Biosynthesis and Release of Thyrotropin-releasing Hormone Immunoreactivity in Rat Pancreatic Islets in Organ Culture

EFFECTS OF AGE, GLUCOSE, AND STREPTOZOTOCIN

L. Ø. DOLVA, J. H. NIELSEN, B. S. WELINDER, and K. F. HANSSEN, *Hormone Laboratory and Medical Department B, Aker Hospital, Oslo, Norway; Hagedorn Research Laboratory, Gentofte, Denmark*

ABSTRACT Thyrotropin-releasing hormone immunoreactivity (TRH-IR) was measured in isolated islets and in medium from rat pancreatic islets maintained in organ culture. TRH-IR in methanol extracts of both islets and culture medium was eluted in the same position as synthetic TRH by ion-exchange and gel chromatography and exhibited dilution curves parallel with synthetic TRH in radioimmunoassay. [^{3}H]Histidine was incorporated into a component that reacted with TRH antiserum and had the same retention time as synthetic TRH on reversed-phase high-performance liquid chromatography.

A continuous release of TRH-IR into the culture medium was observed from islets of both 5-d-old (newborn) and 30-d-old (adult) rats with a maximum on the second day of culture (28.7±7.0 and 13.3±3.6 fmol/islet per d, respectively). The content of TRH-IR was higher in freshly isolated islets from newborn rats (22.4±2.3 fmol/islet) than in adult rat islets, which, however, increased their content from 1.3±0.5 to 7.0±0.5 fmol/islet during the first 3 d of culture. Adult rat islets maintained in medium with 20 mM glucose released significantly more TRH-IR than islets in 3.3 mM glucose medium (13.0±0.7 vs. 4.3±0.3 fmol/islet per d). In contrast, the content of TRH-IR in the islets was reversed (1.4±0.3 vs. 4.7±1.6 fmol/islet). By exposing islets from newborn rats to streptozotocin 0.7 mg/ml for 30 min, a 50% reduction of TRH-IR content in the islets compared with the non-treated islets was seen after subsequent culture for 7 d. The insulin content was reduced by 80%, while glucagon was slightly elevated.

In conclusion, these results indicate that TRH is synthesized in rat pancreatic islets, and that the release is stimulated by glucose.

INTRODUCTION

Thyrotropin-releasing hormone immunoreactivity (TRH-IR)[1] has been found in the pancreas of man (1, 2) and rat (3, 4), and seems to be located mainly in the islets of Langerhans (5, 6). The amount of TRH-IR in the rat pancreas and islets is highest during the first 24 h after birth, but declines rapidly with age, concomitantly with the appearance of TRH in the hypothalamus (7, 8). Thus, during the first week the pancreas is one of the main sources of circulating TRH-IR in the rat (7).

The origin and the possible role of TRH-IR in pancreatic islets is not known. Various studies in man failed to show any effect of TRH on the pancreatic hormones in peripheral blood (9). In perfused rat pancreas, however, TRH potentiated the arginine-stimulated glucagon release (10). Intraperitoneal administration of streptozotocin to the rat reduced the content of TRH-IR in islets but not in the hypothalamus (5).

To evaluate the regulation of TRH-IR in the islets

Address all correspondence to Dr. Lars Øystein Dolva. His present address is Medical Department A, Rikshospitalet, Oslo 1, Norway.

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[1] *Abbreviations used in this paper:* HPLC, high-performance liquid chromatography; HS, human serum; NBCS, newborn calf serum; TRH, thyrotropin-releasing hormone; TRH-IR, TRH immunoreactivity.

without influence of neural and humoral factors from tissues outside the islets, we have studied the incorporation of [^{3}H]histidine into TRH-IR and the effect of age, glucose, and streptozotocin on the content and secretion of TRH-IR, insulin, and glucagon in rat pancreatic islets maintained in organ culture.

METHODS

Islet isolation and culture. Pancreatic islets were isolated from 5-d-old Wistar rats (newborn) of both sexes and from overnight-fasted 30-d-old male Wistar rats (adult) (Møllegaard, Lille Skensved, Denmark).

