 5\%$ TRH degraded/h for euthyroid nonpregnant adult serum, $14 \pm 2\%$ for cord serum, and $20 \pm 4\%$ for maternal serum. The lack of cross-reaction between the TRH antibody used in these studies and the likely degradation products (9) justifies the use of radioimmunoassay to measure the disappearance of TRH.

In separate experiments, the degradation of TRH and the formation of breakdown products were measured by incubating serum pools with TRH containing a trace amount of [^3H -Pro]TRH and separating undegraded TRH and the degradation products by paper electrophoresis. The rates of TRH degradation were 13.3 pmol TRH degraded/ $\mu\text{l}/\text{h}$ for euthyroid adult serum, 3.00 pmol/ $\mu\text{l}/\text{h}$ for cord serum, and 3.33 pmol/ $\mu\text{l}/\text{h}$ for maternal serum (Table I). The fourfold difference in rates of TRH degradation between cord and euthyroid adult sera and between maternal and euthyroid adult sera is in very good agreement with that measured by the radioimmunoassay procedure.

The formation of proline and proline-containing degradation products is shown in Table I. It can be seen that euthyroid adult, cord, and maternal sera yield the same degradation products. Furthermore, the relative percentages of each of the degradation products are similar for all three sera. Proline is the major degradation product (75–79%) and a dipeptide containing proline (His-Pro, His-Pro-NH₂ or the diketopiperazine derivative of His-Pro-NH₂; product identification is incomplete at this time) comprises 20–24% of the total degradation products. Only trace amounts of prolineamide and deamido-TRH are present. No other peaks of radioactivity have been detected. By contrast, when measured by the same electrophoresis system, the major degradation product of TRH-degrading activity from rat hypothalamus is deamido-TRH, in agreement with previous reports (15–17).

TRH-degrading activity in serum can also be quantitated in terms of proline produced during TRH degradation; a rapid and sensitive procedure has been

² Neary, J. T., C. Nakamura, F. Maloof, and M. Soodak. 1978. TRH-degrading enzyme activity: a sensitive and rapid radiochemical assay procedure. Manuscript in preparation.

TABLE I
*Degradation of TRH and Production of Proline and Proline-Containing Degradation Products as Measured by Paper Electrophoresis**

Sample	Radioactivity†						TRH-degrading activity
	Total on strip	TRH	Proline	Deamido-TRH	Dipeptide(s)	Pro-NH ₂	
							<i>pmol TRH degraded/μl/h</i>
1. Heat-inactivated serum (5 μl)	5,002	4,595	86	37	43	32	—
2. Euthyroid adult serum (5 μl)	5,508	2,888	1,712 (75)	28 (0)	573 (24)	60 (≤1)	13.3
3. Cord serum (10 μl)	5,351	3,958	825 (76)	39 (0)	270 (23)	51 (≤1)	3.00
4. Maternal serum (10 μl)	5,516	3,965	995 (79)	34 (0)	276 (20)	49 (≤1)	3.33
5. Rat hypothalamus (2 μl of 10% homogenate, wt/vol)	5,477	3,663	166 (5)	667 (46)	550 (36)	212 (13)	20.8

* The conditions of incubation and electrophoresis are described in Methods.

† Numbers in parentheses are the percentages of the sum of the degradation products.

developed to separate proline from TRH in a batchwise Dowex procedure.² The differences in rates of TRH-degrading activity between cord and euthyroid adult sera and between maternal and euthyroid adult sera, as measured by the Dowex procedure, is in very good agreement with that measured by the radioimmunoassay and paper electrophoresis methods. For males, the mean rates (\pm SD) of proline production were 2.07 ± 0.38 in cord serum and 9.81 ± 5.10 pmol proline/ μ l/h in euthyroid adult serum (Table II). For females, the rates of proline production were 2.12 ± 0.90 in cord serum, 9.14 ± 3.99 in euthyroid adult serum, and 3.19 ± 1.30 pmol proline/ μ l/h in maternal serum (Table II). The differences between cord and euthyroid adult and between maternal and euthyroid adult are highly

significant ($P < 0.001$). No significant differences have been observed between male and female sera within groups.

