

Triiodothyronine Down-Regulates Thyrotropin-Releasing Hormone (TRH) Synthesis and Decreases pTRH-(160–169) and Insulin Releases from Fetal Rat Islets in Culture*

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ABSTRACT

TRH is localized with insulin in β -cells. It is synthesized as a prohormone containing five copies of TRH and seven cryptic peptides, including pro (p)-TRH-(160–169). Thyroid hormone regulates the expression of several genes encoding peptide hormones. We found that circulating T_3 concentrations were inversely correlated with TRH levels in two physiopathological situations. There are low concentrations of circulating thyroid hormone and very high concentrations of TRH and pTRH-(160–169) during development, and experimental hypothyroidism results in higher concentrations of prepro (pp)-TRH messenger RNA (mRNA) and TRH content in the adult rat pancreas than are present in the euthyroid pancreas.

The present study was carried out to investigate the interaction between T_3 and pancreatic TRH during the functional maturation of islets in culture and to validate the data obtained *in vivo*.

T_3 decreases ppTRH mRNA in islets in a dose-dependent manner. The primary impact of T_3 on islet function may be mediated by ppTRH mRNA, as short term T_3 treatment had no effect. Long term T_3 treatment reduced the islet TRH content and the amounts of pTRH-(160–169) and insulin released. This secretory pattern and coordinated regulation of pTRH-(160–169) and insulin suggests that pTRH-(160–169) plays a specific role in the regulation of insulin secretion. (*Endocrinology* 140: 4113–4119, 1999)

TRH WAS ORIGINALLY isolated from the hypothalamus (1, 2), but it is also synthesized in the islets of Langerhans and localized in insulin-containing cells (3–5). The TRH prohormone contains five copies of the TRH progenitor sequence, Gln-His-Pro Gly, linked by connecting peptides (6, 7). One such connecting peptide, pro (p)-TRH-(160–169), is reported to be biologically active (8, 9) and has been detected in the islets, but its secretory pattern is unknown (10). Unlike major islet hormones, however, the highest concentrations of TRH and pTRH-(160–169) are detected during the early development of neonatal rats (2–3 days after birth) (11). This period coincides with a marked growth of the β -cell population (12). This suggests that TRH is involved in the regulation or growth of fetal islets in an as yet undefined way. TRH and pTRH-(160–169) are, therefore, candidate phenotypic markers for monitoring the growth characteristics of islet β -cells. In man, the pancreatic TRH concentrations are highest between 6–8 weeks gestation, and they peak before insulin levels (13). Thus, this study may also be relevant to human islet development.

Hypothalamic TRH stimulates TSH secretion (1, 2). Pancreatic TRH is involved in the stimulation of glucagon secretion and the inhibition of exocrine pancreatic secretion (14, 15). However, despite its biological contribution as a regu-

latory peptide in the adult pancreas, the physiological significance of TRH in islet development remains an open question. Hence, a clear picture of the hormonal control of TRH gene expression may shed light on this point. We previously studied the regulation of islet TRH gene expression *in vivo* after chemical thyroidectomy. Experimentally induced hypothyroidism was associated with an increase in TRH messenger and peptide in rat islets (16–18).

Fetal islet cultures (19) provide the only available model for investigating the regulation of islet TRH gene expression and the possible impact of this regulation on islet development. We have therefore investigated the direct effect of T_3 on islet ppTRH messenger RNA (mRNA) concentrations, the TRH and pTRH-(160–169) contents and secretions, together with the concomitant regulation of insulin synthesis and secretion.

Materials and Methods

Preparation of islets

Fetal islets were prepared as described by Hellerström *et al.* (19). Briefly, fetuses were removed from pregnant Wistar rats at 21 days gestation. Day 0 was defined as the day on which mating occurred. Fetal pancreases were removed aseptically, placed in cold HBSS supplemented with 100 U/ml penicillin and 100 μ g/ml streptomycin, and minced. HBSS (4 ml) containing 6 mg/ml collagenase CLS 4 (Worthington Biochemical Corp., Freehold, NJ) was added to each of 4 centrifuge tubes, each containing 10–12 pancreases. The tubes were incubated in a shaking water bath at 37 C for 8 min. The resulting digested tissue was washed 3 times with cold HBSS, and the pellets were pooled and suspended in 500 μ l HBSS. Aliquots of this suspension (100 μ l) were placed in 50-mm plastic culture dishes and cultured for 5 days in 5 ml RPMI 1640 medium containing 11 mM glucose, 10% heat-inactivated

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FCS, 100 U/ml penicillin, and 100 µg/ml streptomycin. The culture dishes were kept at 37 C in a humidified atmosphere of 5% CO₂ and 95% air. The growth medium was replaced every day. The islets attached to the bottom of the culture dishes were then gently blown free with a sterilized Pasteur pipette under a stereomicroscope. The detached islets were cultured free floating in 50-mm petri dishes, which did not permit cell attachment (Falcon 1007, Falcon Plastics, Los Angeles, CA), in complete RPMI 1640 medium supplemented with 10% FCS that was changed every other day. The term culture refers to the free floating islets in this study.

Chronic exposure and cumulative release experiments. The islets were collected, washed with PBS, distributed in dishes (100 islets/dish), and cultured in RPMI medium supplemented with charcoal-stripped FCS (ch-FCS; 1% charcoal for 24 h at 4 C) and 1 mM bacitracin, with increasing concentrations of T₃ for 48 h in a humidified atmosphere of 5% CO₂-95% air.

Short term release experiments. The islets were collected and washed three times with PBS, and batches of 10 islets were incubated for 3 h at 37 C with various concentrations of T₃ in 1 ml HBSS supplemented with 0.1% BSA, 1 mM bacitracin, and 2.8 or 16.7 mM glucose.

At the end of chronic and short term release experiments, the islets and media were separated and treated for peptide or RNA determination. One thousand to 1500 neofomed islets were recovered from 10–15 pancreases. The number of islets per batch was routinely determined from the total insulin content per batch and the insulin content per islet, as previously described (10).

Islet extraction for TRH, pTRH-(160–169), and insulin determinations

After chronic or short term exposure to T₃ (48 or 3 h), an inhibitor cocktail (phenylmethylsulfonyl fluoride, 2-iodoacetamide, and EDTA; 1 mM each) was added, and the medium was removed. The islets were disrupted by sonication. The supernatant from the sonicated islets and the medium were then separately acidified with 1.7 M acetic acid, lyophilized, and kept for TRH, pTRH-(160–169), and insulin RIAs.