The excised pancreas was digested by the collagenase method and the islets were isolated after gradient centrifugation on Percoll™ (11). Isolated islets were placed in 5-cm plastic petri dishes (Nunc, Roskilde, Denmark). Each dish contained ~100 islets in 5 ml medium RPMI 1640 (Flow Laboratories, Irvine, UK) containing 11 mM glucose and 10% heat-inactivated newborn calf serum (NBCS) (Gibco Laboratories, Paisly, UK). The dishes were incubated at 37°C in a humidified atmosphere containing 5% carbon dioxide. Medium and islets were withdrawn at various intervals as indicated in the figures. Islets were homogenized in distilled water by sonication. Hormones were measured in both medium and islets.

Glucose stimulation. Islets from adult rats were preincubated for 1 d in RPMI 1640 medium containing 10% NBCS and 11 mM glucose and then transferred to RPMI 1640 medium containing 0.5% human serum (HS) and either 3.3 or 20 mM glucose. After 1 d in 3.3 mM glucose one group of islets was transferred to 20 mM glucose, while others were maintained in either 3.3 or 20 mM glucose. The medium was changed daily and hormones were measured in medium and islets.

Streptozotocin treatment. After preincubation for 2 d in RPMI 1640 medium with 10% NBCS and 11 mM glucose, newborn islets were exposed to 0, 0.3, 0.4, 0.5. 0.6, and 0.7 mg/ml streptozotocin (Upjohn Co., Kalamazoo, MI) in saline. After 30 min the islets were transferred to RPMI 1640 containing 0.5% HS and 11 mM glucose. The islets' content of TRH-IR, insulin, glucagon, and DNA, and TRH-IR and insulin in the medium were measured.

Insulin and glucagon measurements. Insulin was determined in proper dilution of culture medium and of 20% acetic acid extracts of islet homogenates, by radioimmunoassay with crystalline rat insulin (Novo, Bagsværd, Denmark) standard. Glucagon was measured in the same samples by radioimmunoassay (12).

DNA determination. The DNA content in the islet homogenates was measured by a fluorometric method using 3.5-diaminobenzoic acid (Fluka AG, Buchs, Switzerland) as fluorochrome (13). Calf thymus DNA (Sigma Chemical Co., St. Louis, MO) was used as standard.

TRH-IR measurement. Extraction procedure: The frozen sample (−60°C) was extracted in 90% methanol. After centrifugation (2,500 *g*, 15 min, 4°C) the supernatant was dried in a 37°C water bath under a stream of nitrogen. The residue was then dissolved in assay buffer and TRH-IR measured. The TRH radioimmunoassay has previously been described in detail (2). The TRH antiserum is specific for alterations in pyroglutamyl and proline-NH_2 moieties, but cross-reacts with histidine-modifications (15). No cross-reactivity was seen with glucagon or insulin up to 2.5×10^{-9} mol/tube or histidyl-prolyl-diketopiperazine up to 2.5×10^{-11} mol/tube. Addition of NaCl up to 1 M did not interfere with the assay. pH was adjusted to 7.4 by NaOH in the unknown samples. The nonspecific binding was 2.4±0.3%. The intraassay variation was for the lower part of the standard curve (5.5–55 fmol) 11%, the middle part (55–450 fmol) 8%, and the upper part (450–1,800 fmol) 8%. The interassay variation was 20, 8, and 9%, respectively. The lower detection limit plus 2 SD calculated from 12 observations in the absence of unlabeled hormone was 5.5 fmol/tube.