All of the cord samples ($n = 15$) contained levels of TRH-degrading activity below those in adult nonpregnant individuals. The range of TRH-degrading activity was smaller in cord serum (1.31–3.87 pmol proline/ μ l/h) than in euthyroid adults (4.1–20.7 pmol proline/ μ l/h). In the adult samples studied, there was no correlation between TRH-degrading activity and the age of the individual (18–77 yr for females and 26–73 yr for males). The wide range of TRH-degrading activity in euthyroid adult serum confirms the observation of Bassiri and Utiger (8) and Dupont et al. (18). The elevated TSH and low T₃ levels in cord serum (Table II) are consistent with what has been reported (1–3).

A wider range of TRH-degrading activity was found in maternal sera (1.21–5.23 pmol proline/ μ l/h) than in cord sera. Of the 11 maternal patients studied, 3 were in the low region of the euthyroid adult range and 8 were below the euthyroid adult range. The normal levels of TSH and T₃ in maternal serum are consistent with previous reports (1–3).

In an attempt to explore possible reasons for the decreased levels of TRH-degrading activity in cord and maternal sera, the following experiments were conducted. (a) Cord and maternal sera pools were dialyzed exhaustively at 4°C against 100 mM Na phosphate, pH 7.4. TRH-degrading activity in cord sera was 1.98 before dialysis and 1.76 pmol proline/ μ l/h after dialysis. In maternal sera, TRH-degrading activity was 2.99 before dialysis and 2.88 pmol proline/ μ l/h after dialysis. Under similar conditions, TRH-degrading activity in euthyroid adult serum was 9.40 before dialysis and 9.25 pmol proline/ μ l/h after dialysis. (b) Maternal or cord serum pools were mixed separately with an equal volume of a euthyroid adult serum pool before assay. In the

TABLE II
TRH-Degrading Activity, TSH, and T₃ in Human Cord, Maternal, and Euthyroid Adult Sera (Mean \pm SD)

Serum*	TRH-degrading activity	TSH†	T ₃ ‡
	<i>pmol proline/μl/h</i>	μ U/ml	ng%
Cord, male (6)	$2.07 \pm 0.38^{\text{II}}$	10.4 ± 8.1	26.0 ± 6.9
Cord, female (9)	$2.12 \pm 0.90^{\text{II}}$	5.18 ± 1.35	23.3 ± 4.7
Euthyroid adult, male (19)	9.81 ± 5.10	1.26 ± 0.81	110 ± 30
Euthyroid adult, female (20)	9.14 ± 3.99	1.31 ± 1.05	110 ± 30
Maternal (11)	$3.19 \pm 1.30^{\text{II}}$	1.5 ± 0.90	120 ± 25

* Number of samples is in parentheses.

† TSH adult normal range = 0.2 to 3.5 μ U/ml.

‡ Total T₃ adult normal range = 75–175 ng/100 ml.

^{II} Compared to euthyroid adult, $P < 0.001$.

mixed maternal-euthyroid adult sample, a total of 52.8 pmol proline was produced during a 90-min incubation at 37°C; the sum of the maternal and euthyroid adult pools assayed separately was 51.6 pmol proline. Similar results were obtained when cord serum was mixed with euthyroid adult serum. (c) Pools of cord, maternal, or euthyroid adult sera were incubated with 5 μ M pGlu-His and 5 μ M proline, containing a trace amount of [³H]proline, and the reaction mixtures were analyzed by paper electrophoresis; no peaks of radioactivity were detected in the regions corresponding to deamido-TRH or TRH.

DISCUSSION

TRH-degrading activity in serum has been assessed by measuring both substrate (TRH) disappearance and product (proline) formation. The results of three independent assay procedures are in very good agreement. Cord and maternal sera degrade TRH at 25 to 33% the rate of euthyroid adult serum; concomitantly, cord and maternal sera form degradation products at one-quarter to one-third the rate of euthyroid adult serum.

