Paracrine GABA and insulin regulate pancreatic alpha cell proliferation in a mouse model of type 1 diabetes

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Abstract

Aims/hypothesis This study aimed to elucidate the mechanism of increased proliferation of alpha cells in recent-onset type 1 diabetes. Pancreatic beta cells express GAD and produce γ-aminobutyric acid (GABA), which inhibits alpha cell secretion of glucagon. We explored the roles of GABA in alpha cell proliferation in conditions corresponding to type 1 diabetes in a mouse model and in vitro.

Methods Type 1 diabetes was induced by injecting the mice with streptozotocin (STZ). Some of the STZ-injected mice were treated with GABA (10 mg/kg daily) for 12 days. Isolated pancreatic islets were treated with STZ or STZ together with GABA for 2 days. The effects of GABA treatment on STZ-induced alpha cell proliferation in vivo and in vitro were assessed. The effect of muscimol, a GABA receptor agonist, on αTC1-6 cell proliferation was also examined.

Results STZ injection substantially decreased levels of GAD, GABA and insulin in pancreatic beta cells 12 h after injection; this was followed by an upsurge of phosphorylated mechanistic target of rapamycin (p-mTOR) in the alpha cells at day 1, and a significant increase in alpha cell mass at day 3. Treating STZ-injected mice with GABA largely restored the immunodetectable levels of insulin and GAD in the beta cells and significantly decreased the number of aldehyde dehydrogenase 1 family, member A3 (ALDH1a3)-positive cells, alpha cell mass and hyperglucagonaemia. STZ treatment also increased alpha cell proliferation in isolated islets, which was reversed by co-treatment with GABA. Muscimol, together with insulin, significantly lowered the level of cytosolic Ca²⁺ and p-mTOR, and decreased the proliferation rate of αTC1-6 cells.

Conclusions/interpretation GABA signalling critically controls the alpha cell population in pancreatic islets. Low intraislet GABA may contribute to alpha cell hyperplasia in early type 1 diabetes.

Keywords Alpha cell · Beta cell · Diabetes · GAD · Glucagon · Insulin · Proliferation · Streptozotocin · Transdifferentiation · γ-Aminobutyric acid

Introduction

Pancreatic alpha cells are located close to and operate cooperatively with beta cells in the islets of Langerhans. Under physiological conditions, these two types of cell secrete the
‘counterregulatory’ hormones glucagon and insulin, respectively, ensuring normoglycaemia. Type 1 diabetes mellitus is caused by autoimmune destruction of the beta cells [1, 2]. In patients [3] and animal models [4–7] of recent-onset type 1 diabetes, there is a significant increase in the number of alpha cells in the islets. However, the mechanisms underlying this increase in alpha cells in recent-onset type 1 diabetes remain unclear [8].

The increase in alpha cells in recent-onset type 1 diabetes may result from a paucity of paracrine factors released from the beta cells. Early studies showed that beta cells produce γ-aminobutyric acid (GABA) via the activity of GAD [9, 10], which is localised around GABA-containing microvesicles [11]. In addition, type A GABA receptors (GABAARs), a class of chloride channels, are expressed in both beta and alpha cells [12–15]. Due to the disparity in expression levels of chloride-intruding and chloride-extruding transporters, the intracellular chloride concentration is high in beta cells but low in alpha cells [16, 17]. Therefore stimulation of GABAARs in the beta cells causes chloride efflux and membrane depolarisation, increasing Ca2+ entry and insulin secretion [18–20], whereas activation of GABAARs in alpha cells results in chloride influx and membrane hyperpolarisation, decreasing Ca2+ entry and glucagon release [12, 21].