Characterization of TRH-IR. Dried extracts of islets, culture medium, or whole pancreas, were dissolved in (*a*) 0.01 M ammonium acetate buffer pH 3.5 and purified on a cation-exchange chromatography column (SP Sephadex C25, Pharmacia, Uppsala, Sweden) 8 × 1 cm and in (*b*) 2 ml buffer consisting of 50 mM KH_2PO_4, 110 mM NaCl, 7.7 nM NaN_3, and 0.2% human serum albumin (Behringwerke AG, Marburg-Lahn, GFR), pH 7.4, and purified on a gel chromatography column (Bio-Gel P2, Bio-Rad Laboratories, Richmond, CA) 20 × 1.5 cm. The cation exchange chromatography column was eluted with 0.01 M ammonium acetate buffer pH 3.5 containing 2% bovine serum albumin. Elution was performed with this buffer by a stepwise pH gradient: 40 ml pH 3.5, 40 ml pH 5.2, and 60 ml pH 7.4. Fractions of 2 ml were collected and pH adjusted to 7.4 by NH_4OH before measured in the TRH assay. The gel chromatography column was washed and eluted with assay buffer pH 7.4. Fractions of 2 ml were collected and the TRH-IR determined in the TRH assay. The recovery of added TRH was >90% in the gel chromatography column and ~40% in the cation-exchange chromatography column.

Recovery of synthetic TRH. Degradation of synthetic TRH in the culture medium was followed by addition of TRH to the culture medium, incubated at 37°C. TRH was determined in samples drawn daily for 5 d. Degradation of endogenous TRH-IR in islet homogenates was followed after storage for 1, 2, and 20 h at 24°C.

Biosynthesis of TRH-IR. 500 islets from newborn rats were incubated in medium RPMI 1640 modified by omission of histidine (Gibco Biocult, Paisly, UK) and supplemented with human serum albumin 1 mg/ml and 50 μCi [2,5-^{3}H]histidine (40–60 Ci/mmol) (Radiochemical Centre, Amersham, UK). After incubation for 20 h the islets were extracted in 90% methanol and subjected to reversed-phase high-performance liquid chromatography (HPLC) (Waters Associates, Millipore Corp., Milford, MA) on a Hamilton PRP-1 resin (Hamilton, Bonanduz, Switzerland) eluted with ammonium bicarbonate, 0.1 M, pH 7.0, and a linear acetonitrile gradient from 2.5 to 17.5% for 60 min. Synthetic TRH (Novo) 15 μg was added as carrier. The radioactivity was measured in each fraction after addition of Aquasol-2 (New England Nuclear, Drelich, GFR) in a liquid scintillation counter.

Immunoprecipitation of the lyophilized methanol extracts was performed as described for insulin (14) by addition of 200 μl TRH antiserum diluted 1:10 in glycin buffer pH 8.8, and incubated overnight at 4°C. The sample was precipitated with protein A-Sepharose (Pharmacia). After washing twice the precipitate was extracted by acidification with 1 N HCl and addition of 15 μg synthetic TRH as carrier. The supernatant was analyzed by HPLC as described above.

Histology. 100 formalin-fixed hematoxylin and eosin-stained pancreatic islets were examined by light microscopy for ganglionic cells or exocrine tissue.

Statistical calculations. Wilcoxon's test for paired observations was used for statistical calculations (16). Values are expressed as mean±SEM and $P < 0.05$ was considered significant.

RESULTS

Characterization of TRH-IR. Extracts of both pancreatic islets, culture medium, and whole rat pancreas exhibited dilution curves parallel to synthetic TRH and TRH-IR was coeluted with synthetic TRH by both cation exchange and gel chromatography (2). Disappearance of TRH added to the culture medium containing 10% heat-inactivated calf serum followed first order kinetics with $T_{1/2}$ = 5 d. There was no detectable loss of TRH-IR in homogenized pancreatic islets stored at room temperature up to 20 h. Ganglionic or exocrine cells were not detected by light microscopic examination of the freshly isolated islets.

Biosynthesis of TRH-IR. A methanol extract of islets labeled with [^{3}H]histidine showed a radioactivity peak at the same position as synthetic TRH on HPLC (Fig. 1 *a*, *b*). After immunoprecipitation with a surplus of TRH-antiserum and protein A-Sepharose followed by acid extraction of the precipitate a peak was coeluted with synthetic TRH (Fig. 1 *c*). There was no peak of radioactivity at that position when extracts of islets labeled with tritiated leucine, phenylalanine, or tryptophane were analyzed (data not shown). The radioactivity peak from the [^{3}H]histidine-labeled islets extracts, which appeared without retention, coeluted with free histidine. The peak appearing after 40 min was not precipitated by the TRH-antiserum.