TRH was measured, using a specific antiserum, 4-B-18 (10), and [¹²⁵I]TRH (2200 Ci/mmol; New England Nuclear Corp., Boston, MA). The sensitivity of the assay was 5 fmol/tube, and the intra- and inter-assay coefficients of variation were 4% and 6%, respectively. pTRH-(160–169) was assayed using a specific antibody (7, 10). The sensitivity of the assay was 3.3 fmol/tube, and the intra- and inter-assay coefficients of variation were 3.5% and 7%, respectively. Insulin was measured using an antibody directed against a mixture of porcine and bovine insulin, with purified rat insulin as a standard (Novo Research Industries, Copenhagen, Denmark). [¹²⁵I]Insulin was prepared by the lactoperoxidase method and purified by reverse phase HPLC (10). The assay sensitivity was 15 pg/tube, and the coefficient of variation within and between assays was 10%. Results are expressed as femtomoles per islet for TRH and pTRH-(160–169) and nanograms per islet for insulin.

Extraction of islet RNA

Total RNA was extracted from islets by a single step method (20). The islets were washed twice in PBS, suspended in guanidine thiocyanate-phenol-chloroform (50:25:25, vol/vol/vol), and precipitated twice with isopropanol. The RNA was then dissolved in distilled water. The integrity and yield of the RNA extracts were checked by absorbance at 260 and 280 nm and by electrophoresis in 1% agarose gel containing 0.01% ethidium bromide under nondenaturing conditions. The recovery of total RNA was 10 µg/400 islets.

Northern blot analysis

Samples of total RNA (4 µg) were electrophoresed under denaturing conditions in 1% agarose gels containing 2.2 M formaldehyde. The nucleic acids were vacuum blotted (Vacuum Blotter, Appligene, Strasbourg, France) to Hybond-N nylon membranes and cross-linked by UV irradiation. The membranes were hybridized with complementary DNA (cDNA) probes labeled by random priming to a specific activity of 10⁹ cpm/µg (21). Membranes were prehybridized at 65 C in 7% lauryl sulfate, 200 mM phosphate buffer (pH 7.2), 1 mM EDTA, and 1% BSA and

then hybridized with the ³²P-labeled ppTRH cDNA probe (10⁷ cpm/ml) for 16 h at 65 C. The membranes were then washed three times for 15 min each time at 65 C in 0.5 × SSC (standard saline citrate) containing 0.1% SDS and autoradiographed using Kodak XAR 5 film (Eastman Kodak Co., Rochester, NY) at –80 C. Blots were stripped by washing at 95 C with 0.01 × SSC-0.1% SDS, 1% glycerol, and 1 mM EDTA for 30 min and were reprobated with the ³²P-labeled proinsulin (pIns) cDNA (10⁶ cpm/ml). A cDNA probe encoding 18S RNA was used to normalize the intensity of the hybridization signals and to allow for minor differences in recovery. The relative densities of the bands were determined using a computerized image analysis system, with Image 1.57 (NIH, public domain).

Probe synthesis and labeling

Prepro (pp)-TRH cDNA. A 1241-bp *EcoRI*-*Pst*I fragment of ppTRH cDNA inserted into the plasmid vector pSP65 was excised with *EcoRI* and *Hind*III.

pIns cDNA. A 300-bp fragment of proinsulin cDNA inserted into pUC was excised with *Bam*HI and *EcoRI* (3, 22).

18S cDNA. A 1975-bp fragment of 18S cDNA inserted into pSP64 was excised with *Sal*I and *EcoRI* (23).

Each insert was separated electrophoretically in a 1% low melting point agarose gel (BRL, Gaithersburg, MD). An aliquot (60 ng) of purified insert was used as a template to prime DNA synthesis *in vitro* with [³²P]deoxy-CTP and the Klenow fragment of DNA polymerase (Prime-a-Gene Labeling System, Promega Corp., Madison, WI).

Statistical analyses

All results are the mean ± SE. The statistical significance of the differences was determined by one-way ANOVA and Student's *t* test.

Results

In pilot experiments, we tested the viability, insulin and TRH contents, and secretory capacity of fetal islets grown in various conditioned media. Islets maintained for 24 or 48 h in RPMI supplemented with 10% ch-FCS had insulin and TRH contents similar to those of islets maintained under standard culture conditions. Similarly, islets maintained for 24 h in serum-free medium containing 0.1% BSA had insulin and TRH contents similar to those of islets cultured in standard medium. In contrast, insulin and TRH contents of islets incubated for 48 h in serum-free medium containing 0.1% BSA were much lower than those of islets maintained in standard culture conditions (Table 1).

The secretory capacity and content of the islets maintained in RPMI supplemented with ch-FCS were compared with those of islets grown under standard culture conditions (Fig 1). Batches of 10 islets were incubated for 3 h at 37 C in HBSS containing 2.8 or 16.7 mM glucose. The content of the islets

TABLE 1. Insulin and TRH content of islets maintained for 24 and 48 h in RPMI containing FCS, charcoal-stripped FCS (ch-FCS), or 0.1% BSA

	Insulin (ng/islet)		TRH (fmol/islet)	
	24 h	48 h	24 h	48 h
10% FCS	19.2 ± 1.5	23.1 ± 3.3	15.9 ± 1.3	25.0 ± 3.0
10% ch-FCS	20.1 ± 2.2	21.7 ± 1.6	14.3 ± 1.1	20.9 ± 2.5
0.1% BSA	20.1 ± 2.2	10.5 ± 0.3	17.9 ± 3.3	8.2 ± 0.3

The preparation of islets, experimental conditions, and extraction procedures were described in *Materials and Methods*. Insulin and TRH contents were measured by specific RIAs. Results are the mean ± SE for five to seven independent determinations.

