Immunoreactive thyrotrophin-releasing hormone like material (IR-TRH LM) in human cord blood: high circulating serum levels but possible non-identity with hypothalamic TRH

J. M. Kaufman, A. Elewaut and A. Vermeulen

Department of Internal Medicine, Section of Endocrinology, Academic Hospital, State University of Gent, De Pintelaan 185, B-9000 Gent, Belgium

Abstract. Levels of immunoreactive thyrotrophin-releasing hormone like material (IR-TRH LM) were measured in paired maternal and umbilical cord serum samples (n = 45) and in serum of non-pregnant women (n = 63), using a sensitive and specific radioimmunoassay. In all pairs IR-TRH LM in venous cord serum (mean 91.3 pg/ml, range 20–270) was markedly elevated as compared to maternal serum (mean 13.5 pg/ml, range 0–37, P < 0.001, the maternal levels being similar to those in non-pregnant women (mean 12.0 pg/ml, range 0–39, P > 0.1). In 23 cases IR-TRH LM was also measured in arterial cord serum: arterial and venous cord levels were highly significantly correlated (r = 0.904) with higher arterial levels in 22 out of 23 cases (mean 117.8 pg/ml, range 32–280 and 90.6 pg/ml, range 20–270, P < 0.001), suggesting that cord IR-TRH LM is not of placental origin. There was no correlation between individual levels of IR-TRH LM and either TSH, T4, T3 or FT4 levels in either maternal or cord serum.

Agar gel electrophoresis and equilibrium dialysis of adult and cord serum pre-incubated with [125I]TRH or [3H]TRH revealed no protein binding. Cord IR-TRH LM was immunologically, as well as in paper electrophoresis and in gel filtration, indistinguishable from synthetic and hypothalamic TRH. In vitro degradation of synthetic TRH was much slower in cord serum as compared to maternal and control serum (P < 0.001). In vitro degradation of cord IR-TRH LM could not be demonstrated, neither by cord serum itself nor by adult serum, this in contrast to hypothalamic extracted TRH which was readily degraded by adult serum. Neither the presence of an inhibiting substance, nor methodological factors seem to account for the apparently different behaviour of cord IR-TRH. It is suggested that circulating IR-TRH could be closely related, but still different from tissue extracted and synthetic TRH.

The striking differences in foetal, perinatal and adult thyroid physiology, with drastic changes in hormonal levels occurring in the early postnatal period, are well documented (Fisher et al. 1977; Fisher 1978).

A physiologic role of thyrotrophin-releasing hormone (TRH) in the regulation of foetal and perinatal thyroid function is not yet established. The main purpose of the present study was to gain information on the levels of circulating IR-TRH in neonates and their possible relation to thyroid function.

Material and Methods

Subjects and sampling procedures

Umbilical cord and maternal blood was collected immediately after delivery following normal fullterm pregnancy in 45 healthy women. In all subjects paired samples were obtained by simultaneous puncture of the umbilical cord vein (UCV) and of the maternal femoral artery (MFA); whenever possible (n = 23) an additional
sample was obtained by puncture of an umbilical cord artery (UCA). Venous blood samples were also obtained from non-pregnant women (n = 63) with normal thyroid function, as assessed by determination of T₄, T₃, FT₄ and TSH.

Blood was collected in test-tubes kept on chilled ice, centrifuged at 4°C and the serum frozen at -20°C until further processing. Under similar conditions, all manipulations being performed strictly at 4°C, the recovery of synthetic TRH added to samples of cord blood, maternal blood, or blood from non-pregnant controls, was constantly over 90%. This sampling procedure was further validated by radioimmunoassay of serum samples obtained from adult volunteers at different time intervals following iv administration of synthetic TRH: there were no significant losses in immunoreactivity in comparison with samples collected in syringes containing inhibitors of enzymatic activity.

The described method was preferred to the use of inhibitors such as mixtures of 8-hydroxyquinoline sulphate and Tween 20 or 2, 3 dimercapto 1-propanol (BAL) as they in our hands, although effective in preventing enzymatic degradation of TRH, seemed to interfere with the initial binding of the tracer in the TRH assay. Paired samples were always assayed within the same assay-run.

Hypothalamic tissue from 6 subjects, 2 women and 4 men, aged 56–74 years, was dissected at autopsy (12 to 48 h post-mortem) and immediately extracted with acidified ethanol according to Parker et al. (1981).

TRH-radioimmunoassay

High affinity TRH-antibodies (8.23 × 10⁹ M⁻¹) were obtained by immunization of rabbits with synthetic TRH (UCB, Belgium) conjugated to bovine serum albumin (BSA). BSA was coupled to the histidyl residue of synthetic L-pyroglu-L-his-L-pro-NH₂ (TRH) by means of bis-diazotized benzidine (Bassiri & Utiger 1972).