The low levels of TRH-degrading activity in cord and maternal sera, as compared to nonpregnant individuals, cannot be explained by the presence of a low-molecular weight inhibitor of degradation or by the absence of an activator of degradation. Neither dialysis of the sera nor mixing cord or maternal sera with serum containing normal levels of TRH-degrading activity gave an increase in TRH-degrading activity. Furthermore, it is unlikely that the decreased levels of TRH-degrading activity in cord and maternal sera result from a reversal of TRH degradation. A reverse reaction has been reported in hypothalamic tissues, but not in serum (15). When an inhibitor of TRH degradation, pGlu-His-OCH₃, was incubated with hypothalamic extracts and [¹⁴C]proline, deamido-TRH production was observed (15). However, no accumulation of deamido-TRH was observed when porcine serum was incubated with [³H-Pro]TRH and pGlu-His-OCH₃ (15). We were unable to detect a reverse reaction between pGlu-His and proline in cord, maternal, or euthyroid adult sera. Thus, it appears that serum degradation of TRH differs from that of brain tissue (15).

There is no difference in the types of radioactive degradation products formed by cord, maternal, and euthyroid adult sera. Proline is a major product in all three sera (Table I). The identity of proline has been confirmed by thin-layer chromatography.² Proline is also a major product of TRH degradation in porcine and rat sera (15, 19). Furthermore, the relative amounts of proline and a secondary product, a dipeptide-containing proline, are identical for all three serum pools (Table I). Since two possible degradation products, Glu-His-Pro-NH₂ and Glu-His-Pro, have not been

utilized in these studies, it is possible that one of these tripeptides may be the minor product shown in Table I. At present, there is no conclusive evidence for either of these tripeptides as serum degradation products. Redding and Schally (20) injected radioactive TRH into rats and, after 1 and 6 min, a radioactive product was found in serum which co-chromatographed with Glu-His-Pro. By contrast, Knigge and Schock (19) did not detect Glu-His-Pro after incubating [³H-Pro]TRH with rat serum for 20–60 min. Studies are now in progress to elucidate the structure of the proline-containing minor degradation product and to determine whether it is an end-product of TRH degradation or an intermediary in the formation of proline.

The significance of the low levels of TRH-degrading activity in cord and maternal sera is not clear. The role of TRH and factors affecting its activity during the perinatal period have not been defined. TRH is present in the fetal hypothalamus (21) and in the placenta (22). It is also of interest that TRH (23–25), TRH receptors (26), and TRH-degrading activity (16) are widely distributed in the central nervous system. These findings suggest that TRH may function in neuronal processes, possibly as a neurotransmitter. One of the factors which may affect TRH activity is degradation in the brain and in serum. Support for this concept comes from recent studies which demonstrate that TRH-degrading activity in serum varies with changes in the thyroid status of both man and rat (18, 27, 28). When TSH is elevated, TRH-degrading activity may be decreased, thereby leading to a longer half-life for TRH. The low level of TRH-degrading activity in cord serum is consistent with elevated TSH (3) and decreased T₃ (1, 3). However, a similar relationship between TRH-degrading activity, TSH, and T₃ does not exist in maternal serum. Thus, further studies are needed to determine what role, if any, serum degradation of TRH plays in thyroid physiology.

ACKNOWLEDGMENTS

We wish to thank Dr. Susan Leeman, Department of Physiology, Harvard Medical School, Boston, Mass. for reviewing this manuscript; W. F. White, Abbott Laboratories, North Chicago, Ill. for providing TRH, deamido-TRH, and pGlu-His; and Dr. J. Burton, Cardiac Unit, Massachusetts General Hospital, for synthesizing His-Pro and His-ProNH₂. We are also grateful to Ms. Linda Lorenz, Ms. Kathryn C. Kern, and Panfilo Federico for conducting the TSH, T₄, and T₃ radioimmunoassays and to Ms. Ruth DiBlasi and Sharon Melanson for their expert secretarial assistance.

This research was supported by United States Public Health Service grant AM-13916.

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