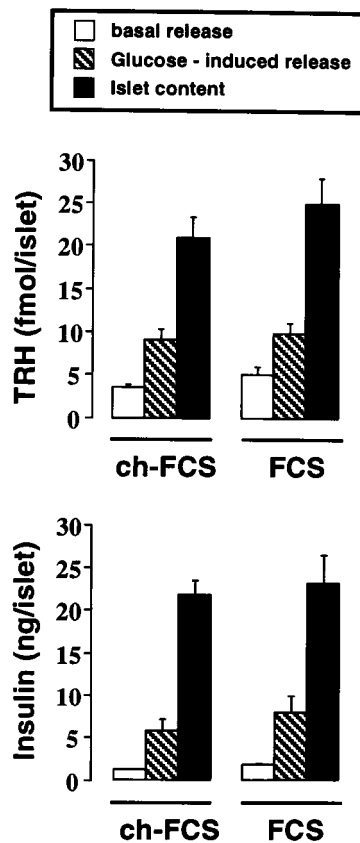


FIG. 1. TRH and insulin release and content of fetal islets cultured for 48 h in medium supplemented with either FCS or ch-FCS. The free floating islets were precultured for 5 days and then for 2 days in RPMI medium supplemented with 10% FCS. The islets were switched from standard culture medium to RPMI supplemented with ch-FCS for 2 additional days, but the intraexperiment controls were kept in standard medium. The islets were rinsed with PBS, and batches of 10 islets were incubated for 3 h at 37 C in HBSS containing 2.8 or 16.7 mM glucose, a cocktail of protease inhibitors was added, and the islets were separated from the release medium. The islets were sonicated, and sonicates and medium were acidified with 1.7 M acetic acid, lyophilized, and assayed for TRH and insulin. The contents of the islets remained unchanged. Values are the mean \pm SE for 15 observations from 3 independent experiments.

remained unchanged. The TRH release pattern was similar to that of insulin, and both were stimulated by 16.7 mM glucose (Fig. 1). The viability of the islets maintained in medium supplemented with ch-FCS for up to 6 days was tested. The patterns of insulin content over time for islets in medium supplemented with either ch-FCS or normal FCS were similar (data not shown). Therefore, islets treated for 48 h and used for peptide extraction were switched from standard medium to RPMI medium supplemented with ch-FCS, whereas those treated for 24 h and used for RNA extraction were routinely switched to serum-free medium containing 0.1% BSA, except in pilot experiments, in which RPMI supplemented with ch-FCS was also tested, so that the results could be compared.

Effects of T₃ on the steady-state contents of ppTRH and pIns mRNAs in islets cultured for 2 and 7 days (Fig. 2). Islets cultured for 2 and 7 days (d-2 and d-7) were maintained for 24 h with or without T₃ (10⁻⁸ M) in RPMI medium containing 0.1% BSA

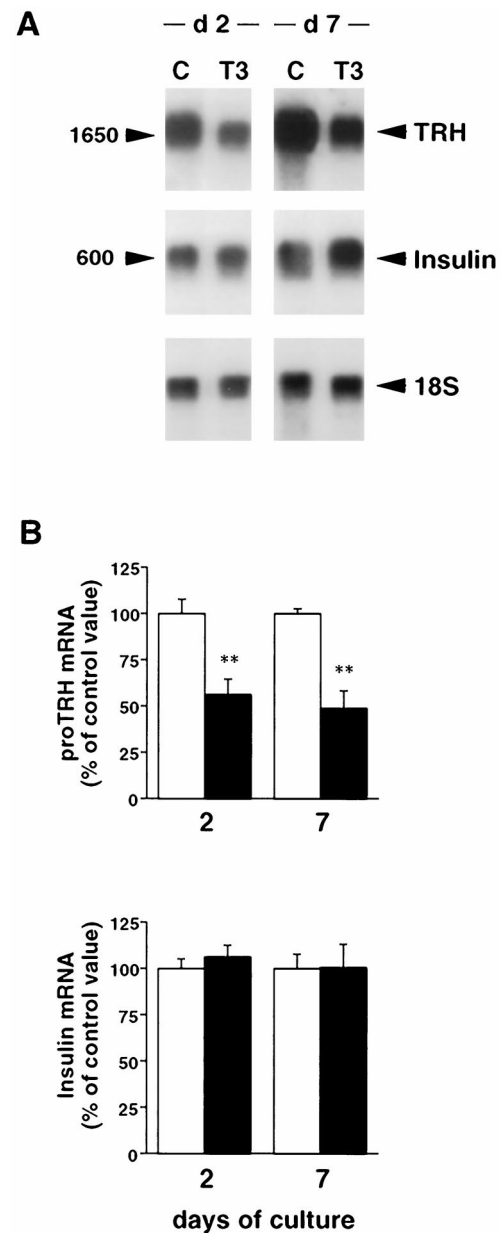


FIG. 2. Effect of T₃ on the steady-state levels of TRH and insulin mRNAs from neofomed fetal rat islets in d-2 and d-7 cultures. Neofomed islets were cultured free floating for 2 (d-2) or 7 days (d-7) in petri dishes, which did not permit cell attachment, in complete RPMI medium changed every other day. The islets were rinsed with PBS and maintained for the last 24 h in RPMI supplemented with 10% ch-FCS or in serum-free RPMI containing 0.1% BSA with or without T₃ (10⁻⁸ M). Total islet RNA was extracted, and samples (4 μ g) were loaded and size-separated on denaturing agarose gels, transferred to Hybond-N nylon membranes, and hybridized successively with random primed ³²P-labeled cDNA encoding TRH, insulin, and 18S RNAs. The membranes were autoradiographed with Kodak XAR 5 film at -80 C for 48 h for ppTRH and 6 h for pIns mRNA. A, The autoradiograph shown is representative of five other blots. B, Densitometric analysis of the hybridization signals for TRH and insulin from d-2 and d-7 islet culture with (black columns) and without (white columns) T₃. The optical densities of the hybridization signals for TRH and insulin from independent experiments were averaged and corrected for 18S mRNA. The relative densities of the bands were determined by computerized image analysis (Image 1.57, NIH, public domain). Data are the mean \pm SE for three to five independent experiments. **, P < 0.001 vs. control (no T₃ added).

or 10% ch-FCS. The control levels of ppTRH and pIns mRNAs and the magnitude of the decrease in ppTRH mRNA were similar regardless of the medium used or the duration of the treatment (24 or 48 h). Adding 10^{-8} M T₃ to d-2 and d-7 islets reduced the amounts of ppTRH mRNA to below the control values. Expressed as a percentage of the control value (100%), the magnitude of the decreases in d-2 and d-7 islets were similar: 56 ± 8 for d-2 and 48 ± 10 for d-7 islets. The amount of pIns mRNA was not affected.