Activation of GABAARs inhibits neural stem cell proliferation [22] and promotes neuroprogenitor differentiation [23] through the Ca2+-dependent kinase mechanistic target of rapamycin (mTOR) [24]. Moreover, GABA facilitates pancreatic beta cell generation [14] and insulin regulates beta cell proliferation via the mTOR signalling pathway [7, 25, 26]. This study sought to investigate the roles of intraislet GABA and insulin in regulating alpha cell proliferation, as well as the attendant signalling mechanism(s), in recent-onset type 1 diabetes.

Methods

Type 1 diabetes model, animal groups and treatments
Male C57BL/6 mice (8–10 weeks old; Charles River Laboratories, Senneville, QC, Canada) were used in this study, and they were handled in line with the Guide for the care and use of laboratory animals, published by the U.S. National Institutes of Health (NIH publication no. 85-23, revised 1996). In the first batch of experiments, conditions of type 1 diabetes were induced in 38 mice by a single i.p. injection of streptozotocin (STZ; 150 mg/kg in 0.2 ml saline [154 mmol/l NaCl]). Another 12 mice injected with saline alone served as controls. Mice were euthanised at 2 h (n = 5), 6 h (n = 5), 12 h (n = 6), 1 day (n = 5), 2 days (n = 6), 3 days (n = 5) and 2 weeks (n = 6) after treatment.

The second batch of experiments employed four groups of mice. The control group (n = 6) received 0.2 ml saline daily for 12 days. The mice in the STZ group (n = 10) were treated daily with saline for the first 4 days, a single dose of STZ (150 mg/kg, i.p.) on day 5, and saline daily for another 7 days. The GABA group mice (n = 8) were treated with GABA (10 mg/kg, i.p.) daily for 12 days. Finally, the mice in the STZ + GABA group (n = 8) were treated with GABA (10 mg/kg) daily for the first 4 days and a single dose of STZ (150 mg/kg) on day 5, followed by GABA daily for another 7 days. All mice were euthanised on day 19, and pancreatic tissues were collected for analysis.

Blood glucose and glucagon assays
Two weeks after the STZ injection, mice in the second batch of experiments were fasted for 16 h before tail blood glucose was measured using a glucose meter (OneTouch Ultra2, LifeScan, Wayne, PA, USA). Blood was then collected by cardiac puncture under anaesthesia to obtain serum. Serum glucagon was measured using the Glucagon EIA Kit (Sigma-Aldrich Canada, Oakville, ON, Canada), following the manufacturer’s instructions.

Immunohistochemistry, image analysis and endocrine cell mass measurement
As previously reported [20], pancreatic tissues were serially cut into 5 μm sections at 50 μm intervals. After serum blocking, tissue sections were incubated at 4°C overnight with specific primary antibodies against insulin, glucagon-like peptide 1 (GLP-1), Ki67 (Abcam, Toronto, ON, Canada), glucagon, GAD (Sigma-Aldrich, G5163), the α1 (α1GABAAR) and α4 (α4GABAAR) subunits of GABAAR, aldehyde dehydrogenase 1 family, member A3 (ALDH1a3; Novus Biologicals, Oakville, ON, Canada), p-mTOR and KIT proto-oncogene receptor tyrosine kinase (cKit; R&D Systems, Minneapolis, MN, USA). After primary antibody fostering, sections were simmered with Cy3- or FITC-conjugated secondary antibody. Omission of primary antibody was used as a negative control. Nuclei were stained with DAPI (5 μg/ml). Confocal microscopic images were taken from each section.