Labelled TRH was obtained by iodination of 5 µg of synthetic TRH with 1 µCi [¹²⁵I] according to Greenwood et al. (1963) and subsequently purified by gel filtration on Sephadex G-10 (1 × 15 cm). The calculated specific activity of the [¹²⁵I]TRH was 120–158 µCi/µg: re-purification by gel filtration was always performed just before use.

One ml serum samples were extracted with 5 ml methanol, evaporated to dryness at 40°C under nitrogen and re-dissolved in 200 µl of 0.01 M phosphate buffer containing 0.15 M NaCl and 0.25% BSA (PBS-buffer; pH 7.5) for subsequent RIA. The extraction efficiency was found to be over 95% as assessed by both addition of [¹²⁵I]TRH and recovery experiments with known amounts of synthetic TRH.

Synthetic TRH was used as standard for the calibration curve; standards in 200 µl of PBS-buffer were pre-incubated with dried methanol extracts of 1 ml (charcoal treated) TRH-free serum.

Twohundred µl standard or extract of unknown sample, 100 µl of diluted antibody (final dilution of 1:320000) and 100 µl [¹²⁵I]TRH (± 3000 CPM) were incubated for 48 h at 4°C. The bound [¹²⁵I]TRH was precipitated by addition of 1 ml ice-cold n-propanol and 25 µl 1:5 diluted human serum, followed by centrifugation (2500 g for 30 min), the precipitate being counted for 4 min in a gamma-counter.

The specificity of the assay was assessed by determination of the cross-reactivity with different related peptides, including TRH analogues and putative TRH degradation products, amino acids, hypothalamic hormones, pituitary and thyroid hormones, the immunoreactivity of synthetic TRH at 50% of the initial binding being arbitrarily taken as 100%: of the 25 tested substances, only L-pyroglu-L-tyr-L-pro NH₂ (214%) and L-pyroglu-L-3-MeHis-L pro NH₂ (66%) had significant cross-reactivity.

These two synthetic TRH-analogues differ from L-pyroglu-L-his-L-pro NH₂ by modification of the histidyl residue. When large amounts (up to 10 ng) of synthetic or hypothalamus extracted TRH were pre-incubated with adult human serum for a time sufficiently long (up to 360 min at 37°C) to allow complete enzymatic degradation of the TRH, the incubation medium was found to be free of immunoassayable IR-TRH.

Interference of urea was excluded by cross-reactivity experiments (up to 10 mg urea/assay tube; i.e. 10 g/l serum) and by IR-TRH measurements in adult serum, cord serum and serum from patients with terminal renal insufficiency, both before and after incubation of the samples with urease (Merck Darmstadt, W-Germany; 10 mg/ml serum; 120 min at 37°C). IR-TRH levels (adult serum and cord serum) extracted with methanol alone were not different from those extracted with diethyl ether prior to the methanol extraction; the dried ether fractions re-dissolved in PBS-buffer contained no IR-TRH. This indicates that serum lipids do not interfere with the assay. Interference of bilirubin and red blood cell haemolysate was also excluded.

The intra-assay coefficient of variation was 12.0%, 5.9% and 3.9%, respectively for the low (30.4 pg/ml), middle (85.1 pg/ml) and upper (254.0 pg/ml) portion of the standard curve. The inter-assay coefficient of variation for the middle portion of the standard curve (78.7 pg/ml) was 10.8%. The recovery of 100 pg synthetic TRH added to extracts of 2 ml pooled cord serum was 94.6 ± 5.1%, indicating that cord serum does not alter values of standards. Assay detection limit was 2 pg/ml.

Elution pattern of IR-TRH on gel chromatography

Gel chromatography was performed on a Sephadex G-10 (60 x 1 cm) column prepared and eluted with PBS-buffer.

Fractions of 2 ml were collected and assayed for IR-TRH (RIA directly on a 200 µl sample).
Migration of IR-TRH on paper electrophoresis

Electrophoresis (300 V, 22 mA for 120 min) was performed on Whatman 3 MM paper strips using phosphate buffer 0.05 M, pH 7.5. Each strip (15 × 3 cm) was cut into 1 cm segments which were extracted with methanol. The dried methanol extracts were re-dissolved in 200 µl PBS-buffer and assayed for IR-TRH.

