Glucagon-like peptide-1 stimulates type 3 iodothyronine deiodinase expression in a mouse insulinoma cell line

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Aims: The pathophysiological roles of thyroid hormones in glucose metabolism remain uncertain. Type 3 iodothyronine deiodinase (D3) converts thyroxine (T4) and 3,5,3′-triiodothyronine (T3) to 3,3′,5′-triiodothyronine (T3′) and 3,3′-diiodothyronine (T2), respectively, inactivating thyroid hormones in a cell-specific fashion. In the present study, we identified D3 expression in MIN6 cells derived from a mouse insulinoma cell line and examined the mechanisms regulating D3 expression in these cells.

Main methods: We characterized D3 activity using HPLC analysis, and examined the effect of GLP-1 or exendin-4 on D3 expression and cAMP accumulation in MIN6 cells. We also measured insulin secretion from MIN6 cells exposed to GLP-1 and T3.

Key findings: We identified enzyme activity that catalyzes the conversion of T3 to T3′ in MIN6 cells, which showed characteristics compatible with those for D3. D3 mRNA was identified in these cells using RT-PCR analysis. Forskolin rapidly stimulated D3 mRNA and D3 activity. Glucagon-like peptide-1 (GLP-1) increased D3 expression in a dose-dependent manner, and this effect was inhibited by the protein kinase A (PKA) inhibitor H-89. Exendin-4, a GLP-1 receptor agonist, also stimulated D3 expression in MIN6 cells. These results suggest that a cAMP-PKA-mediated pathway participates in GLP-1-stimulated D3 expression in MIN6 cells. Furthermore, GLP-1 stimulated insulin secretion was suppressed by the addition of T3 in MIN6 cells.

Significance: Our findings indicate that GLP-1 regulates intracellular T3 concentration in pancreatic β-cells via a cAMP-PKA-D3-mediated pathway that may also regulate β-cell function.

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Introduction

The physiological roles of thyroid hormones in glucose homeostasis remain debatable (Lenzen and Bailey, 1984; Chidakel et al., 2005). Systemic hypothyroidism promotes fasting hyperglycemia and decreases plasma insulin levels in mice following glucose stimulation (Taguchi et al., 2010). 3,5,3′-Triiodothyronine (T3) enhances function and survival of rat pancreatic islets (Verga Falzacappa et al., 2010), and thyroid hormone promotes postnatal rat pancreatic β-cell development and glucose-responsive insulin secretion (Aguayo-Mazzucato et al., 2013). Plasma free T3 concentrations are positively associated with insulin secretion in euthyroid individuals (Ortega et al., 2008). Thyroid hormone improves insulin resistance by acting on brown adipose tissue (Skarulis et al., 2010) and modulates hepatic glucose production (Klieverik et al., 2009). In contrast, systemic hyperthyroidism decreases glucose tolerance and β-cell insulin secretory capacity (Lenzen et al., 1975; Ximenes et al., 2007). Reduced thyroid hormone signaling in pancreatic β-cells is important for normal islet function and glucose homeostasis (Medina et al., 2011). In rats, thyroxine (T4) is able to induce apoptosis in pancreatic β-cells (Jörns et al., 2002). These observations suggest that plasma thyroid hormone concentrations are related to both insulin production and resistance.

T3, a major thyroid gland product, is converted to biologically active T3′ by iodothyronine deiodinase (Blanco et al., 2002). There are 3 types of iodothyronine deiodinase: type 1 (D1), type 2 (D2), and type 3 (D3) (Blanco et al., 2002). Both D1 and D2 remove iodine from the T3 outer ring to form T3′. D1 activity in rats is present in the thyroid, liver, kidney and many other tissues, and likely plays a role in maintaining circulating T3′ levels (Blanco et al., 2002). D2 activity in rats is present in only a few tissues, including the brain, anterior pituitary, and brown adipose tissue (Blanco et al., 2002). Because D2 activity increases and D1 activity decreases in the hypothyroid state, D2 plays a significant role in providing intracellular T3′ where it exists (Blanco et al., 2002). In contrast, D3 removes iodine from the T4 and T3′ inner rings to form the inactive thyroid hormones 3,3′,5′-triiodothyronine (rT3′) and 3,3′-diiodothyronine (T2′), respectively (Gereben et al., 2008). D3 is expressed in the placenta, central nervous system, and fetal liver, and its expression is stimulated...
by a cAMP-mediated pathway (Lamirand et al., 2008). It is noteworthy that D3-mediated thyroid hormone inactivation promotes insulin secretion from pancreatic β cells (Medina et al., 2011).