TRH-IR in islets from newborn rats. In the culture medium the daily release of TRH-IR increased during the first 2 d, whereafter it declined (Fig. 2). Release of TRH-IR could still be measured on day 8. The daily release of glucagon and insulin was almost constant during the whole period. The content of TRH-IR in the freshly isolated islets (22.4±2.2 fmol/islet) remained unchanged the first 3 d but was reduced on day 8. No decrease in the contents of glucagon and insulin in the islets were observed during the culture period.

TRH-IR in islets from adult rats. The release of TRH-IR was ~50% of the release from newborn rat islets (Fig. 2). The release increased during the first 2 d and declined thereafter, but was still present after 10 d of culture. The daily release of glucagon and insulin to the culture medium was almost constant during the whole period. The content of the TRH-IR in the freshly isolated islets from adult rats (1.3±0.5

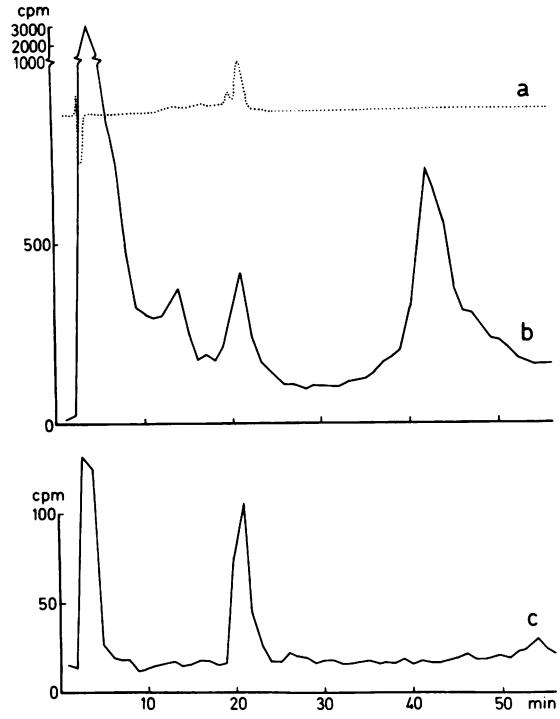

FIGURE 1 Reversed-phase HPLC on a Hamilton PRP-1 resin of synthetic TRH (*a*); methanol extract of islets labeled with [^{3}H]histidine (*b*); and acid extract of immunoprecipitated islet extract (*c*). The column was eluted with 0.5 M ammonium bicarbonate pH 7.0 and a linear acetonitrile gradient from 2.5 to 17.5% during 60 min.

