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RESEARCH PAPER

Inhibition of glucagon secretion from pancreatic α-cells by the bile acid TUDCA involves a S1PR2-PI3K pathway

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Abstract

The global prevalence of type 2 diabetes (T2D) continues to rise, and predictions indicate alarming records in coming decades. Although pancreatic β-cell dysfunction and insulin resistance are key factors in the etiology of T2D, the impairment of α-cells has been also implicated. Hyperglucagonemia and altered suppression of glucagon release can be frequently found in individuals with T2D, contributing to hyperglycemia. Bile acids have emerged as novel signaling molecules that regulate metabolism. A wealth of evidence shows that oral treatment with the bile acid TUDCA has therapeutic benefits in T2D, primarily by improving both insulin release from β-cells and insulin sensitivity in peripheral tissues. However, it is unknown whether TUDCA could affect other processes involved in the control of glucose metabolism. Here, we show that acute administration of TUDCA exerts a glucagonstatic action on mouse pancreatic islets and glucagon-releasing αTC1-9 cells. Pharmacological and/or molecular inhibition of the sphingosine-1-phosphate receptor 2 (S1PR2) and the PI3K pathway blunted the TUDCA effect on glucagon release. Additionally, TUDCA increased the activity of ATP-sensitive K^+ (K_{ATP}) channels, decreased action currents and inhibited Ca^{2+} signaling in α-cells without directly affecting the exocytotic process. Glucose-induced suppression of glucagon secretion was found to be compromised under hyperglycemic conditions, yet TUDCA was able to inhibit α-cell function, highlighting its glucagonstatic effect in a pathological context. Collectively, these findings suggest that TUDCA-induced inhibition of glucagon secretion involves the opening of α-cell K_{ATP} channels and activation of the S1PR2/PI3K pathway, expanding the repertoire of potential therapeutic benefits of TUDCA in diabetes treatment.

Keywords: Pancreatic alpha-cell; Pancreatic beta-cell; Glucagon secretion; Islet of Langerhans; Bile acid; TUDCA.

1. Introduction

According to the International Diabetes Federation, more than 850 million people will suffer from diabetes by 2050 [1]. Diabetes carries numerous long-term complications, including heart disease, retinopathies, neuropathies and nephropathies, among others, but also life-threatening acute conditions such as hypoglycemia and hyperosmolar hyperglycemic state [2]. Insulin resistance is a key factor in the etiology of type 2 diabetes (T2D). Under conditions of decreased peripheral insulin sensitivity, the endocrine pancreas enhances insulin secretion to maintain a normoglycemic state. However, if this situation is not adequately compensated, hy-

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perglycemia develops, further contributing to impaired β-cell function, and eventually, apoptosis, resulting in diminished plasma insulin levels and excessive glucose concentrations [2].

Pancreatic α-cells are also key players in glucose homeostasis in both health and disease. Glucagon secretion counteracts insulin actions, and importantly, constitutes the first line of defense against hypoglycemia. At low glucose concentrations, α-cells increase glucagon release, which acts primarily in the liver to promote hepatic glucose output, thereby restoring normoglycemia. As in β-cells, changes in plasma glucose result in cytosolic metabolic signals that regulate electrical activity, Ca^{2+} signaling, and exocytosis in α-cells [3]. However, due to the different ion channel configuration and physiological properties of these two cell types, insulin or glucagon release occurs at opposite plasma glucose levels. Several mechanisms have been proposed for the control of glucagon secretion [3]. In this context, the ATP-dependent K^+ (K_{ATP}) channel plays an important role in the regulation of α-cell secretion by glucose and paracrine factors [4–7]. For instance, the paracrine effect of insulin on α-cells involves the activation of the PI3K/AKT signaling pathway, leading to the opening of K_{ATP} channels, membrane hyperpolarization and inhibition of Ca^{2+} signals, thereby reducing glucagon secretion [8–13].

In addition to β-cell defects, T2D is also characterized by relative or absolute basal hyperglucagonemia and impaired suppression of glucagon secretion at high glucose levels, which can result in enhanced hepatic glucose output, contributing to hyperglycemia [14–17]. Accordingly, attenuation of glucagon release or action has been explored as an antidiabetic strategy [18]. Indeed, the antagonism of the glucagon receptor has been shown to improve glucose homeostasis in both animal models of diabetes and clinical studies. However, several side-effects limit their use as adjuvant therapy in T2D, thus requiring further research for clinical development [18,19]. Instead, strategies aimed at reducing glucagon secretion could also be effective for the treatment of hyperglucagonemia and hyperglycemia in T2D [20].