Dose-dependent effects of T₃ (Fig. 3). The dose-dependent effects of T₃ treatment on ppTRH and pIns mRNAs were studied using d-2 islets kept for 24 h in serum-free RPMI containing 0.1% BSA. T₃ dose-dependently reduced the steady-state concentrations of ppTRH mRNA, as estimated by Northern blot analysis. Adding T₃ to the islet culture medium for 24 h produced much lower ppTRH mRNA concentrations than those in the control culture. The densitometric analysis was performed on six blots, and the results were expressed as the mean \pm SE. The maximal decrease was about 50% of the control value.

Long-term T₃ treatment and the regulation of TRH, pTRH-(160–169), and insulin (Fig. 4). The islets were randomly distributed (100 islets/dish) and maintained for 48 h in medium containing 10% ch-FCS and various concentrations of T₃. The media and islets were separately treated for peptide measurement. Each point is the mean \pm SE of six independent determinations.

Changes in cumulative release. TRH release (femtomoles per dish/48 h) was similar for all concentrations except the highest dose of T₃ (10^{-7} M): $61.9 \pm 13.7\%$ of the control value, for six determinations.

Adding T₃ to the islet culture medium for 48 h produced a dose-dependent decrease in cumulative pTRH-(160–169) release (femtomoles per dish/48 h). Expressed as a percentage of the control value (100%), the decrease in cumulative pTRH-(160–169) was 74.9 ± 6.4 for 10^{-13} M T₃ ($P < 0.05$), 60.9 ± 5.5 for 10^{-11} M T₃ ($P < 0.01$), 50.5 ± 5.3 for 10^{-9} M T₃ ($P < 0.005$), and 36.7 ± 3.7 for 10^{-7} M T₃ ($P < 0.005$). T₃ caused a smaller, but significant, decrease in cumulative insulin release (nanograms per dish/48 h). The percent decreases from the control value (100%) were 79.1 ± 3.8 for 10^{-13} M T₃ ($P < 0.05$), 72.9 ± 2.1 for 10^{-11} M T₃ ($P < 0.005$), 74.6 ± 1.4 for 10^{-9} M T₃ ($P < 0.005$), and 62.6 ± 3.1 for 10^{-7} M T₃ ($P < 0.001$).

Changes in islet TRH, pTRH-(160–169), and insulin contents. Adding T₃ to the islet culture medium for 48 h resulted in a dose-dependent decrease in TRH content. The percent decreases in TRH content from the control value (100%) were 85.3 ± 9.5 for 10^{-13} M T₃, 59.1 ± 9.4 for 10^{-11} M T₃ ($P < 0.05$), 54.8 ± 7.6 for 10^{-9} M T₃ ($P < 0.05$) and 41.1 ± 4.3 for 10^{-7} M T₃ ($P < 0.01$). The pTRH-(160–169) content was unaffected, and that of insulin was decreased to $78.4 \pm 6.0\%$ of the control value ($P < 0.05$) only by the highest dose (10^{-7} M) of T₃.

Short-term T₃ treatment and the regulation of TRH, pTRH-(160–169), and insulin (Fig. 5). Batches of 10 untreated islets were used to study the effect of T₃ on TRH, pTRH-(160–169), and insulin releases and contents. They were incubated for 3 h at

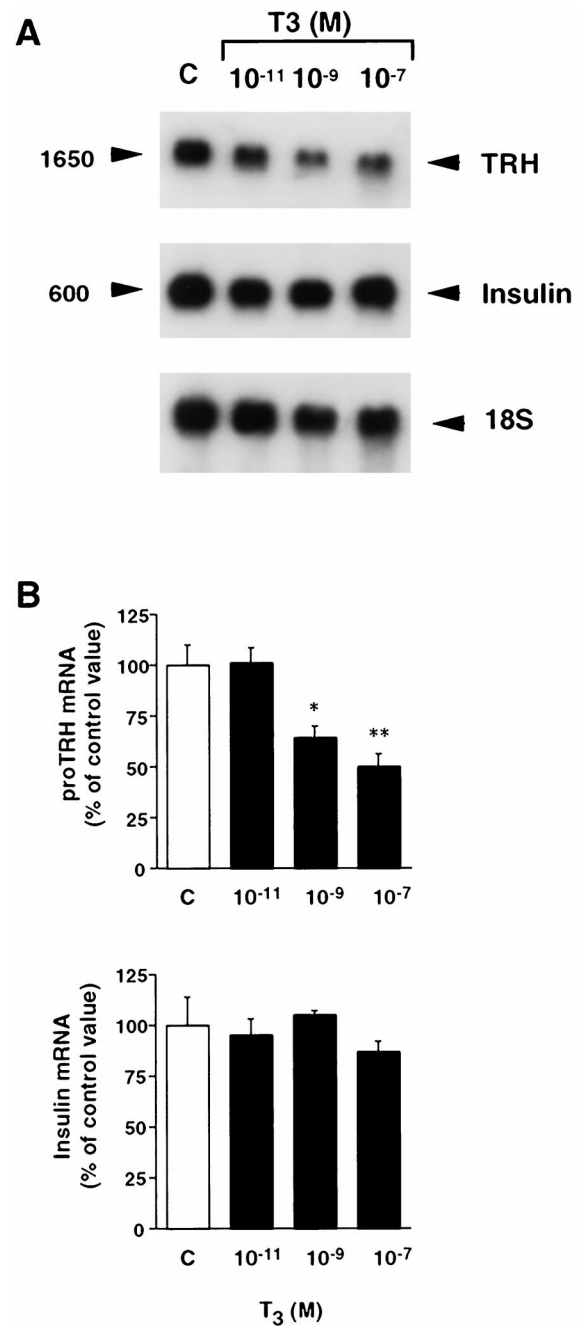


FIG. 3. Dose-dependent effect of T₃ on islet ppTRH and pIns mRNA levels. Precultured islets were cultured for 2 days in total RPMI medium containing 10% FCS. The islets were maintained for 24 h in serum-free RPMI containing 0.1% BSA and various concentrations of T₃. Total islet RNA was then extracted, and samples (4 μ g) were loaded and electrophoresed onto 1% denaturing agarose gels, transferred to Hybond-N nylon membranes, and hybridized successively with random primed ³²P-labeled cDNAs encoding TRH, insulin, and 18S RNAs. Autoradiographs were prepared using Kodak XAR 5 film at -80 C. The exposure time was 48 h for ppTRH and 6 h for pIns mRNA. A, The autoradiograph shown is one of six similar blots. B, Densitometric analysis of the hybridization signals for ppTRH and pIns mRNAs. The optical densities of the hybridization signals of TRH and insulin were averaged and corrected for 18S mRNA. The relative densities of the bands were determined by computerized image analysis (Image 1.57, NIH, public domain). Results are the mean \pm SE of six independent determinations. *, $P < 0.01$; **, $P < 0.001$ [vs. control (without T₃)].