Images were analysed using Image-J (https://imagej.nih.gov/ij/) as previously described [20, 27]. To assess each of the immunostained proteins, we analysed between 18 and 68 islets in pancreatic slices prepared from five or six mice in each group. The area of individual islets and the total area of glucagon-positive or insulin-positive cells in the islet were quantified as a measure of alpha and beta cell mass, respectively. As previously described [28], the mass of alpha cells and beta cells was calculated by dividing the total area of glucagon- and insulin-expressing cells, respectively, by the total pancreatic area and then multiplying by the pancreatic weight. The averaged intensity of protein immunofluorescence in individual islets was reported in arbitrary units. Mander’s coefficient of co-localisation of stained proteins was evaluated using the intensity correlation analysis [29].
Pancreatic islet isolation, culture and treatment Pancreatic islets were isolated from C57BL/6 mice (n = 8) using liberase RI digestion as previously described [28] with modifications. Briefly, under a surgical microscope, the major duodenal papilla and common hepatic duct were ligated, while a 30G needle attached to a 10 ml syringe was cannulated into the common bile duct. The pancreas was perfused with 50 μg/ml liberase RI (5.0 ml; Roche Diagnostics, Laval, QC, Canada) and then carefully removed. The pancreas was digested at 37°C for 16–18 min with vigorous shaking every 4 min. The digested samples were filtered through a 0.5 mm wire mesh and centrifuged at 400 g for 2 min at 4°C. The pellets were resuspended in 10 ml RPMI1640 with 10% se-

QC, Canada) and then carefully removed. The pancreas was perfused with a needle attached to a 10 ml syringe was cannulated into the same medium in an atmosphere of 95% air and 5% CO2 at 37°C. Islets were separated into groups (16–20 per group) and treated with either 2.0 mmol/l STZ, 1.0 mmol/l GABA or 2.0 mmol/l STZ + 1.0 mmol/l GABA for 48 h. Test islets were fixed in 4% paraformaldehyde and then triple-stained for insulin, glucagon and DAPI. Images of the stained islets were taken using confocal microscopy.

Experiments on cultured αTC1-6 cells Mouse pancreatic alpha cell line αTC1-6 cells [30] were cultured in regular dishes for immunoblotting immunostaining or growth assay. The cells in each group were treated with insulin (10 nmol/l), the GABAAR agonist muscimol (50 μmol/l) and rapamycin (1.0 μmol/l) alone or in combination. The concentration of muscimol used for the group tests was determined by dose–response analyses (see electronic supplementary material [ESM] Fig. 1).

Using a Countess Automated Cell Counter kit (Life Technologies, Burlington, ON, Canada), the number of αTC1-6 cells was analysed 6 h and 30 h after seeding. Cell lysates were immunoblotted using the following primary antibodies: anti-p-Ser2448 of mTOR and anti-mTOR (Cell Signaling Technology, Danvers, MA, USA; catalogue nos 2971 and 2972) and anti-β-actin (Sigma-Aldrich). Blot band densities were calculated using the Quantity One program (Bio-Rad, www.bio-rad.com/en-uk/product/quantity-one-1-d-analysis-software) and normalised to that of β-actin. Immunocytochemistry was performed as previously described [27, 31, 32]. Briefly, αTC1-6 cells were incubated with rabbit anti-Ki67 (ab15580; Abcam), rabbit anti-GABAARx1 (no. 06-868; Millipore, Peel, ON, Canada) or anti-p-Ser2448 of mTOR (sc-101,738; Santa Cruz Biotechnology, Dallas, TX, USA) at 4°C overnight and sub-
sequently with Cy3- or FITC-conjugated secondary antibo-
dies at room temperature for 1 h. Immunofluorescence was viewed under a Zeiss LSM 510 confocal microscope, and images acquisition was performed using AxioVision software (Zeiss Canada, North York, ON, Canada). Following a previously described procedure [33], cytosolic Ca2+ levels in αTC1-6 cells were recorded by dual-wavelength fura-2 microfluorometry and analysed using ImageMaster software (Photon Technology International, Birmingham, NJ, USA).

Statistical analysis Statistical analysis was performed using one-way ANOVA followed by the Student–Newman–Keuls test for multigroup comparisons. The unpaired Student’s t test was used for comparing data between two groups. Data were expressed as mean ± SD. A p value <0.05 was considered statistically significant.