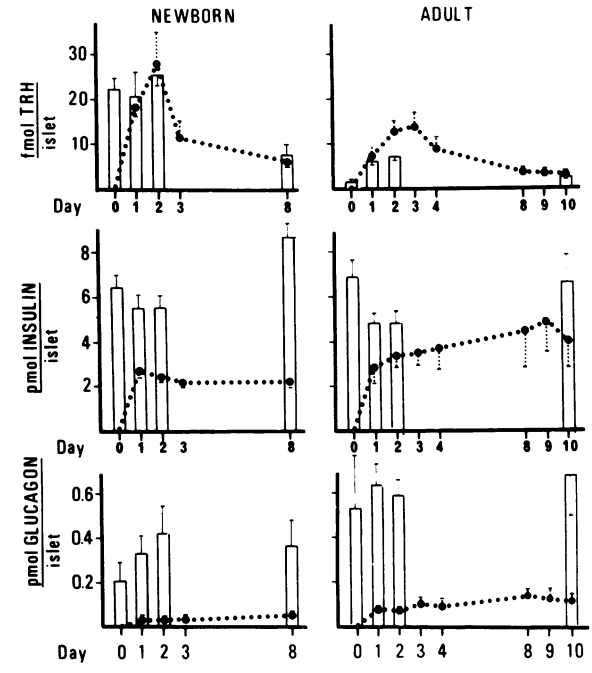

FIGURE 2 Content and release of hormones from newborn (*left* panel) and adult (*right* panel) rat islets maintained in organ culture. The content of TRH-IR, insulin, and glucagon in the pancreatic islets (columns) and the daily release (femtomoles per islet per day) (dotted lines) to the culture medium consisting of RPMI 1640 supplemented with 10% heat-inactivated calf serum and 11 mM glucose (mean±SEM of four to eight experiments).

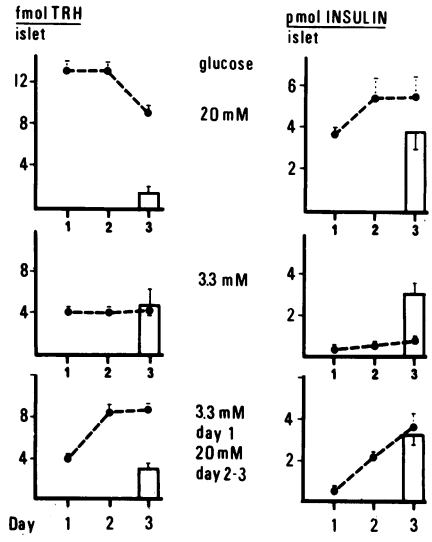

FIGURE 3 Effect of glucose on release and content of TRH-IR and insulin from adult rat islets in organ culture. The daily release to the medium (broken lines) and the islet content of TRH-IR and insulin (columns) in islets from adult rats maintained in medium RPMI 1640 supplemented with 0.5% HS and 20 or 3.3 mM glucose. In the *lower* panel the glucose concentration in the medium was changed from 3.3 to 20 mM after 1 d (mean±SEM of six observations).

fmol/islet) was lower compared with the islets from newborn rats. In contrast to the latter, there was a significant increase in the content of TRH-IR in the islets from adult rats during the first 3 d culture ($P < 0.05$). The content of TRH-IR in the islets on day 10 was at the same level as in freshly isolated islets. There was no significant change in the content of insulin and glucagon between the freshly isolated islets and the islets cultured for 10 d, although a transitory decrease in the insulin content was seen on day 1 and 2.

Effect of glucose concentration. Variation of the glucose concentration in the culture medium of adult rats islets from 3.3 to 20 mM increased the mean TRH-IR concentration in the medium from 4.3±0.3 fmol/islet per d to 13.0±0.7 fmol/islet per d ($P < 0.05$) (Fig. 3). In the experiments where the glucose concentration was increased between first and second day, the TRH-IR in the medium likewise increased. No significant change occurred during the 3 d in 3.3 mM glucose, whereas TRH-IR measured in the medium with 20 mM glucose was significantly lower on day 3 compared with day 1 and 2 ($P < 0.05$).

In contrast to the release, the TRH-IR content of the islets decreased from 4.7±1.6 fmol/islet to 1.4±0.3 fmol/islet after culture in 3.3 and 20 mM glucose, respectively ($P < 0.05$).

Streptozotocin treatment. Streptozotocin in a concentration > 0.4 mg/ml significantly reduced the TRH-IR content in the islets (Fig. 4) ($P < 0.05$). The most pronounced effect was obtained with 0.7 mg/ml streptozotocin (2.4±0.5 fmol/islet vs. 5.6±0.4 fmol/islet) in the control islets. The islet contents of insulin and DNA were significantly reduced with all doses of streptozotocin, whereas the glucagon content was increased at the highest dose (Fig. 4) ($P < 0.05$).

