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Thyrotropin-releasing hormone (TRH) reverses hyperglycemia in rat

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ABSTRACT

Hyperglycemia in thyrotropin-releasing hormone (TRH) null mice indicates that TRH is involved in the regulation of glucose homeostasis. Further, TRH levels in the pancreas peak during the stages of late embryonic and early neonatal β cell development. These observations are consistent in linking TRH to islet cell proliferation and differentiation. In this study, we examined the effect of TRH administration in damaged pancreatic rat (streptozotocin, STZ) to determine whether TRH could improve damaged pancreatic β cells function. We hypothesize that TRH is able to reverse STZ-induced hyperglycemia by increasing pancreatic islet insulin content, preventing apoptosis, and potentially induce islet regeneration. It was found that following intra-peritoneal (ip) injection, TRH (10 μg/kg body weight (bwt)) reverses STZ (65 mg/kg bwt)-induced hyperglycemia (TRH given 3 days after STZ injection). Increased circulating insulin levels and insulin content in extracted pancreas suggests that TRH reversed STZ-induced hyperglycemia through improving pancreatic islet β cell function. Further studies show a significantly lower level of apoptosis in islets treated with TRH as well as the presence of proliferation marker nestin and Brdu, suggesting that the TRH has the potential to prevent apoptosis and stimulate islet proliferation.

Lack of insulin production from pancreatic islets is the major cause of hyperglycemia in Type I diabetes. Though immune response and genetic tendencies are often considered the primary causes of islet induced hyperglycemia [1], studies have suggested that other mechanisms such as peptide disposition could also attribute to the same effect [2].

Thyrotropin-releasing hormone (TRH), a tripeptide first found in the hypothalamus, in stimulating the pituitary–thyroid axis, has been shown to have an active role in regulation blood glucose [3–6]. Though it was first believed that these effects occurred via mediation of the central nervous system (CNS), later experiments with hypophysectomized animals showed to have the same effect [7] suggests a direct involvement of TRH on pancreatic function.

We believe TRH in the pancreas does not attribute its effects through the mediation of thyroid hormone. This is due to the observation that targeted disruption of the TRH gene in mice causes hyperglycemia and hypothyroidism but compensatory administration of thyroid hormone for the resulting in hypothyroidism does not reverse the hyperglycemia, suggesting that hyperglycemia occurs in TRH null mice through the absence of TRH *per se* and not thyroid hormone [8]. TRH receptor knock out mice also showed hyperglycemia further supports this theme [9]. Furthermore, the reduction of glycodeoxycholic acid-induced pancreatic damage by TRH [10] suggests that TRH has a protective function against toxins in the pancreas.

The goal of this study is to understand the therapeutic effects of TRH *in vivo* and to identify the morphological effects on pancreatic islets. In the present study, a streptozotocin (STZ)-induced hyperglycemia model in the rat [11] was used to evaluate the effect of TRH in counteracting hyperglycemia and determine whether TRH may have a potential role in stimulating recovery of pancreatic islet β cell function from pancreatic injury. Pancreatic tissues were tested for insulin content, apoptosis, and regeneration markers to understand the in depth effect of TRH on islet morphology.

Materials and methods

Animals. A group of 8-week-old male Sprague–Dawley rats (200 ± 10 g) were given streptozotocin (STZ: 65 mg/kg bwt) [11] or normal saline followed by TRH (pGlu-His-ProNH2: 10 μg/kg bwt) only once, on day 1 or 3 after the initial STZ or saline injection. All injections were given ip.Accordingly, four groups of animals were studied: (1) STZ alone, (2) STZ followed by TRH, (3) TRH alone, and (4) saline control. Each group contained six animals. On day 7 after 12 h fasting, the rats were sacrificed and pancreases were collected. The animals were anesthetized with single dose of ip injection of sodium pentobarbital solution (60 mg/kg bwt, Sigma, St. Louis, MO), blood samples and the whole pancreas were collected. The pancreas was snap frozen with liquid nitrogen and

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immediately stored at −80 °C until use for protein preparations and insulin assays. The Institutional Animal Welfare Committee (Roger Williams Hospital) approved the animal studies.