Results

STZ injection caused sequential changes in GAD and insulin in beta cells, mTOR activity in alpha cells and alpha cell mass We first examined the immunofluorescence levels of GAD and GABAAR subunits, mTOR activity and beta and alpha cell mass at different time points before (control) and after STZ injection. High levels of GAD65/67 immunofluorescence (Fig. 1a) and GABA (not shown) were detected in insulin-positive beta cells in control mice. GAD immunoreactivity significantly declined 12 h after STZ injection (Fig. 1a, b), and this was followed by a significant drop in the number of insulin-positive beta cells 24 h after STZ. As a control, GAD immunoreactivity in the adrenal medulla of STZ-treated mice was comparable to that of control mice (not shown). Two weeks after STZ injection, the immunofluorescence of GAD and insulin had largely faded away (Fig. 1a, b), although there were a few GAD-positive cells scattered in the islets (Fig. 1a). Immunofluorescence of the α1 and β2/3 subunits of GABAAR was demonstrated in beta cells, and this declined in the same temporal order as that of GAD after STZ treatment (ESM Fig. 2). The immunoreactiv-
ity of α4GABAARs was detected in both the alpha and beta cells of control mice (Fig. 1c). α4GABAAR immunoreactivity declined significantly in beta cells 12 h after STZ injection (Fig. 1b) but stayed at a high level in alpha cells 2 days after STZ injection (Fig. 1c).

In control pancreases, immunoreactivity of p-mTOR (ac-
tive mTOR) was mainly associated with insulin-positive beta cells (Fig. 2b) but marginally correlated with glucagon-positive alpha cells (Fig. 2c, d). One day after injection, however, p-mTOR immunoreactivity primarily co-localised with alpha cells (Fig. 2c, d).

The alpha cell mass in control pancreases was 0.12 ± 0.03 mg/g (Fig. 3a, b), whereas the beta cell mass was 1.2 ± 0.12 mg/g (Fig. 3a, c). The beta cell mass fell significantly, to 0.14 ± 0.04 mg/g, 2 days after STZ injection, and partially recovered, to 0.26 ± 0.14 mg/g, 2 weeks after STZ had been given (Fig. 3c). In contrast, the mass of alpha
cells significantly increased 3 days after STZ and reached 0.74 ± 0.17 mg/g 2 weeks after STZ injection (Fig. 3b).

Beta cells expressing Ki67, a cell proliferation marker [34], were frequently observed in the islets of control mice (ESM Fig. 3a), but few alpha cells expressed Ki67 (Fig. 3d). Notably, the number of Ki67-expressing alpha cells significantly increased 2 days after STZ administration (Fig. 3d, e), and the proportion of Ki67-positive alpha cells stayed significantly higher than those of controls even 2 weeks after STZ injection (Fig. 3d, e). Alpha cells also displayed high immunoreactivity for GLP-1; the proportion of GLP-1-positive cells significantly increased 2 days after STZ and was maintained 2 weeks after treatment (Fig. 3g). The proportion of Ki67-positive beta cells also increased in islets 2 weeks after STZ administration, although it was not significantly different from control values (Fig. 3h, i).

**GABA treatment reduced alpha cell hyperplasia and hyperglucagonaemia of STZ-treated mice**

Administration of GABA (10 mg/kg, daily) to the STZ-treated mice for 12 days restored islet GAD immunoreactivity (ESM Fig. 3b) in STZ-treated mice to a level comparable to that in control mice. Importantly, GABA treatment prevented the STZ-induced changes in alpha (Fig. 4a, b) and beta (Fig. 4a, c) cell mass.

As previously reported [35], STZ treatment greatly increased the expression of cKit, a progenitor cell marker [36], in most beta cells (not shown) and some alpha cells (ESM Fig. 4). Interestingly, a large population of ALDH1a3-positive cells that represent phenotypically dedifferentiated and functionally failing beta cells [37] also appeared in the islets of STZ-treated mice (Fig. 4d, e), in which serum glucagon (Fig. 4f) and blood glucose (Fig. 4g) significantly increased. Remarkably, administering GABA to the STZ-treated mice not only significantly decreased the number of pancreatic cKit-positive cells (ESM Fig. 4) and ALDH1a3-positive cells (Fig. 4d, e), but also fundamentally lowered serum glucagon (Fig. 4f) and blood glucose (Fig. 4g) concentrations.