GLP-1 is produced in pancreatic α-cells and enteroendocrine L-cells from proglucagon, which is encoded by a glucagon gene, and is critical to glucose homeostasis (Stoffers et al., 2000; Baggio and Drucker, 2007). Intestinal endocrine cells secrete GLP-1 when nutrients such as carbohydrates, lipids, and proteins are digested. GLP-1 affects β-cells by amplifying glucose-induced insulin secretion, promoting proliferation, and inhibiting apoptosis (Li et al., 2003; Baggio and Drucker, 2007). GLP-1 increases intracellular cAMP in β-cells via the GLP-1 receptor and G-protein coupling pathway that activates PKA-dependent and -independent pathways (e.g., cAMP-guanine nucleotide exchange factor pathway) (Kwon et al., 2004). GLP-1 also inhibits α-cell postprandial glucagon secretion (Brubaker and Drucker, 2004; Baggio and Drucker, 2007).

Whether GLP-1 regulates iodothyronine deiodinase activity remains unknown, although GLP-1 increases intracellular cAMP which may regulate iodothyronine deiodinase expression in pancreatic β-cells. Here we identified D3 expression in mouse insulinoma cell line-derived cultured MIN6 cells and examined the mechanisms underlying GLP-1-mediated D3 expression.

Materials and methods

Materials

[125I]T2 and [125I]T3, were purchased from NEN Life Science Products Corp. (Boston, MA). LH-20, ECL reagent, and HYPERfilm-ECL were from GE Healthcare Bio-Sciences Corp. (Piscataway, NJ). AG 50W-X2 resin and protein assay kits were from Bio-Rad Laboratories, Inc. (Hercules, CA). Glucagon-like peptide-1 (7–36)-amide (GLP-1) was obtained from the Peptide Institute (Osaka, Japan). All other chemicals at the highest quality were obtained from Sigma-Aldrich Corp. (St. Louis, MO) or Wako Pure Chemical Industries, Ltd. (Osaka, Japan) unless otherwise indicated.

Cell culture

MIN6 cells were kindly provided by Dr. Masahiro Hosaka and Dr. Toshiyuki Takeuchi (Institute for Molecular and Cellular Regulation, Gunma University, Maebashi, Japan). MIN6 cells were cultured as previously described (Miyazaki et al., 1990). Briefly, MIN6 cells (passages 18–25) were cultured in DMEM (containing 15% heat-inactivated fetal bovine serum, 7 μL/L 2-mercaptoethanol) in culture flasks under a humidified atmosphere of 95% air and 5% CO2 at 37 °C, and the culture medium was changed every 3 days. The MIN6 cells were passaged once weekly following detachment using 0.25% trypsin-ethylenediamine tetra-acetate (EDTA) solution when the cells reached 80% confluence. All experiments using pseudo-islets were conducted 6 times weekly following detachment using 0.25% trypsin-EDTA solution when the cells reached 80% confluence.

Measurement of D3 and D1 activities

For the measurement of D3 activity, MIN6 cells in each well were washed twice with PBS, scraped off the plate, and transferred into 1-ml ice-cold assay buffer [100 mM potassium phosphate (pH 7.0) containing 1 mM EDTA and 10 mM dithiothreitol (DTT)]. After centrifugation at 3000 rpm for 10 min at 4 °C, the resultant precipitates were sonicated in assay buffer and incubated in a total volume of 50 μL with [125I]T2, which was purified using LH-20 column chromatography on the day of the experiment, and in the presence or absence of 1 μM 6-propyl-2-thiouracil (PTU) or 1 mM iopanoic acid (IOP) with several concentrations of unlabeled T3. The reaction products were analyzed using HPLC (Hitachi Co., Tokyo, Japan). The incubation mixtures were treated for 2 volumes of absolute ethanol, evaporated, dissolved in acetonitrile/water (32:68), and applied to the C18 column (Shimazu Co., Kyoto, Japan), and eluted with acetonitrile/water/phosphoric acid (32:68:0.1). The flow rate was 1 mL/min, and each 0.5-min fraction was collected and counted for radioactivity. The protein concentration was determined by Bradford’s method using BSA as a standard. The deiodinating activity was calculated as femtomoles of T2 produced/mg protein/h from T3 (Bessho et al., 2010).