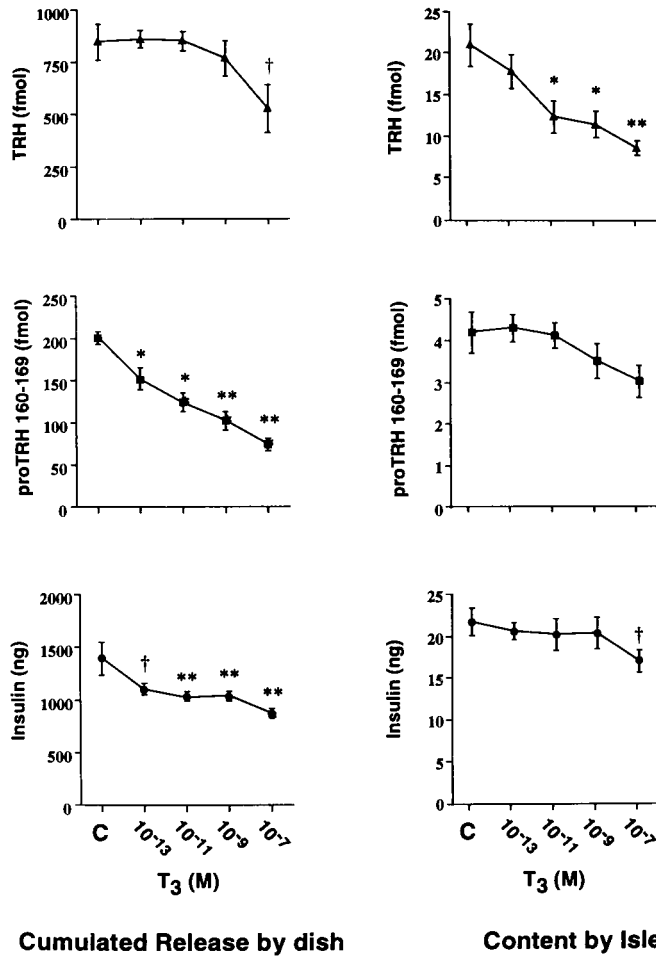


FIG. 4. Effect of chronic T₃ treatment on the cumulative releases and contents of TRH, pTRH-(160-169), and insulin of islets in culture. Precultured islets were cultured free floating in total RPMI medium supplemented with 10% FCS for 48 h, rinsed twice with PBS, distributed randomly in dishes (100 islets/dish), and maintained in RPMI medium containing 10% ch-FCS and various concentrations of T₃ for an additional 48 h. The media and islets were separated by centrifugation, acidified, lyophilized, and assayed for TRH, pTRH-(160-169), and insulin. Each point is the mean ± SE (n = 6). †, P < 0.05; *, P < 0.01; **, P < 0.005; ***, P < 0.001.

37 C in HBSS containing 2.8 or 16.7 mM glucose and various concentrations of T₃. The TRH, pTRH-(160-169), and insulin contents were unaffected. The molar ratio of TRH/pTRH-(160-169) in the islets was about 5. The contents and releases of the peptides were expressed as the mean ± SE of the indicated number of determinations.

TRH content was 17.7 ± 2.1 fmol/islet (n = 12). The basal and glucose-induced TRH releases were 4.5 ± 0.2 (n = 5; basal) and 10.6 ± 0.5 (n = 11; glucose-induced) fmol/islet·3 h; the release/content ratios were 0.25 (basal) and 0.60 (glucose-induced) regardless of whether T₃ was present or absent.

The pTRH-(160-169) content was 3.7 ± 0.6 fmol/islet (n = 23). The basal and glucose-induced pTRH-(160-169) releases were 0.6 ± 0.1 (n = 10; basal) and 2.1 ± 0.4 (n = 10; glucose-induced) fmol/islet·3 h; the release/content ratios were 0.16 (basal) and 0.55 (glucose-induced) regardless of whether T₃ was present or absent.

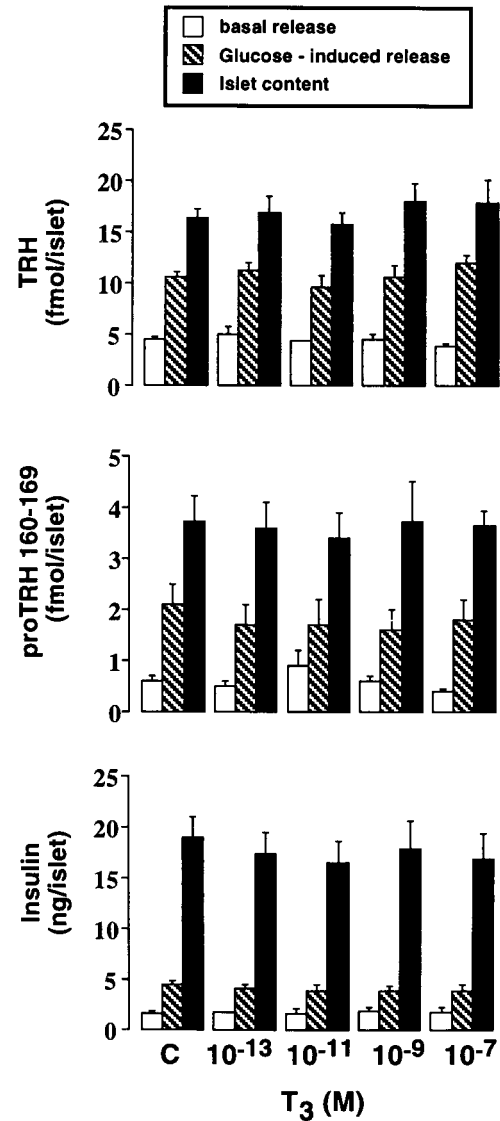


FIG. 5. Effect of T₃ on the acute release of islet TRH, pTRH-(160-169), and insulin. Precultured islets were cultured free floating in RPMI medium containing 10% FCS for 2 days. Batches of 10 islets were then incubated for 3 h at 37 C in HBSS containing 2.8 or 16.7 mM glucose. The medium and islets were separated, acidified, lyophilized, and assayed for TRH, pTRH-(160-169), and insulin. Values are the mean ± SE (n = 10-15 for three independent experiments).