Hyperglycemic rats and monitoring blood glucose. Hyperglycemia within 24 h was induced by ip injection of STZ. Blood glucose was monitored daily. Approximately, 10 μl of peripheral blood was obtained from the tail vein. Blood glucose was measured using an Accucheck Blood Glucose Meter (Roche Diagnostics Corporation, IN) after 12 h fasting.

ELISA for insulin measurement. Insulin concentrations in the specimen (serum or tissue extracts) were measured using Ultra Sensitive Rat Insulin ELISA Kit (Crystal Chem, Inc., Downer Grove, IL) according to the vendor's instruction. Briefly, insulin standards and appropriately-diluted (1:50–1:500) samples were added to an insulin antibody-coated 96-well microplate and incubated for 2 h at 4 °C. After washing five times, anti-rat insulin enzyme conjugate was added to the wells and incubated for 30 min at room temperature. Enzyme substrate solution developed color was read at 450 nm under μQuant microplate reader (Bio-Tek Instruments, Inc., Winooski, VT) and concentrations were calculated by KC Junior® microplate reader software (Bio-Tek Instruments, Inc.).

Pancreatic islets assessment. Each pancreas was placed in head (central) to tail (peripheral) orientation and immediately snap frozen and kept at −80 °C. The special orientation-treated tissue gave an opportunity for sectioning of equal surface areas when freeze-cutting the tissue. In this case, the pancreatic slide section included every part of the pancreas in parallel level avoiding variations caused by sectioning from different pancreatic areas. The hematoxylin and eosin (H&E) histological staining was performed in a serial frozen slide 5 μm thick. The number of islets was determined by point counting morphometry on the slide and standardized by correcting for tissue area. For each block of pancreas, 10 sections were scored systematically at a magnification of 20× using a 20-point grid and a total of 60 sections were analyzed for each group (6 rats/group). The final result was calculated from the sum of the number of islets in the specific region divided by total pancreatic islet tissue area (islets/mm^2) [12].

Islet insulin staining. Immunoperoxidase staining of paraformaldehyde-fixed slides was performed using avidin–biotin-peroxidase detection (ABC Elite, Vector Laboratories, Burlingame, CA). Normal goat blocking serum at 1:100 dilutions in 3% PBS–BSA (Sigma Chemical Co., St. Louis, MO) was applied. The primary mouse anti-insulin monoclonal antibody 1:50 in 1% PBS–BSA (CHEMICON, Temecula, CA) were incubated with the section overnight at 4 °C. After washing, the sections were incubated with the biotinylated secondary antibody (1:5000 dilution) for 30 min at room temperature, or fluorescent conjugation second antibody for fluorescent staining, followed by avidin–biotin-peroxidase complex (diluted 1:25 in PBS) for 60 min. 3,3′-Diaminobenzidine tetrahydrochloride and hydrogen peroxide (Sigma, St. Louis, MO) provided the peroxidase. Slides were incubated with methyl green RT 8 min for nuclear staining and rinsed clear with distilled H_2O.

In vivo BrdU labeling and detection. A working solution of BrdU in PBS at 10 mg/ml was made and injected (ip) into the rats (100 mg/kg bwt). Four hours after the injection, the pancreases were collected and sectioned. The sections were performed with monoclonal antibody to BrdU (DAKO Corp., CA) as described above.

Pancreatic islet apoptosis assay. The detection of apoptotic nuclei using the TdT-mediated dUTP nick end labeling (TUNEL) method was performed using a Clone ApoAlert™ DNA Fragmentation assay kit BD (Clontech) according to the protocol specified by the manufacturer. Briefly, pancreatic tissue, fixed as described previously, was placed in 3% paraformaldehyde and incubated in 0.3% H_2O_2 in methanol for 5 min to block endogenous peroxidase. Slides were incubated with TdT incubation buffer for 2 h at 37 °C and terminated with 2× SSC. The cell mean intensity was analyzed by the Image Analysis System (Biovision Image Analysis, Perceptics, TN) without knowing the treatment protocols. The mean intensity was calculated from the measurement of more than 10 islets picked randomly from each slide; four slides were measured from one sample and twenty four slides were designated as one group.