**GABA prevented the STZ-induced alpha cell proliferation in isolated pancreatic islets**

Immunostaining assays identified both insulin-expressing beta cells and glucagon-expressing alpha cells in isolated pancreatic islets.
The proportion of alpha cells to total cells in control islets (Fig. 5a) was 0.062 ± 0.017 (n = 11 islets). Forty-eight hours after adding 2.0 mmol/l STZ to culture media, the immunoreactivity of insulin (Fig. 5a, b) declined, while the proportion of alpha cells to total cells increased to 0.11 ± 0.03 (n = 13 islets; Fig. 5a, c). Remarkably, treating isolated islets with STZ and GABA (1.0 mmol/l) effectively restored the immunoreactivity of insulin (Fig. 5a, b) and significantly decreased the proportion of alpha cells to total cells to 0.061 ± 0.014 (n = 10 islets) in single islets (Fig. 5a, c).

Activating GABA_ARs decreased proliferation, cytosolic Ca^{2+} and mTOR activity of αTC1-6 cells

Thirty hours after plating, the αTC1-6 cells in the control dishes showed a doubling in the number of cells displaying active proliferation (Fig. 6a). The quantity of cells that had been treated with muscimol (50 μmol/l) was comparable to that of control cells, whereas the number of cells treated with insulin (10 nmol/l) increased significantly as reported [7]. Consistent with a previous report [12], insulin treatment increased αLGABA_A_R immunoreactivity in the cell membranes (not shown). Notably, the total number of cells treated with insulin + muscimol was not significantly different from the control (Fig. 6a). In cells treated with rapamycin (1.0 μmol/l), however, the insulin effect vanished, the proliferation rate declined (Fig. 6b) and the percentage of Ki67^+ cells decreased (Fig. 6c). Notably, 30 h after combined treatment with insulin and muscimol, the population of Ki67^+ cells also decreased to a level similar to that of rapamycin-treated group (Fig. 6c, d).

Immunoblotting assays of αTC1-6 cells showed that insulin increased the phosphorylation level of both mTOR and P70S6K, a downstream kinase of mTOR, whereas insulin + muscimol significantly decreased p-mTOR (Fig. 7a, b) and p-P70S6K (ESM Fig. 5a). In contrast, insulin alone or insulin +
muscimol affected the expression level of the tumour suppressor p53 (ESM Fig. 5b).

Ca²⁺-imaging analyses showed that bath perfusion of the insulin-treated αTC1-6 cells with GABA (1.0 mmol/l for 60 s) significantly decreased intracellular Ca²⁺ (Fig. 7c,d), which returned to the basal level when GABA was washed out of the bath solution (Fig. 7c).

Discussion

GABA prevents STZ-induced alpha cell hyperplasia in vivo and in vitro

Intraperitoneal injection of STZ is commonly used to induce the condition of diabetes in animal models of type 1 diabetes. Although it has been shown that STZ lowers intraislet GAD/GABA and results in alpha cell hyperplasia [4–7], the present study is the first to examine STZ-induced temporal changes in intraislet insulin and GAD, mTOR activity and alpha cell proliferation in the same model. Specifically, this study demonstrated that the STZ-induced increased alpha cell proliferation may be initiated by a decline in intraislet GABA and insulin, as a result of beta cell injury.