Insulin content was 16.2 ± 4.5 ng/islet (n = 32). The basal and glucose-induced insulin releases were 1.60 ± 0.22 (n = 12; basal) and 4.53 ± 0.32 (n = 20; glucose-induced) ng/islet/3 h. The release/content ratios were 0.10 (basal) and 0.27 (glucose-induced).

T₃ had no acute (3 h) effect on the content or release of TRH, pTRH-(160-169), or insulin.

Discussion

We have examined the chronic and dose-dependent effects of T₃ on TRH and insulin gene expression in fetal islets in culture as a first step toward defining the mechanisms involved in the thyroid hormone-dependent regulation of TRH and insulin synthesis and release. These experiments were

also designed to validate *in vivo* observations made on hypothyroid rat pancreas (16–18) and to gain insight into the effect of T₃ on islet TRH synthesis.

Fetal islets in culture are a valuable experimental model of β -cell function. The characteristics of fetal islets in culture have been extensively described. The culture of fetal cells makes it possible to prepare large numbers of islets free of exocrine tissue contamination and fibroblast-like cells (19). A histological description of these islets has been reported (24). The endocrine cells are first attached as a monolayer and then progressively reorganize into islets (25). The neoformed fetal islets are functionally immature, and so have a developing, rather than adult islet hormone secretory pattern. Their fetal character is indicated by their low sensitivity to glucose (26). Unlike intact, vascularized islets *in situ*, they can only undergo interstitial (paracrine-like) interactions. Studies of this primary organ culture may provide insight into islet development.

Fetal islets in culture have a higher proportion of β -cells than age-matched, neonatal islets *in situ* (27). Interestingly, they also have a persistent high TRH concentration throughout the culture period (28). They are, therefore, suitable for studying β -cell function. We find that T₃ reduces TRH gene expression in cultured fetal islets. The decrease is similar when RNA from d-2 or d-7 islet cultures is assayed. We, therefore, performed all studies on islets cultured for 2 days to obtain the highest proportion of β -cells and to reduce the corrective effect of non- β -cells, especially on the secretory pattern of insulin (24, 26). The reduction in the steady-state concentrations of ppTRH mRNA and TRH were dose dependent, whereas the levels of pIns mRNA and insulin were unaffected by T₃.

Treatment for 48 h with various doses of T₃ resulted in a lower TRH content and lower amounts of cumulative pTRH-(160–169) and insulin released. To our knowledge, the secretory pattern of pancreatic pTRH-(160–169) has not been studied before, and its biological effects on islet function has not previously been reported. In agreement with a previous report, the molar ratio of TRH to pTRH-(160–169) was 5% in fetal islets (10). The molar ratio of TRH to insulin contents was about 0.5%, and that of pTRH-(160–169) to insulin was 0.1%. This study also shows that pTRH-(160–169) is secreted and that its secretory pattern is similar to that of insulin. The release of pTRH-(160–169) is glucose sensitive: 16.7 mM glucose stimulated its release, giving 3 times more release in short term experiments.

We demonstrated here that T₃ down-regulates TRH gene expression in the cultured fetal islets. To increase the sensitivity of the islets to thyroid hormone, media devoid of T₃ were used during the treatment. Low levels of T₃ are indeed sufficient to promote a decrease in TRH gene expression. The reduction in steady-state concentrations of ppTRH mRNA was dose dependent, whereas the levels of pIns mRNA were unaffected by T₃.

The release of pTRH-(160–169) is, like that of TRH and insulin, unaffected by short term T₃ treatment. Chronic T₃ treatment dose dependently inhibited pTRH-(160–169) and insulin release and selectively affected TRH content. The inhibition of insulin release is not due to a decrease in the insulin store (unchanged) or to a membrane effect (unaffected), as shown by the short term release experiments. The similarity of the secre-

tory patterns of pTRH-(160–169) and insulin suggests that pTRH-(160–169) is involved in insulin secretion. A direct test is now needed to address this question. Taking into account the elevated concentrations of endogenous pTRH-(160–169), one appropriate approach may be immunoneutralization experiments using anti-TRH-(160–169) serum. This has been used to determine the effect of islet TRH on glucagon secretion (14). pTRH-(160–169) has also been detected in pituitary cells and has been reported to potentiate the action of TRH on TSH and PRL secretion (8, 9).

T₃ selectively down-regulates the expression of the TRH gene, but has no effect on pIns mRNA or insulin contents. In contrast to its effect on islets, thyroid hormone up-regulates the cell contents of ppTRH mRNA and TRH in anterior pituitary cells in culture (29), suggesting tissue- or cell-specific regulation of the TRH gene.

The T₃-dependent inhibition of TRH gene expression in cultured islets is consistent with recent data obtained with hypothyroid rats. In this *in vivo* experimental model, the steady-state concentrations of islet ppTRH mRNA and TRH contents markedly increased, and T₃ replacement restored the euthyroid levels (16). We also found twice as much basal secretion of TRH in isolated perfused pancreas from hypothyroid rats as in euthyroid pancreas, but significantly less insulin secretion (data not shown). Therefore, the regulation of TRH in fetal islets culture by T₃ mirrors that of the adult islets from hypothyroid rats, except for the secretory patterns. This difference may be due to the functional immaturity of fetal islets. The regulation of islet TRH release by glucose is also the opposite pattern to that of insulin in the adult islets, but similar to that of insulin in the neonatal islets *in situ* and fetal islets in culture (10). Experimental hypothyroidism is associated with increased ppTRH mRNA (30, 31) and TRH release (32, 33) in the rat hypothalamus. These thyroid status-associated effects are all reversible by T₃ replacement, indicating that they were mainly due to the circulating thyroid hormone. Consistent with this, unilateral implants of T₃ directly into the hypothalamus regulate TRH synthesis by negative feedback (34).