Statistical analysis. In the text, table and figures, all data are presented as means ± SEM. All experiments were repeated three times. For figures and tables, the results of a single representative experiment are shown. Blood glucose and insulin data used for graphical presentation and statistical analysis are expressed as per experimental time periods. The data for blood glucose, insulin and islet were analyzed by ANOVA statistical program using one and two factor analysis of variance of repeated measures. p values for all F tests are $p < 0.01$. Tukey's test was used for *post hoc* comparisons among individual means.

Results

Effect of TRH on pancreatic function in response to STZ treatment

Reversal of hyperglycemia

On the third day after STZ injection, when animals had blood glucose levels about 400 mg/dL, TRH (10 μg/kg bwt ip) was administered, with a control group receiving saline. Additional controls included an STZ only group, without administration of TRH, and a group given just TRH alone. Seven days after TRH injection (10th day following STZ injection), animals were sacrificed under anesthesia with collection of blood and pancreatic tissue samples. On daily monitoring the blood glucose fell from 432 ± 20.42 mg/dL, 3 days after TRH injection, to 98 ± 48.32 mg/dL (6 and 9 days following TRH and STZ injections, respectively) (Fig. 1A). Serum Insulin levels were significantly increased by TRH alone (5.094 ± 1.569 ng/ml) and TRH plus STZ groups (3.600 ± 1.373) compared with STZ alone (0.633 ± 0.192, $p < 0.05$) and slightly higher than control (2.113 ± 0.488 ng/ml) (Fig. 1B).

Mechanism for recovery of STZ-induced hyperglycemia

Effect on pancreatic β cell insulin, serum insulin and islet structure

Pancreatic β cell insulin level was detected by both immunohistochemistry and ELISA (pancreatic insulin content) (Fig. 1C). The blood glucose level fell and serum insulin level enhance in parallel with enhanced pancreatic insulin content and islet insulin staining following TRH administration to the STZ-treated animals (Fig. 2A). Pancreatic islet assessment and morphological study (Fig. 2B) indicated that the number of islets was increased in the TRH group vs. control (109.36 ± 11.22 vs. 53.94 ± 10.23 islets/mm^2; $p < 0.05$) and STZ plus TRH vs. STZ alone (40.77 ± 9.82 vs. 24.24 ± 5.12; $p < 0.05$) (Fig. 2C).

Induction of pancreatic islet cell proliferation

In vivo Brdu labeling studies indicated that there was a significant increase of Brdu positive staining in both the TRH alone and in the STZ plus TRH-treated groups (Fig. 3A), which indicated cell differentiation in the TRH-treated animal's pancreas.

Further studies with a nestin antibody were carried out using immunohistochemistry to identify these pancreatic tissues. Nestin is expressed in neuronal progenitor cells and has also been suggested as a marker for multipotent pancreatic progenitor cells. The results indicated that nestin positive cells were clearly localized around islets, vessels and ducts (Fig. 3B).

Reversal of pancreatic islet cell apoptosis

The seven day period for TRH administration to affect recovery of pancreatic endocrine function suggests that there are multiple mechanisms involved in its action. TRH anti-apoptosis could occur