DISCUSSION

It has previously been shown that the beta cell function of the islets can be preserved for several months under tissue culture condition (17). In the present study insulin, glucagon, and TRH-IR have been measured simultaneously in islets and in culture media from both newborn and adult rats. The presence of TRH-IR in whole rat pancreas and in islets of Langerhans has previously been described and appears to be identical to TRH (6–8). In our study, TRH-IR in extracts from the culture medium as well as rat pancreas and islets behaved like synthetic TRH by dilution and on cation-exchange and gel chromatography. In addition, demonstration of incorporation of [^{3}H]histidine into a molecule that could bind to TRH antiserum and appeared with the same elution time as synthetic TRH on HPLC (Fig. 1), strongly suggests that TRH-IR is synthesized in the islets and that the material is identical to TRH.

Synthetic TRH is rapidly degraded in serum (18). Heat inactivation of calf serum has been shown to prevent degradation of insulin (17) and $T_{1/2}$ for the degradation of TRH in the culture medium containing 10% heat-inactivated calf serum was 5 d. No reduction in the TRH-IR content in homogenized pancreatic islets stored at room temperature for 20 h was observed. Since no correction for the degradation of TRH-IR in the medium has been made, the values are somewhat underestimated.

The content of TRH-IR in pancreatic rat islets has previously been shown to be considerably higher in newborn than in adult rats (8). This was confirmed in the present study (Fig. 2), and, in addition, the rate of release was found to be ~50% higher in the newborn rat islets. At the culture condition with 11 mM glucose there was a pronounced increase in TRH-IR release in islets from both newborn and adult rats during the first 2–3 d, whereafter the rate of release declined. This time course was not observed for the release of glucagon and insulin release. The release of TRH-IR to the medium was not simply accompanied by a reduction in the content of TRH-IR in the islets. In the newborn islets the content was constant for the first 3 d, while the islets from the adult rats showed a significant increase, suggesting a net biosynthesis of TRH-IR. Since

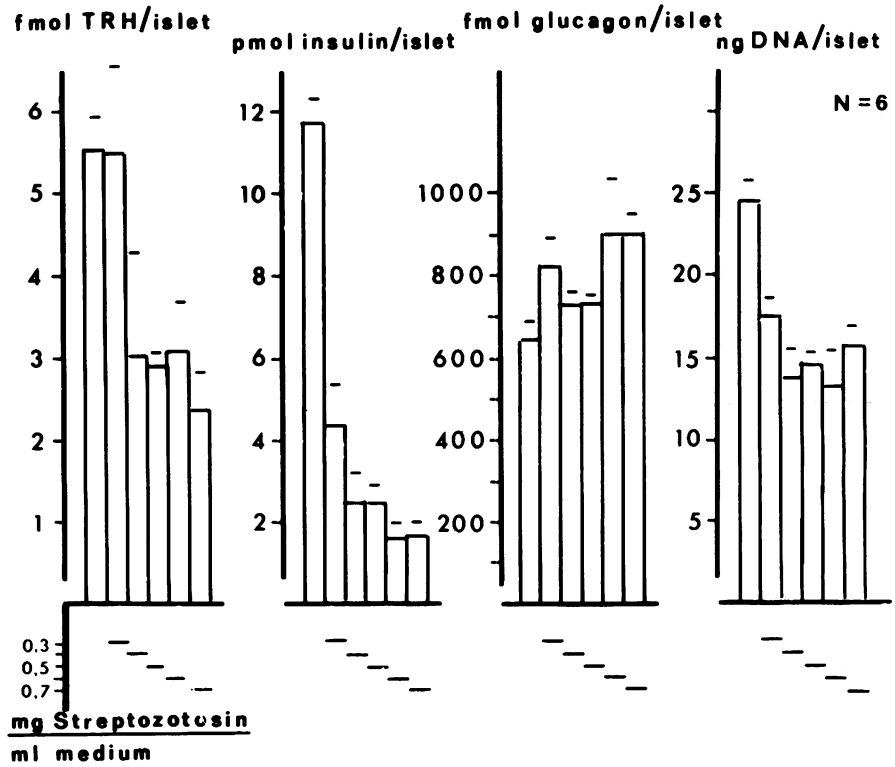

FIGURE 4 The content of TRH-IR, insulin, glucagon, and DNA in islets from newborn rats, exposed 30 min to medium with various concentrations of streptozotocin and subsequently cultured for 7 d in medium RPMI 1640 supplemented with 0.5% HS and 11 mM glucose (mean±SEM of six observations).

no ganglion cells or exocrine tissue was observed by histological examination, the TRH-IR biosynthesis most probably occur in the endocrine cells.