D1 activity was measured as previously described (Miyashita et al., 1995). MIN6 cells were sonicated in ice-cold assay buffer [100 mM potassium phosphate (pH 7.0) containing 1 mM EDTA and 0.5 mM DTT], and incubated in a total volume of 50 μL with 0.5 μM [125I]T3 for 5 min at 37 °C. The reaction was terminated by the addition of 100 μL 2% BSA and 800 μL 10% trichloroacetic acid. After centrifugation at 3000 rpm for 10 min at 4 °C, the supernatant was applied to a column packed with AG 50W-X2 resin (bed volume, 1 mL) and then eluted with 2 mL 10% glacial acetic acid. Separated 125I was counted with a γ-counter. Nonenzymatic deiodination was corrected by subtracting 125I released in control tubes without cell sonicates. The deiodinating activity was calculated as picomoles of 1 released/mg protein/min, after multiplication by a factor of two to correct random labeling at the equivalent 3' and 5' positions.

Reverse transcription and real-time polymerase chain reaction (PCR)

We performed quantitative real-time PCR to examine D3 and D1 mRNA expression in MIN6 cells (Bessho et al., 2010; Ogiwara et al., 2013). Total RNA was isolated from MIN6 cells using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. After treatment with deoxyribonuclease I (Invitrogen) to remove contaminating DNA, CDNA was synthesized with TaqMan reverse transcription reagent (Applied Biosystems, Foster City, CA) using 1-μg RNA as a template and random hexamers as primers in a total reaction volume of 20 μL. Subsequently, 5 μL of the CDNA was amplified in a TaqMan 7500 real-time PCR apparatus using mouse D3 (Di3 mRNA probe Mm00548953_s1), mouse D1 (Dio1 mRNA probe Mm00839358_m1), and mouse G3PDH (GAPDH probe Mm99999915_g1) TaqMan gene

Fig. 1. Characterization of D3 activity in MIN6 cells. Deiodinating activity was measured in MIN6 cell sonicates. Cell sonicates were incubated for 1 h with various T3 concentrations in the presence of 10 mM DTT. Using the double reciprocal plot of 1 and Vmax = 115 femtoles T2 produced/mg protein/h.
expression assays (Applied Biosystems) (Wang et al., 2010). Messenger RNA levels were expressed as arbitrary units after correcting for mouse G3PDH.

Measurement of cAMP accumulation

Cyclic AMP accumulation in the MIN6 cells was quantified using previously described methods (Al-Majed et al., 2004). MIN6 cells were plated into 6-well plates with a final density of approximately $5 \times 10^5$/well and incubated with DMEM containing forskolin, GLP-1 or exendin-4. After incubating for 15 min at 37 °C, the medium was removed and stored at $-20$ °C until the cAMP assay. Cyclic AMP concentration was measured by a cAMP RIA kit (Yamasa Co., Choshi, Japan) (Murakami et al., 1995).

Measurement of insulin concentration

MIN6 cells were plated into 12-well plates with a final density of approximately $5 \times 10^5$/well. Before the experiments, MIN6 cells were preincubated in the presence or absence of T3 for 30 min at 37 °C in 0.1% BSA added glucose-free Krebs–Ringer bicarbonate HEPES buffer (KRBH):129 mM NaCl, 4.8 mM KCl, 5.0 mM NaHCO_3, 1.2 mM KH_2PO_4, 1.2 mM MgSO_4, 1.26 mM CaCl_2, 5.0 mM glucose, and 10 mM HEPES, pH 7.4. MIN6 cells were then incubated with 25 mM glucose containing GLP-1 and/or T3 for 15 min. Insulin concentration in the

![Graph](image-url)

**Fig. 2.** Effects of forskolin on D3 mRNA and D3 activity in MIN6 cells. MIN6 cells were incubated with forskolin ($10^{-5}$ M) for 3 h to perform a real-time PCR analysis of D3 mRNA and 6 h to measure D3 activity. D3 mRNA and D3 activity are represented by the mean ± SE of 3 wells. *p < 0.05 (compared with control).