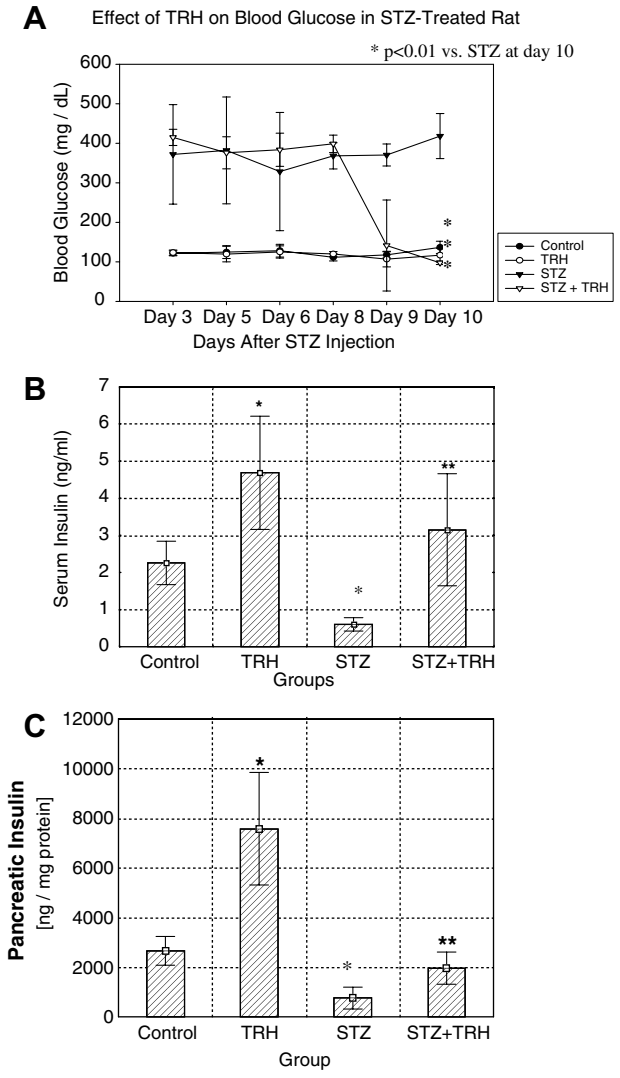

Fig. 1. TRH reverses STZ induced high blood glucose. (A) After 3 days STZ injection ip induced hyperglycemia, TRH (10 μg/kg body weight, ip) was administered to rats. On the day 8, TRH-treated STZ animal glucose started to decline to reach a normal level ($^{*}p<0.01$ vs. STZ group, $n=6$); (B) TRH reversed the decline in serum insulin level resulting from STZ treatment on day 10 ($^{*}p<0.05$ vs. control; $^{**}p<0.05$ vs. STZ $n=6$). (C) Quantities of insulin content by ELISA from rat pancreas extract on day 10 (simultaneously collected in frozen section for immunohistochemistry, Fig. 2A) (results normalized by total protein, $^{*}p<0.05$ vs. control; $^{**}p<0.05$ vs. STZ $n=6$).

in islet β cell and/or pancreatic other cells. The transferase-mediated dUTP nick end labeling (TUNEL) assay showed that apoptotic signals were markedly increased in β cells of islet (indicated by anti-insulin antibody red) in STZ-treated animals vs. both controls and the TRH treated alone group, but were markedly reduced by addition of TRH (STZ plus TRH group) (All p <0.01; Fig. 4).

Discussion

The studies reported in this manuscript show that TRH administration reverses STZ-induced diabetes in the rat. These findings suggest that the neuropeptide TRH, which is expressed in the islets of Langerhans, may be an important factor in the modulation of insulin secretion and consequently carbohydrate metabolism.

The ontogeny of TRH expression and production corresponds to that of insulin in the developing pancreas [13] during embryonic development suggests that TRH might be involved in pancreatic β cell development [14–16]. This possibility is further strengthened