Assessment of possible TRH-protein binding by agar gel electrophoresis and equilibrium dialysis

0.6 µCi [125I]TRH or 6.25 µCi [3H]TRH (New England Nuclear Corp., Boston, MA; 250 µCi/µg) were incubated with 100 µl cord serum or adult serum at 4°C during 30 min. After incubation, 4 µl serum was subjected to agar gel electrophoresis according to Wieme (1965). One ml cord serum pre-incubated (60 min at 4°C) with 0.04 µCi [125I]TRH or 0.25 µCi [3H]TRH was subjected to equilibrium dialysis.

TRH-degradation by serum

Serum degradation experiments were performed by in vitro incubation at 37°C of synthetic TRH (200 pg), hypothalamic extracted IR-TRH (± 200 pg equivalents), or serum extracted IR-TRH (± 200 pg equivalents) in 200 µl PBS-buffer with 300 µl fresh serum. Incubation was terminated by addition of 5 ml cold methanol and immediate extraction, TRH-RIA being subsequently performed. The results are expressed as per cent remaining IR-TRH from the control incubation (0 min incubation).

In other experiments 1 ml serum samples were directly incubated at 37°C before extraction and subsequent RIA.

Results

Individual and mean (±SD) level of IR-TRH LM in maternal and umbilical cord blood and in euthyroid non-pregnant controls, are shown in Fig. 1. Maternal levels of IR-TRH LM were similar to those in non-pregnant controls (P > 0.1). In each of the 45 pairs studied, IR-TRH LM in venous cord blood was markedly elevated as compared to maternal blood.

Whenever in addition to umbilical vein blood a sample of arterial cord blood was obtained (n = 23), IR-TRH LM in arterial cord blood was higher than in venous cord blood (with one single exception), the individual arterial and venous IR-TRH levels being highly significantly correlated (r = 0.904; P < 0.001). The mean (±SD) venous and arterial cord IR-TRH LM for these 23 pairs was 90.6 ± 51.3 (range 20–270) and 117.8 ± 62.6 pg/ml (range 32–280), respectively (P < 0.001).

The findings for TRH, TSH, T4, T3 and FT4 are summarized in Table 1. There was no significant correlation between individual IR-TRH levels and either TSH, T4, T3, or FT4 levels in either maternal or umbilical (arterial or venous) cord blood. No correlation could be established between cord blood IR-TRH LM and either birth weight, sex, APGAR score or eventual administration of oxytocin or prostaglandin PgE2 during labour. Agar gel electrophoresis did not reveal any binding of [125I]TRH, [3H]TRH or degradation products to serum proteins in either adult or cord serum: electrophoretic migration of [125I]TRH and [3H]TRH in serum was always identical to that of [125I]TRH or [3H]TRH in buffer (the migration being very slow towards the cathode by buffer electro-osmosis, together with the slowest migrating serum immunoglobulin fractions).
Table 1.
Serum TRH, TSH, T₃, T₄ and FT₄ levels in paired umbilical cord and maternal blood samples.

<table>
<thead>
<tr>
<th></th>
<th>Maternal (arterial)</th>
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<th>Venous umbilical cord</th>
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<th>Arterial umbilical cord</th>
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<tr>
<td></td>
<td>n</td>
<td>Mean ± SD (range)</td>
<td>n</td>
<td>Mean ± SD (range)</td>
<td>n</td>
<td>Mean ± SD (range)</td>
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<tr>
<td>TRH (pg/ml)</td>
<td>45</td>
<td>13.5 ± 12.8 (0–37)</td>
<td>45</td>
<td>91.3 ± 49.4¹ (20–270)</td>
<td>23</td>
<td>117.8 ± 62.6¹,² (32–280)</td>
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<td>TSH (mU/l)</td>
<td>34</td>
<td>2.39 ± 1.33 (0.8–6.4)</td>
<td>34</td>
<td>8.76 ± 5.8¹ (3.0–31.4)</td>
<td>17</td>
<td>9.78 ± 7.96¹ (3.3–36.7)</td>
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<td>T₃ (ng/dl)</td>
<td>34</td>
<td>220.8 ± 45.2 (141–312)</td>
<td>34</td>
<td>49.1 ± 24.0¹ (9–97)</td>
<td>17</td>
<td>47.5 ± 27.9¹ (10–106)</td>
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<tr>
<td>T₄ (µg/dl)</td>
<td>34</td>
<td>13.5 ± 2.98 (8.04–18.5)</td>
<td>34</td>
<td>11.24 ± 2.40¹ (7.3–16.4)</td>
<td>17</td>
<td>11.07 ± 27.9¹ (6.9–15.2)</td>
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<tr>
<td>FT₄ (ng/dl)</td>
<td>25</td>
<td>1.05 ± 0.24 (0.7–1.7)</td>
<td>25</td>
<td>1.35 ± 0.23¹ (0.9–2.0)</td>
<td>13</td>
<td>1.35 ± 0.24¹ (1.1–1.9)</td>
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1 P < 0.001 vs paired maternal samples. 2 P < 0.005 vs paired venous cord samples.
3 P < 0.001 vs paired venous cord samples.