![Graph](image-url)

**Fig. 3.** Effect of GLP-1 on D3 mRNA and D3 activity in MIN6 cells. a) MIN6 cells were incubated for 3 h with various concentrations of GLP-1. D3 mRNA is represented by the mean ± SE of 3 wells. *p < 0.05 (compared with control). b) MIN6 cells were incubated for 3 h with various concentrations of GLP-1. D3 activity is represented by the mean ± SE of 3 wells. *p < 0.05 (compared with control). c) MIN6 cells were incubated for a varying number of hours with GLP-1 ($10^{-8}$ M). D3 mRNA expression is represented by the mean ± SE of 3 wells. *p < 0.05 (compared with control). d) MIN6 cells were incubated for a varying number of hours with GLP-1 ($10^{-8}$ M). D3 activity is represented by the mean ± SE of 3 wells. *p < 0.05 (compared with control).
incubation buffer was measured using an insulin RIA kit (Eiken Chemical, Tokyo, Japan) (Nakagawa et al., 2009).

Statistical analysis

All values are expressed as means ± standard errors (SEs). Statistical differences between groups were evaluated by ANOVA or Student’s t-test.

Results

Characteristics of D3 activity in MIN6 cells

After incubating MIN6 cell sonicate with [125I]T3 in the presence of 10 mM DTT, HPLC analysis revealed only 2 clear peaks that corresponded to T3 and T2. Monodeiodination of T3 was dependent on the protein concentration and incubation period for up to 2 h. Incubation at 0 °C or preheating the cell sonicate at 56 °C for 30 min completely abolished the deiodination. The deiodinating activity was not influenced by 1 mM PTU, but it was completely inhibited by 1 mM IOP. Using a double reciprocal plot with several T3 concentrations, we calculated the T3 kinetic constants and found that Km was equal to 3.5 nmol/L and that the maximum velocity (Vmax) was equal to 115 fmoles T2 produced/mg protein/h (Fig. 1). These results indicate that T3 deiodinating activity is present in MIN6 cells and that the characteristics of this activity are compatible with those of D3.

D3 mRNA expression and the effects of forskolin on D3 expression in MIN6 cells

Real-time PCR demonstrated D3 mRNA expression in MIN6 cells. As shown in Fig. 2, 3-h and 6-h incubation with 10-5 M forskolin markedly increased D3 mRNA and D3 activity, respectively, in MIN6 cells. Forskolin stimulation increased Vmax, with no effect on the Km of T3 deiodinating activity (data not shown). In addition, intracellular cAMP accumulation in MIN6 cells increased with 10-5 M forskolin treatment (data not shown). These results indicate that a cAMP-mediated pathway regulates D3 expression in MIN6 cells.

Effects of GLP-1 on D3 mRNA and D3 activity in MIN6 cells

Because GLP-1 increases intracellular cAMP through the GLP-1 receptor and G-protein coupling pathway in MIN6 cells (Yamato et al., 1997), we examined whether GLP-1 affected D3 mRNA and D3 activity in MIN6 cells. We observed that D3 mRNA and D3 activity in MIN6 cells increased with GLP-1 in a dose-dependent manner (Fig. 3a,b). D3 mRNA and D3 activity increased by 10-8 M GLP-1 and reached peak levels at 3 h (Fig. 3c) and 6 h (Fig. 3d), respectively, in MIN6 cells. Using the double reciprocal plot with various T3 concentrations, we calculated the T3 kinetic constants for control and 10-8 M GLP-1 treatments. These calculations were as follows: Km = 2.1 nmol/L and 1.83 nmol/L and Vmax = 192 fmoles T2 produced/mg protein/h and 435 fmoles T2 produced/mg protein/h, respectively, in MIN6 cells (Fig. 4).

GLP-1 and exendin-4 affected D3 mRNA, D3 activity, and cAMP production in MIN6 cells

We examined whether the GLP-1 receptor agonist exendin-4, which is widely used to treat patients with diabetes (Wei et al., 2012), affected D3 expression in MIN6 cells. As indicated in Fig. 5a, both GLP-1 and exendin-4 significantly stimulated D3 mRNA and D3 activity in MIN6 cells.