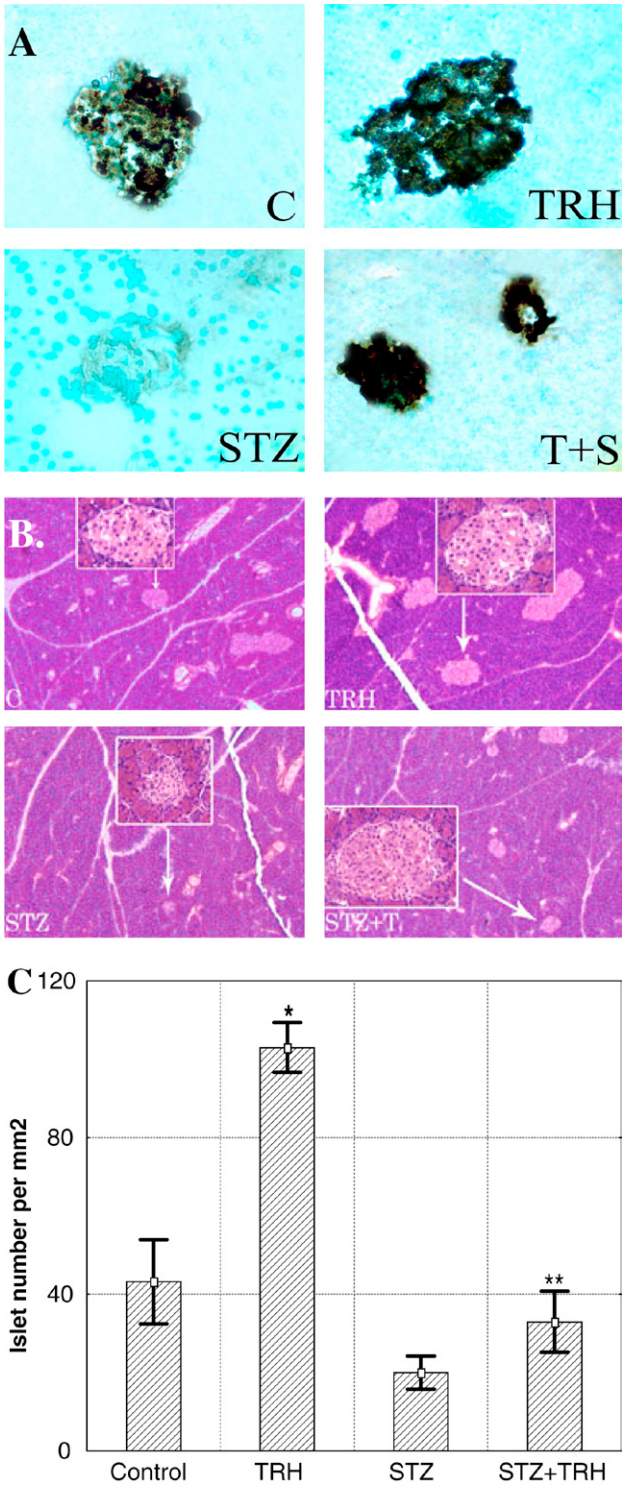

Fig. 2. TRH preserves pancreatic β cell function against STZ damage. (A) Immunohistochemistry of pancreatic sections showed that TRH increased insulin staining in the islet and partially reversed the reduction following STZ treatment on day10 (C = control; T + S = TRH and STZ), magnification 400×. (B) TRH reversed STZ-induced pancreatic islet loss (histological studies, H&E staining; bottom left STZ; bottom right STZ + TRH) magnification 100×. The islets were also shrunken as shown by a decreased nuclear staining in magnification images. Arrows indicate islets corresponding to the magnification images (400×) (C = control; T + S = TRH and STZ). (C) Pancreatic islets were counted as described previously after animals were sacrificed on day 10. TRH significantly increased the number of islets in pancreas vs. both STZ and control groups ($^{*}p<0.05$ vs. control; $^{**}p<0.05$ vs. STZ $n=12$).

by that Dexamethasone (DEX) treatment results in an increase of pancreatic weight and retardation of the peak pancreatic TRH