Equilibrium dialysis further confirmed the absence of TRH protein binding in cord serum.

In RIA binding-inhibition curves, dilutions of pooled cord blood extract showed good parallelism with synthetic TRH standard-dilutions, with dilutions of extract of pooled plasma obtained from normal adult subjects 20 min after iv administration of synthetic TRH, as well as with dilutions of hypothalamic extract (Fig. 2).

IR-TRH LM extracted from cord blood and synthetic TRH showed a similar elution pattern on Sephadex G-10 gel chromatography (Fig. 3) and a similar migration on paper electrophoresis (Fig. 4).

In vitro, umbilical cord blood was shown to degrade synthetic TRH only slowly, the TRH-degrading activity being markedly lower in cord serum as compared to maternal serum or control serum from adult non-pregnant women (Fig. 5).

In vitro degradation of endogenous IR-TRH LM present in cord serum could not be demonstrated, neither by cord serum itself (Table 2A), nor by adult donor serum (Table 2B). In opposition to the findings for cord IR-TRH LM, hypothalamus extracted IR-TRH was shown to be degraded by adult serum to a similar extend as synthetic TRH (Table 2D). When incubated together, the cord serum extract did not prevent degradation of synthetic TRH by adult serum (Table 2C), indicating...
Fig. 3.
Chromatography of an umbilical cord serum (20 ml) methanolic extract and synthetic TRH (10 ng) on a Sephadex G-10 column (60 x 1 cm) prepared and eluted with PBS-buffer (0.01 M; 0.15 M NaCl; pH 7.5). Radio-immunoassay performed on a 200 µl sample of each fraction.

Fig. 4.
Paper electrophoresis of an umbilical cord serum (3 ml) extract and synthetic TRH (1 ng) on Whatman 3 MM (0.05 M phosphate-buffer, pH 7.5; 300 V, 22 mA for 120 min).

Fig. 5.
Synthetic TRH (%) remaining in the incubation medium after incubation with cord serum, maternal serum or control serum from euthyroid non-pregnant women. Incubation of 200 pg TRH in 200 µl PBS-buffer (pH 7.5) with 300 µl serum at 37°C for 15, 30 and 60 min. Mean ± SD of 6 separate, but contemporaneous, incubations for each incubation-time Asterisks indicate significance levels for differences between cord serum, maternal serum and control serum; * P < 0.001 vs maternal and control serum; ** P < 0.005 vs control serum.
Table 2.

<table>
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<tr>
<th>Incubation experiment (120 min incubation; 37°C)</th>
<th>% remaining IR-TRH¹</th>
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<tr>
<td>A. Unextracted cord serum (~100 pg equiv. IR-TRH LM/ml)</td>
<td>102.3 ± 22.4 (n = 6)</td>
</tr>
<tr>
<td>B. Cord serum extracted IR-TRH LM (~200 pg equiv.) with adult serum</td>
<td>96.1 ± 5.7 (n = 7)</td>
</tr>
<tr>
<td>Synthetic TRH (200 pg) with adult serum</td>
<td>17.7 ± 2.0* (n = 7)</td>
</tr>
<tr>
<td>C. Cord serum extracted IR-TRH LM (~200 pg equiv.) + synthetic TRH (100 pg) with adult serum</td>
<td>77.8 ± 5.4*² (n = 6)</td>
</tr>
<tr>
<td>D. Hypothalamus extracted IR-TRH (~200 pg equiv.) with adult serum</td>
<td>7.1 ± 4.7* (n = 8)</td>
</tr>
<tr>
<td>Synthetic TRH (200 pg) with adult serum</td>
<td>8.5 ± 2.3* (n = 8)</td>
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¹ % of the IR-TRH initially present in 0 min (mean ± SD).
² % of total IR-TRH (cord serum IR-TRH LM + synthetic TRH).
* significant degradation (P < 0.001).

that the lack of degradation of cord IR-TRH LM is not due to the presence of an inhibiting substance in cord serum.

In an additional control experiment circulating IR-TRH LM (~200 pg equivalent) present in serum samples obtained from adult volunteers between 20 and 40 min following an iv injection of synthetic TRH was shown to be degraded during in vitro incubation: 55.2 ± 8.2% (n = 6) and 32.2 ± 3.1% IR-TRH remaining after direct incubation of unextracted serum and incubation of extracted TRH-IR with donor serum, respectively.