Interestingly, the hypothyroidism produced by targeted disruption of the ppTRH gene also involves a significant decrease in insulin secretion, but thyroid hormone replacement does not correct the deficit in insulin secretion (35).

It is not known whether T₃ regulates TRH gene expression transcriptionally, by changing ppTRH mRNA stability, or both. There are, however, several arguments for a classic genomic action of T₃: 1) T₃ causes parallel decreases in TRH, pTRH-(160–169), and ppTRH mRNA levels, indicating that the effect is probably not posttranslational; 2) T₃ has no acute effect on the content or/and release of TRH and pTRH-(160–169) during short term incubations, suggesting that T₃ acts primarily at the transcriptional level; and 3) we have previously shown that there are unpaired TRH-degrading activity (TRH-DA) and high concentrations of TRH in the hypothyroid rat pancreas (17, 18). We have also shown that administering T₃ *in vivo* increases TRH-DA and decreases the TRH content (36, 37). Similar decreases in TRH content are observed with purified fetal islets, almost free of degrading enzymes, so the initial impact of T₃ is probably at the transcriptional level, although fine regulation of the TRH concentration by TRH-DA cannot be excluded. The decrease in

islet ppTRH mRNA mirrors at least partly that of the islet TRH and pTRH-(160–169) contents and releases. 4) The presence of nuclear T₃ receptors in the pancreas (38) suggests that T₃ has a direct effect on the islet TRH gene. Furthermore, T₃ receptor-binding site consensus sequences have been identified in the TRH promoter that may be the target of the direct action of T₃ (39–41). T₃ may differentially regulate TRH gene expression by inhibiting or stimulating a *trans*-acting factor (42) or by tissue-specific regulation of separate thyroid hormone receptor subspecies (43). The mechanisms underlying this differential regulation have yet to be investigated. The pathophysiological relevance of the effect of thyroid hormone on islet function and development is unknown.

This study, by documenting the thyroid-dependent regulation of developing islets, provides the basis for examining the nuclear T₃ effect on the ontogeny of pancreatic hormones. This concept has been investigated separately using fetal islets transfected with TRH regulatory sequences.