Cyclic AMP accumulation increased significantly after 15 min of incubation with 10-8 M GLP-1 and 10-8 M exendin-4 in MIN6 cells (Fig. 5b). These results indicate that GLP-1 and exendin-4 stimulate D3 expression in MIN6 cells via a cAMP-mediated pathway.
The deiodinating activity is represented by the mean ± SE of 3 wells. * compared with control.

**Effects of T3 on GLP-1 stimulated insulin secretion in MIN6 cells**

Because D1 mRNA and D1 activity were detected in MIN6 cells, we examined the effects of GLP-1 on D1 expression in MIN6 cells. In contrast to D3 expression, incubation with 10^{-8} M GLP-1 did not affect D1 mRNA and D1 activity in MIN6 cells (Fig. 7). **Effect of T3 on GLP-1 stimulated insulin secretion in MIN6 cells**

In order to investigate the physiological role of thyroid hormones in the regulation of insulin secretion, we studied the effect of T3 on the GLP-1 stimulated insulin secretion in MIN6 cells (Fig. 8). The addition of 10^{-7} M T3 to the incubation medium significantly suppressed 10^{-8} M GLP-1 stimulated insulin secretion.

**Protein kinase A inhibitor affected GLP-1-stimulated D3 mRNA and D3 activity in MIN6 cells**

To determine the extent to which the cAMP-PKA-mediated pathway regulated D3 expression in MIN6 cells, we examined whether the PKA inhibitor H-89 affected GLP-1 stimulated D3 mRNA and D3 activity in MIN6 cells. The increase in D3 mRNA and D3 activity that followed 3-h incubation with 10^{-8} M GLP-1 in MIN6 cells decreased in the presence of 10^{-5} M H-89 (Fig. 6a, b). These results suggest that D3 expression is pretranslationally regulated by GLP-1 through a GLP-1 receptor-cAMP-PKA-mediated pathway.

**Effects of GLP-1 on D1 mRNA and D1 activity in MIN6 cells**

Because D1 mRNA and D1 activity were detected in MIN6 cells, we examined the effects of GLP-1 on D1 expression in MIN6 cells. In contrast to D3 expression, incubation with 10^{-8} M GLP-1 did not affect D1 mRNA and D1 activity in MIN6 cells (Fig. 7).

Discussion

Using HPLC analysis, we demonstrated that T3 deiodinating activity was present in a mouse insulinoma cell line. MIN6 cells. T3 deiodinating activity had a low Km for T3 and was not affected by 1 mM PTU. Therefore, the characteristics of T3 deiodinating activity in MIN6 cells are compatible with those of D3 (Bianco et al., 2002; Bessho et al., 2010). We also identified D3 mRNA in MIN6 cells, which confirmed D3 expression in these cells. Forskolin significantly increased D3 mRNA and D3 activity, thus implicating a cAMP pathway in mediating the pretranslational regulation of D3 expression in MIN6 cells.

The physiological roles of thyroid hormones in glucose homeostasis and pancreatic β-cell function remain unclear. Hypothyroidism induces fasting hyperglycemia and decreases plasma insulin levels in mice following glucose stimulation (Taguchi et al., 2010). T3 improves rat pancreatic islet function and survival (Verga Falzacappa et al., 2010), and thyroid hormone promotes postnatal rat pancreatic β-cell development and glucose-responsive insulin secretion (Aguayo-Mazzucato et al., 2013). Plasma free T3 concentrations are positively associated with insulin secretion in euthyroid individuals (Ortega et al., 2008). In addition, T3 is a protective factor against STZ-induced diabetes (Verga Falzacappa et al., 2011), and thyroid hormone receptor α-1 expression occurs in pancreatic islets (Zinke et al., 2003). In contrast, systemic hyperthyroidism can decrease glucose tolerance and the insulin secretory capacity of β-cells (Lenzen et al., 1975; Ximenes et al., 2007). In rats, T4 can induce apoptosis in pancreatic β-cells (Jörns et al., 2002). Recently, D3 expression has been identified in mouse perinatal pancreatic β-cells and human adult pancreatic β-cells, and D3-mediated thyroid hormone inactivation is thought to participate in glucose-stimulated insulin secretion from β-cells (Medina et al., 2011).