Discussion

The present study demonstrating the existence of an important positive foeto-maternal difference in levels of IR-TRH LM, confirms and largely extends the limited available data on this matter, showing elevated neonatal IR-TRH levels in a small number of subjects studied by Lombardi et al. (1978a). In accordance with the findings of Lombardi et al. (1978a) and Amino et al. (1981) for third trimester plasma, we found maternal IR-TRH levels similar to those in non-pregnant euthyroid women, the IR-TRH levels being in the same order of magnitude as those reported for adults in previous studies (Oliver et al. 1974; Saito et al. 1975; Mitsuma et al. 1976; Lombardi et al. 1978b; Andreassen et al. 1979) but lower than those found by Guignier et al. (1981) and by Mallik et al. (1982).

It has been reported that the human placenta contains significant amounts of substance with TRH-immunoreactivity (Shambaugh et al. 1979; Youngblood et al. 1980); our results, showing a positive arterio-venous IR-TRH difference in cord blood, suggest however that the umbilical cord IR-TRH LM is of foetal and not of placental or maternal origin.

Agar gel electrophoresis and equilibrium dialysis of cord serum pre-incubated with [¹²⁵I]TRH or [³H]TRH revealed no protein binding that could have accounted for the foeto-maternal differences in levels of IR-TRH LM.

Elevated cord serum levels of IR-TRH LM could be the result of high TRH production rate and/or low TRH-degrading activity in the foetal compartment.

With regard to production, mention should be made of recent reports showing a high foetal or neonatal TRH-content in some extrahypothalamic brain regions and extraneural tissue (Parker et al. 1981; Koivusalo 1981). As to serum TRH-degrading activity, we confirmed the low TRH-degrading activity in neonatal serum as compared to maternal and adult serum (Aratan-Spire & Czernichow 1980; Amino et al. 1981). However, we could neither demonstrate degradation of endogenous IR-TRH LM from cord serum when incubated with adult donor serum, this in contrast to synthetic and hypothalamus extracted TRH. In this respect it should be stressed that literature data on TRH-degrading activity in human serum always refer to the experiments with synthetic or tissue extracted TRH and not to circulating endogenous TRH.

In vitro serum destruction of circulating endogenous IR-TRH LM, shown in some species (Emerson & Utiger 1975; Jackson & Reichlin 1979), was never demonstrated in man. Recently, Mallik et al. (1982) have shown in vitro enzymatic degradation of affinity chromatographed peripheral (adult) blood TRH-LM by a bacterial pyroglutamate aminopeptidase. Notwithstanding our findings of an identical immunological behaviour, a similar elution pattern on gel chromatography
and a similar migration on paper electrophoresis, the lack of proven degradation of the methanol extracted cord IR-TRH LM by adult donor serum raises questions about the identity between this endogenous circulating IR-TRH LM and synthetic L-pyroglu-L his-L pro NH₂. The failure to demonstrate serum degradation of cord IR-TRH LM could be due to the fact that circulating IR-TRH LM is different from synthetic and hypothalamic TRH. Inadequate experimental conditions or the presence of a methanol extractable inhibitor of serum TRH-degeneration in cord serum seem improbable in view of the control experiments showing clearcut degradation of small quantities of synthetic TRH added to methanolic extracts of cord serum and of circulating IR-TRH LM extracted from serum obtained after parenteral administration of synthetic TRH. Previously Neary et al. (1978) had already shown that the low TRH-degrading activity in cord blood is not due to the presence of a dialysable inhibiting substance.

If different from synthetic and hypothalamic TRH, the measured endogenous IR-TRH LM is, however, not likely to be due to the presence of cross-reacting TRH serum-degradation products: indeed the antiserum used in the present RIA showed no cross-reactivity with the major putative TRH-degradation products tested and moreover all immunoassayable TRH disappears from the incubation medium when synthetic TRH is incubated with adult serum for a sufficiently long time. In the present as well in the previously reported TRH-RIA's (Leppäläuntoo 1976; Spindel & Wurtman 1980), only TRH analogues differing from L-pyroglu-L his-L pro NH₂ by substitution or alteration of the histidyl residue seem susceptible to interfere significantly with the assay and it should be mentioned that such changes in the TRH molecule can interfere with serum degradation without necessarily abolishing bioactivity (Morley et al. 1979).

Cord serum IR-TRH LM may represent a different biological form of TRH or may be a distinct peptide only structurally related to the TRH molecule.

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References


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