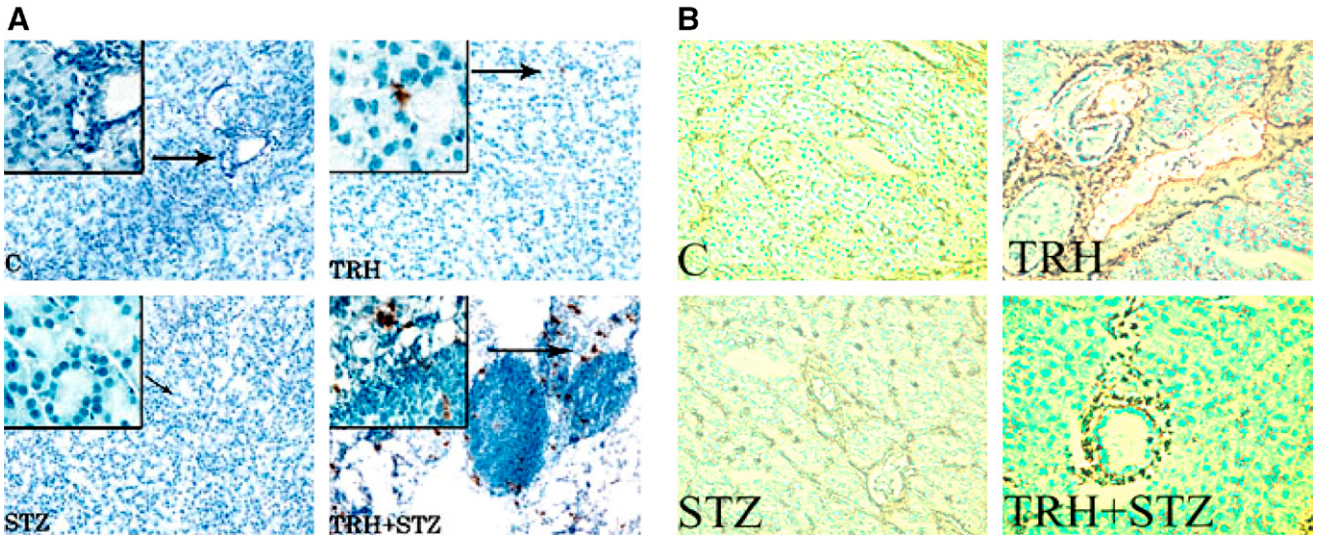

Fig. 3. Effect of TRH on pancreatic tissue regeneration. (A) Brdu staining detected by immunohistochemistry. BrdU (100 mg/kg bwt) was injected ip four hours before collection of tissue. Monoclonal anti-BrdU antibody detects the BrdU in pancreatic nuclei (blue) magnification 100×. Immunohistochemistry indicates a BrdU (brown) strong positive stain, scattered in islets as well as in adjacent exocrine tissue, in the TRH plus STZ and in the TRH-only group; no positive staining was found in the control or STZ groups. Arrows indicate Brdu positive staining corresponding to the magnification images 400×; (B) using anti-nestin antibody, immunohistochemistry of the pancreas revealed that TRH treatment resulted in strong nestin staining (dark spots), located around islets and vessels, in both TRH alone and STZ plus TRH groups. Magnification 400×. (For interpretation of the references in color in this figure legend, the reader is referred to the web version of this article.)