The MIN6 cell line is one of a few pancreatic β-cell lines that retain the insulin-secretory response to glucose and other secretagogues (Miyazaki et al., 1990; Ishihara et al., 1993), and its characteristics are similar to those of islets isolated from mice. Therefore, MIN6 cells are considered particularly useful for analyzing the molecular mechanisms of glucose-stimulated insulin secretion in pancreatic β-cells. The present results indicating D3 expression in MIN6 cells are consistent with those for D3 expression in mouse and human pancreatic β-cells (Medina et al., 2011). Together, these results indicate that the MIN6 cell line is a useful model for studying D3 expression in pancreatic β-cells and D3 contributions to β-cell function.

Forskolin-mediated D3 expression in MIN6 cells is consistent with previous observations that D3 expression is regulated through a cAMP-
mediated pathway in many cells and tissues at the pretranslational level. Furthermore, this phenomenon is consistent with the existence of a cAMP response element in the promoter region of the D3 gene (Lamirand et al., 2008).

In the present study, GLP-1 rapidly stimulated D3 mRNA and D3 activity in dose-dependent manners, although GLP-1 did not affect D1 expression in MIN6 cells. The GLP-1-mediated increases in D3 mRNA and D3 activity were abolished by the PKA inhibitor H-89, implicating pretranslational regulation of D3 expression in MIN6 cells through a GLP-1 receptor-cAMP-PKA-mediated mechanism. Furthermore, exendin-4, a specific GLP-1 receptor agonist, stimulated D3 mRNA and D3 activity in MIN6 cells.

The GLP-1 receptor is widely distributed in the pancreatic islets, hypothalamus, brainstem, lung, heart, kidney, and gastrointestinal tract (Holst, 2007). GLP-1 and its analog have been extensively studied because of the physiological potentiation of glucose-induced insulin secretion (Holst, 2007). It is noteworthy that GLP-1 treatment can inhibit the apoptosis of human islets (Farilla et al., 2003). GLP-1 is released from gut endocrine cells during nutrient digestion, but it is rapidly degraded by dipeptidyl peptidase-4 (DPP4). GLP-1 stimulates glucose-dependent insulin secretion through the GLP-1 receptor, which is expressed in the membranes of pancreatic islet cells (Moens et al., 1996). The GLP-1 receptor is coupled to adenylyl cyclase through a Gsα subunit, which increases cAMP concentration that subsequently activates a PKA-mediated pathway (Nakazaki et al., 2002). GLP-1 potentiates glucose-induced insulin secretion through cAMP/PKA signaling, and this leads to phosphorylation of regulatory proteins associated with secretion in pancreatic β-cells. GLP-1 receptor agonists and DPP4 inhibitors are widely used in clinical settings to treat diabetes. The present results indicate that D3 expression in MIN6 cells is regulated through a GLP-1 receptor-cAMP-PKA-mediated mechanism. Furthermore, exendin-4, a specific GLP-1 receptor agonist, pretranslationally stimulated D3 expression in MIN6 cells. These results suggest that D3 may inactivate T4 and T3 when stimulated by GLP-1 as well as the pharmacological effect of GLP-1 receptor agonists and DPP4 inhibitors.

In the present study, T3 suppressed GLP-1 stimulated insulin secretion in MIN6 cells. These results suggest that the suppression of intracellular T3 concentration by D3 may be involved in the stimulation of insulin secretion by GLP-1. These results support the concept that suppression of thyroid hormone signaling by D3 may be a novel target for therapeutic intervention in diabetes (Medina et al., 2011).

D3 expression in MIN6 cells, which is stimulated by GLP-1 through a GLP-1 receptor-cAMP-PKA-mediated mechanism, suggests that GLP-1 may modulate insulin secretion via T4 -to-rT3 and T3-to-T2 conversion by D3 expressed in pancreatic β-cells (Fig. 9). Because apoptosis of...
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Conflict of interest statement
The authors declare that there are no conflicts of interest.

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Conclusions
In summary, the present study demonstrates that D3 is expressed in MIN6 cells, which is regulated by GLP-1 through a GLP-1 receptor-cAMP-PKA-mediated pathway. The regulation of intracellular T3 concentration by D3 may be involved in the stimulation of insulin secretion by GLP-1.

β-cells is induced by T4 (Jörns et al., 2002), inactivation of thyroid hormones by D3 may participate in GLP-1-mediated inhibition of apoptosis in pancreatic β-cells.

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