References

- Boler J, Enzman F, Folkers K, Bowers CY, Schally AV 1969 The identity of chemical and hormonal properties of the thyrotropin-releasing hormone and pyroglutamyl-histidyl-proline amide. *Biochem Biophys Res Commun* 37:705–710
- Burgus R, Dunn TF, Desiderio D, Guillemin R 1969 Structure moléculaire du facteur hypothalamique hypophysiotrope TRF d'origine ovine: mise en évidence par spectrométrie de masse de la séquence PGA-His-ProNH₂. *C R Acad Sci [D] (Paris)* 269:1870–1873
- Aratan-Spire S, Scharfmann R, Lechan RM, Armen H, Tashjian J 1990 pro-TRH gene expression by fetal pancreatic islets in culture. *Biochem Biophys Res Commun* 168:952–958
- Leduque P, Aratan-Spire S, Wolf B, Dubois P, Czernichow P 1987 Localization of thyrotropin-releasing hormone- and insulin-immunoreactivity in the pancreas of neonatal rats after injection of streptozotocin at birth. *Cell Tissue Res* 248:89–94
- Leduque P, Aratan-Spire S, Scharfmann R, Basmaciogullari A, Czernichow P, Dubois PM 1989 Coexistence of thyrotropin-releasing hormone and insulin in cultured fetal rat islets: a light and electron microscopic immunocytochemical study during islet neofunction. *Biol Cell* 66:291–296
- Lechan RM, Wu P, Jackson IMD, Wolf H, Cooperman S, Mandel G, Goodman RH 1986 Thyrotropin-releasing hormone precursor: characterization in rat brain. *Science* 231:159–161
- Bulant M, Delfour A, Vaudry H, Nicolas P 1988 Processing of thyrotropin-releasing hormone prohormone (pro-TRH) generates pro-TRH-connecting peptides. *J Biol Chem* 263:17189–17196
- Bulant M, Roussel J, Astier H, Nicolas P, Vaudry H 1990 Processing of thyrotropin-releasing hormone prohormone (pro-TRH) generates a biologically active peptide, prepro-TRH-(160–169), which regulates TRH-induced thyrotropin secretion. *Proc Natl Acad Sci USA* 87:4439–4443
- Carr FE, Reid AH, Wessendorf MW 1990 A cryptic peptide from the preprothyrotropin-releasing hormone precursor stimulated thyrotropin gene expression. *Endocrinology* 133:809–814
- Ebiou JC, Bulant M, Nicolas P, Aratan-Spire S 1992 Pattern of thyrotropin-releasing hormone secretion from the adult and neonatal rat pancreas: comparison with insulin secretion. *Endocrinology* 130:1371–1380
- Aratan-Spire S, Wolf B, Portha B, Bailbé D, Czernichow P 1984 Streptozotocin treatment at birth induces a parallel depletion of thyrotropin-releasing hormone and insulin in the rat pancreas during development. *Endocrinology* 114:2369–2373
- Bouwens L, Wang R-N, Blay ED, Pipeleers DG, Klöppel G 1994 Cytokeratins as markers of ductal cell differentiation and islet neogenesis in the neonatal rat pancreas. *Diabetes* 43:1279–1283
- Leduque P, Aratan-Spire S, Czernichow P, Dubois PM 1986 Ontogenesis of thyrotropin-releasing hormone in the human fetal pancreas. *J Clin Invest* 78:1028–1034
- Ebiou JC, Grouselle D, Aratan-Spire S 1992 Antithyrotropin-releasing hormone serum inhibits secretion of glucagon from isolated perfused rat pancreas: an experimental model for positive feedback regulation of glucagon secretion. *Endocrinology* 131:765–771
- Fragner P, Passet O, Bernad N, Martinez J, Aratan-Spire S 1997 A new biological contribution of cyclo(His-Pro) to the peripheral inhibition of pancreatic secretion. *Am J Physiol* 273:E1127–E1132
- Fragner P, Quette J, Aratan-Spire S 1998 Thyroid status and the regulation of thyrotropin-releasing hormone synthesis in rat pancreatic islets. Comparison with insulin regulation. *Biochem Biophys Res Commun* 247:564–568
- Wolf B, Aratan-Spire S, Czernichow P 1984 Hypothyroidism increases pancreatic thyrotropin-releasing hormone concentrations in adult rats. *Endocrinology* 114:1334–1337
- Aratan-Spire S, Wolf B, Czernichow P 1984 Effects of hypo- and hyperthyroidism on pancreatic TRH-degrading activity and TRH concentrations in developing rat pancreas. *Acta Endocrinol (Copenh)* 106:209–214
- Hellerström C, Lewis NJ, Borg H, Johnson R, Freinkel N 1979 Method for large-scale isolation of pancreatic islets by tissue culture of fetal rat pancreas. *Diabetes* 28:769–776
- Chomczynski P, Sacchi N 1987 Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 162:156–159
- Church GM, Gilbert W 1984 Genomic sequencing. *Proc Natl Acad Sci USA* 81:1991–1995
- Villa-Komaroff L, Efstratiadis A, Broome S, Lomedico P, Tizard R, Naber SP, Chick WL, Gilbert W 1978 A bacterial clone synthesizing proinsulin. *Proc Natl Acad Sci USA* 75:3727–3731
- Raynal F 1984 Complete nucleotide sequence of mouse 18S RNA gene comparison with other available homologs. *FEBS Lett* 167:263–268
- Dudek RW, Freinkel N, Lewis NJ, Hellerstrom C, Johnson RC 1980 Morphologic study of cultured pancreatic fetal islets during maturation of the insulin stimulus-secretion mechanism. *Diabetes* 29:15–21
- Mourmeaux JL, Remacle C, Henquin JC 1985 Morphological and functional characteristics of islets neofunctioning during tissue culture of fetal rat pancreas. *Mol Cell Endocrinol* 39:237–246
- Freinkel N, Lewis NJ, Johnson R, Swenne I, Bone A, Hellerstrom C 1984 Differential effects of age versus glycaemic stimulation on the maturation of insulin stimulus-secretion coupling during culture of fetal rat islets. *Diabetes* 33:1028–1038
- Leduque P, Scharfmann R, Chayvialle JA, Aratan-Spire S, Basmaciogullari A, Czernichow P, Dubois PM 1992 Insulin, glucagon and somatostatin in fetal rat islets maintained free-floating in culture: immunocytochemistry and radioimmunoassay. *Biol Cell* 74:179–185
- Scharfmann R, Leduque P, Aratan-Spire S, Dubois P, Basmaciogullari A, Czernichow P 1988 Persistence of peptidylglycine α -amidating monooxygenase activity and elevated thyrotropin-releasing hormone concentrations in fetal rat islets in culture. *Endocrinology* 123:1329–1334
- Bruhn TO, Rondeel JMM, Bolduc TG, Jackson IMD 1994 Thyrotropin-releasing hormone gene expression in the anterior pituitary. III. Stimulation by thyroid hormone: potentiation by glucocorticoids. *Endocrinology* 134:826–830
- Segerson TP, Kauer J, Wolfe HC, Mobtaker H, Wu P, Jackson IMD, Lechan RM 1987 Thyroid hormone regulates TRH biosynthesis in the paraventricular nucleus of the rat hypothalamus. *Science* 238:78–80
- Koller KJ, Wolff RS, Warden MK, Zoeller RT 1987 Thyroid hormones regulate levels of thyrotropin-releasing hormone mRNA in the paraventricular nucleus. *Proc Natl Acad Sci USA* 84:7329–7333
- Rondeel JMM, Greef WJD, Klootwijk W, Visser TJ 1992 Effects of hypothyroidism on hypothalamic release of thyrotropin-releasing hormone in rats. *Endocrinology* 130:651–656
- Dahl GE, Evans NP, Thrun LA, Karsch FJ 1994 A central negative feedback action of thyroid hormones on thyrotropin-releasing hormone secretion. *Endocrinology* 135:2392–2397
- Dyess EM, Segerson TP, Liposits Z, Paull WK, Kaplan MM, Wu P, Jackson IMD, Lechan RM 1988 Triiodothyronine exerts direct cell-specific regulation of thyrotropin-releasing hormone gene expression in the hypothalamic paraventricular nucleus. *Endocrinology* 123:2291–2297
- Yamada M, Saga Y, Shibusawa N, Hirato J, Murakami M, Iwasaki T, Hashimoto K, Satoh T, Wakabayashi K, Taketo MM, Mori M 1997 Tertiary hypothyroidism and hyperglycemia in mice with targeted disruption of the thyrotropin-releasing hormone gene. *Proc Natl Acad Sci USA* 94:10862–10867
- Aratan-Spire S, Moilanen K, Czernichow P 1983 Postnatal developmental pattern of thyrotropin-releasing hormone-degrading activity in rat plasma, hypothalamus and liver: role of triiodothyronine. *J Endocrinol* 97:408–418
- Scharfmann R, Ebiou J-C, Morgat J-L, Aratan-Spire S 1990 Thyroid status regulates particulate but not soluble TRH-degrading pyroglutamate aminopeptidase activity in rat liver. *Acta Endocrinol (Copenh)* 123:84–89
- Lee JT, Lebenthal E, Lee P-C 1989 Rat pancreatic nuclear thyroid hormone receptor: characterization and postnatal development. *Gastroenterology* 96:1151–1157
- Lee SL, Stewart K, Goodman RH 1988 Structure of gene encoding rat thyrotropin releasing hormone. *J Biol Chem* 263:16604–16606
- Stevenin B, Lee SL 1995 Hormonal regulation of the thyrotropin-releasing hormone (TRH) gene. *Endocrinologist* 5:286–296
- Satoh T, Yamada M, Iwasaki T, Mori M 1996 Regulation of the gene for the preprothyrotropin-releasing hormone from the mouse by thyroid hormone requires additional factors in conjunction with thyroid hormone receptors. *J Biol Chem* 271:27919–27926
- Lazar MA, Chin WW 1990 Nuclear thyroid hormone receptors. *J Clin Invest* 86:1777–1782
- Samuels H, Forman B, Horowitz Z, Ye Z-S 1989 Regulation of gene expression by thyroid hormone. *Annu Rev Physiol* 51:623–639