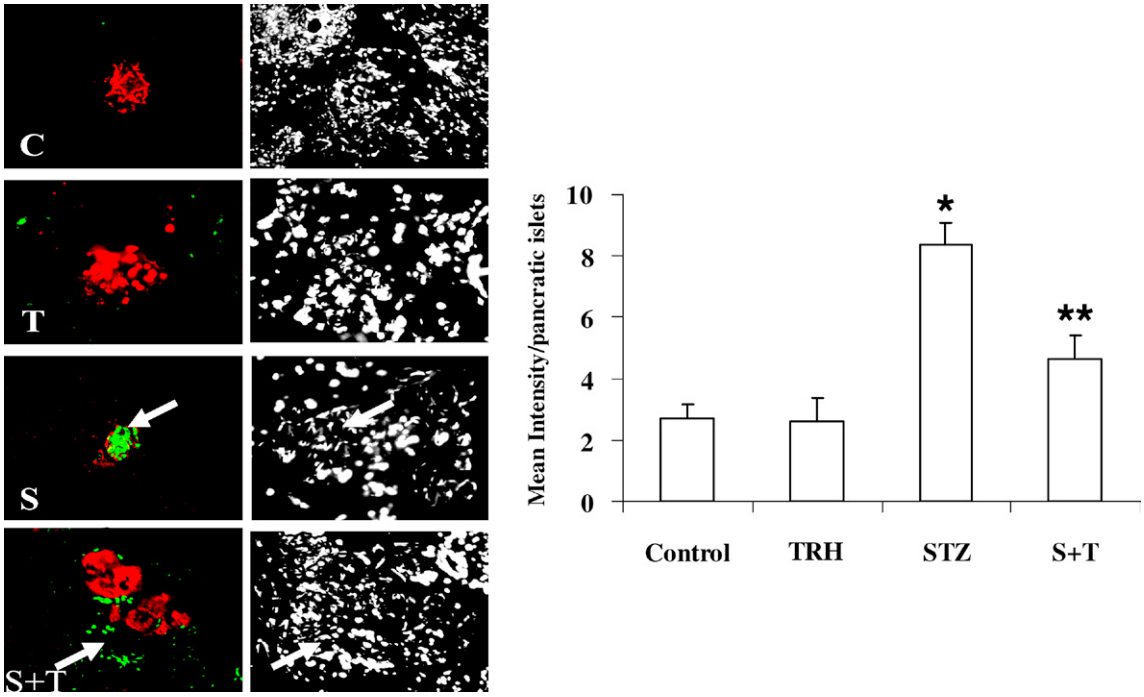

Fig. 4. Effect of TRH on pancreatic TUNEL staining, red = insulin, green = apoptosis. The intensive and scattered positive TUNEL staining (green, arrow indicated) was found in the STZ and STZ plus TRH groups (left panels). The insulin positive staining (red) was significantly reduced in the STZ group while TRH alone and TRH plus STZ groups have strong insulin positive staining (left panel images); right panel represents DAPI staining for nuclei (arrow indicated apoptotic cell's nucleus), magnification 200×. Right graph represents quantity of apoptotic cells image analysis by mean intensity/per area of islet ($^{*}p < 0.01$ vs. control; $^{**}p < 0.01$ vs. STZ. C = control; T = TRH; S = STZ). (For interpretation of the references in color in this figure legend, the reader is referred to the web version of this article.)

concentration and islet development [17,18]. The increase of pancreatic growth induced by chronic TRH administration in rats is due to hyperplasia [19] which may explain TRH administration in normal animal without contribution to alternating blood glucose level. Maternal diabetes, induced by STZ injection, result in a reduction of TRH levels in the pancreases of newborn rats [20]. The associated retardation of insulin secretion is consistent with the importance of TRH for pancreatic β cell development. Therefore, postnatal TRH expression in the pancreas of pups is retarded by maternal diabetes and compensatory TRH over-expression may be necessary for pancreatic islet formation [21]. In adult, TRH may enable to induce β cells regeneration in damaged islet presented in this study.

The significant positive insulin staining in the STZ plus TRH group suggests that the increase in insulin levels in both serum and pancreas could result from improved pancreatic β cell insu-

lin production through initiation of pancreatic β cell regeneration and/or prevention of apoptosis. TRH has the capacity to improve insulin release through regulating cytosol calcium flow [10,22], but no direct evidence linking TRH to stimulation of adult pancreatic β cell regeneration. This possibility is supported by the studies reported here in the STZ diabetic model of the adult rat. The numerous small size pancreatic islets observed in the STZ plus TRH group and the strong positive staining for both Brdu and nestin suggests the possibility of TRH initiating a proliferation mechanism in injured pancreas. In other hand, TRH significantly prevented apoptosis in pancreatic islets indicates that TRH enables to prevent pancreatic β cell from loss [23].

TRH reversal of hyperglycemia was delayed to around 7 days after administration. This delay in response hints at potential downstream mechanisms activated by TRH. The variable blood glucose levels over the first few days of the study suggests that anti-apoptosis could be a mechanism through which TRH restores pancreatic endocrine function in the short term. However, β cell regeneration, which occurs late in the process, improves pancreatic β cell function to stabilize blood glucose in the long term by observation of nestin and BrdU expression in TRH- and STZ-treated pancreatic tissues. TRH affecting the pancreatic microenvironment leading to alteration in the expression of various pancreatic β cell genes [24,25] may contribute the initiation of β cell regeneration. TRH-R1 expression was found in rat-derived β cell lines as well as exocrine pancreatic tissue [26], linked to EGF receptor phosphorylation [25], suggesting that the regulation of pancreatic function by TRH is via autocrine or paracrine mechanisms in pancreas rather than through central nerve system. β cell self-duplication could be linked to the process of islet β cell recovery from damage [27].

In conclusion, we have shown for the first time that adult pancreatic β cell function is recoverable through administration of TRH which is naturally expressed in the islets. TRH recovery of pancreatic β cell function could be through regeneration and/or by reducing apoptosis. Additional mechanistic studies are needed to understand the role of central vs. peripherally acting TRH to further elucidate the mechanisms of action. The findings reported here suggest a significant role for TRH in carbohydrate metabolism and the potential for a therapeutic role in the treatment of diabetes mellitus.

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