Cells expressing high levels of cKit and/or ALDH1a3 display progenitor/stem cell-like features [36, 37]. After STZ treatment, the number of cKit-positive and ALDH1a3-positive cells increased whereas the number of GAD-
positive/insulin-positive cells decreased in the islets, suggesting that some of the injured beta cells may ‘dedifferentiate’ [38] into a progenitor-like phenotype. Treatment with insulin in mice with diabetes induced by STZ neither protects against beta cell injury nor inhibits alpha cell proliferation [39]. In contrast, GABA treatment of mice with STZ-induced diabetes not only reduced ALDH1a3-positive and cKit-positive cells and preserved the insulin-positive-cell population, but also prevented the increase in alpha cell mass and hyperglucagonaemia. Moreover, GABA treatment prevented STZ-induced alpha cell proliferation in isolated islets. Together, these results suggest that increased alpha cell proliferation after beta cell injury possibly results from a decline in intraislet GABA signalling.

The number and mass of alpha cells expand in the first 2–3 weeks after STZ injection and then progressively decline [4, 39]. This phenomenon may be related to spontaneous alpha to beta cell transdifferentiation [40], in which alpha cell-produced GLP-1 [41] plays a critical role [5, 42, 43]. Indeed, in STZ-injected mice, alpha cells expressed high levels of GLP-1, and insulin immunoreactivity was identified in some Ki67-positive cells. Moreover, beta cell mass became larger 2 weeks after STZ injection. These data support the notion that alpha to beta cell transdifferentiation may occur after severe beta cell injury [40].

Activation of GABA_AR signalling restraints alpha cell proliferation in vitro We also examined whether GABA restrained the proliferation of αTC1-6 cells, a widely used mouse pancreatic alpha cell line [44, 45]. We reason that if an increased number of alpha cells in type 1 diabetes results essentially from a decline in GABA and insulin, a GABA_AR
agonist and insulin should decrease αTC1-6 cell proliferation. Our assays showed that the GABAAR agonist muscimol alone had no effect on αTC1-6 cell proliferation, whereas insulin significantly increased the cell proliferation as previously reported [7]. Remarkably, muscimol largely prevented the enhancing effect of insulin. Given that insulin increases GABAARs on the surface of αTC1-6 cells [12], we propose that insulin signals maintain GABA ARs on the surface of alpha cell and GABA, via GABA ARs, retains alpha cell proliferation.

The Ca²⁺-dependent activity [25, 46] of mTOR upregulates the proliferation and renewal of pancreatic endocrine cells [7, 26]. Our assays showed that insulin increased the levels of p-mTOR and p-P70S6K in αTC1-6 cells, which was significantly prevented by muscimol. Of note, treating αTC1-6 cells with insulin and muscimol did not affect p53 expression, suggesting that insulin stimulates alpha cell proliferation by activating a specific mTOR signalling cascade independent of p53 activity. Stimulating GABAARs hyperpolarises alpha cells [12] and hence decreases voltage-gated Ca²⁺ channel activity [47]. Indeed, Ca²⁺-imaging analyses show that GABA significantly lowered cytosolic Ca²⁺. These combined data suggest that activating GABAARs reduces Ca²⁺ entry and lowers mTOR activity.

As illustrated in Fig. 8, we propose that, in normal pancreatic islets, insulin signalling maintains a stable expression of GABAARs on the surface of alpha cells, whereas GABA, through GABAARs, keeps the cells hyperpolarised, hence restraining their cytosolic Ca²⁺, mTOR activity and proliferation. Under conditions of type 1 diabetes, however, severe beta cell injury results in a decline in intraislet insulin and GABA, causing increased alpha cell proliferation. Thus, GABA treatment in rodents with STZ-induced diabetes facilitates beta cell generation [14, 15, 48], improves glucose tolerance [14] and prevents alpha cell proliferation. Severe beta cell injury may also induce alpha to beta cell transdifferentiation [40]. In this regard, two recent studies have demonstrated that long-term stimulation of GABAARs facilitates alpha to beta cell transdifferentiation [49, 50]. However, whether GABA signalling regulates alpha to beta cell transdifferentiation after beta cell injury remains to be investigated in future studies.

Data availability The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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