In acute experiments, Morley et al. (10) could not observe increased TRH-IR in glucose-stimulated perfused rat pancreas. In this study, high glucose increased TRH-IR in the culture medium but reduced TRH-IR in the pancreatic islets, indicating a stimulatory effect of glucose on the TRH-IR release and probably also an increased production since the released TRH-IR exceeded the reduction of TRH-IR in the islets (Fig. 3). The effect of glucose is most likely specific, since islets tolerate wide variations in osmolality without change in hormone secretion (19, 20). The reduced release and content after prolonged culture at 11 mM (Fig. 2) and 20 mM glucose (Fig. 3) may indicate a limited capacity to synthesize TRH-IR or lack of supporting factors in the culture medium. This may be analogous to the supporting effect of hydrocortisone (21) and growth hormone (22) on the insulin production from cultured islets. The difficulties in demonstrating TRH biosynthesis in isolated tissues (23) may be due to general impairment of the enzymatic conversion of peptide precursors at the carboxy-terminal of amidated forms (24, 25).

The marked increase of the TRH-IR content in the islets and in the medium from adult rat islets during the first days may be due to removal of inhibiting or degradating substances when the islets are removed from their natural surroundings in the intact animal. The fact that the same increment does not occur in islets from newborn rats may be due to lack of such inhibiting or degradating properties in these islets. This is supported by the demonstration of TRH-IR in the systemic circulation in newborn, but not in adult rats (7). Increased TRH-IR in the pancreas from starved rats has been described (5), which may correspond to the increased content of TRH-IR in islets cultured in low glucose medium (Fig. 3).

Administration of streptozotocin to rats in vivo resulted in a decreased TRH-IR content of the pancreatic islets (5, 26). In this study, streptozotocin was added directly to the islets for 30 min, resulting in a significant reduction of the islet content of both DNA, TRH-IR, and insulin (Fig. 4). However, the effect on insulin was already seen at the lowest dose of strep-

tozotocin, and a reduction by ~80% was obtained with the higher concentrations, whereas the reduction of the content of TRH-IR was less pronounced at all doses. The fall in insulin and DNA following streptozotocin is probably caused by beta cell destruction (27). Glucagon in the islets was unchanged or even higher following streptozotocin treatment. This is in agreement with other studies where both somatostatin and glucagon content have been shown to increase after administration of streptozotocin in vivo (28, 29).

Koivusalo et al. (30) demonstrated TRH-IR by immunohistochemical staining in the periphery of the pancreatic islets similar to the distribution of glucagon cells, and they found that glucagon and TRH-IR in islet extracts were present in equimolar amounts in the adult Sprague-Dawley rats (30). This relationship was not seen in our study where younger Wistar rats were used. Although it can not be excluded that TRH and glucagon are synthesized in the same cell, the TRH is at least not part of the determined sequence of proglucagon (31). The present results would rather suggest that at least part of the islet TRH-IR is present in the insulin-producing cells, which was also proposed in a recent in vivo study, where the reduction of both insulin and TRH by streptozotocin was prevented by nicotinamide treatment (26).

Recently, however, immunohistochemically stained TRH-IR was in one study reported to be present in all cells in the endocrine pancreas (32) and in another shown to be present in almost all insulin-containing cells and in some glucagon-containing cells but not in somatostatin-containing cells (33). These observations are supported by the present results, where a similar reduction in the islets' contents of DNA and TRH-IR were observed after streptozotocin treatment, in contrast to the more marked fall in insulin content (Fig. 4).

In conclusion, these results indicate that TRH is synthesized in rat pancreatic islets and that the release is stimulated by glucose.

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