Tauroursodeoxycholic acid (TUDCA) is a taurine-conjugated bile acid, endogenously produced in humans, that exhibits diverse therapeutic properties [21]. While this bile acid was initially approved by the U.S. Food and Drug Administration (FDA) for the treatment of liver diseases, ongoing research is extending its potential benefits to various pathologies, including neurodegenerative and metabolic disorders [21,22]. Notably, oral administration of TUDCA has also been linked to various beneficial properties for the management of diabetes. These effects of TUDCA have been attributed to various actions. Acting as a molecular chaperone, TUDCA attenuates endoplasmic reticulum (ER) stress in pancreatic β cells exposed to palmitate, proinflammatory cytokines, or elevated glucose levels, thereby decreasing apoptosis and promoting cell survival [22–25]. Furthermore, TUDCA improves β-cell insulin secretion [26–29] by activation of the bile acid receptor TGR5 (Takeda G protein-coupled receptor 5) [28,29]. Other receptors of bile acids have also been associated with an insulinotropic effect on β-cells [30–32]. These TUDCA actions together with those described in peripheral tissues, such as muscle and liver, may be responsible for the beneficial glycemic results derived from its administration in different animal models of T1D and T2D [22,24,33–35] and in in people with obesity [36]. However, whether TUDCA is able to regulate pancreatic α-cell glucagon secretion is unknown. Here, we show that acute exposure to TUDCA inhibits glucagon secretion from mouse isolated islets and αTC1-9 cells through the activation of the sphingosine-1-phosphate receptor 2 (S1PR2) and the PI3K pathways, and involves the modulation of K_{ATP} channels. This glucagonstatic action adds a new metabolic effect of TUDCA to its beneficial properties on glycemic control and potential for T2D treatment.

2. Material and methods

2.1. Animals

Three-month-old male C57BL/6 mice were housed under controlled and standardized conditions with a 12-hour light/dark cycle, a temperature of 22±1°C, and ad libitum access to food and water. All experimental procedures were approved by the institutional Animal Ethics Committee in compliance with current national and European regulations.

2.2. Islet isolation and cell culture

Mice were euthanized by cervical dislocation, and pancreatic islets were isolated by collagenase digestion, as previously described [16,37]. To obtain isolated cells, islets were digested with trypsin and subsequently cultured for 24 h at 37°C in RPMI 1640 medium supplemented with 10% fetal bovine serum, 0.1 mg/mL streptomycin, 100 IU/mL penicillin, and 11.1 mM D-glucose [16].

2.3. αTC1-9 cell culture

The αTC1-9 cell line (ATCC, Barcelona, Spain; cat. CRL2350) was maintained in a humidified atmosphere with 5% CO_2 and cultured in DMEM (Sigma, D2902) supplemented with 1.5 g/L $NaHCO_3$, 11.1 mM glucose, 15 mM HEPES, 2 mM L-glutamine, 0.1 mM non-essential amino acids, 100 IU/mL penicillin, 0.1 mg/mL streptomycin, and 10% fetal bovine serum (FBS) until reaching 60–80% confluence.

2.4. Glucagon secretion

For static glucagon secretion measurements, pancreatic islets (15 islets per well) or αTC1-9 cells (1×10^6) were first incubated for 2 h at 37°C in extracellular solution containing: 115 mM NaCl, 10 mM $NaHCO_3$, 5 mM KCl, 1.1 mM $MgCl_2$, 2.5 $CaCl_2$, 25 mM HEPES and 1.2 mM Na_2HPO_4 supplemented with 5.6 mM glucose and 0.25% bovine serum albumin (BSA), pH=7.4. Islets or αTC1-9 cells were then pre-incubated for 1 h with Krebs-Ringer solution containing: 120 mM NaCl, 5.4 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM MgSO4, 2.4 mM $CaCl_2$, 20 mM HEPES, supplemented with 5.6 mM glucose and 0.3% BSA (pH=7.4). After preincubation, the solution was replaced with fresh medium containing the experimental treatments and incubated for 1 h. Then, supernatants were collected in borosilicated tubes containing aprotinin and stored at −80°C for subsequent glucagon quantification by ELISA (Mercodia, Sweden). For total glucagon and protein content analysis, pancreatic islets or αTC1-9 cells were lysed in ethanol/HCl buffer with continuous agitation for 24 h [16]. Total protein content was determined using the Bradford dye assay [16]. Glucagon secretion experiments using S1PR2-silenced cells were conducted 48 h post-siRNA transfection. For the in vitro assay in conditions of hyperglycemia, pancreatic islets were maintained for 48 h with RPMI 1640 medium supplemented with 10% FBS, 100 IU/mL penicillin, 0.1 mg/mL streptomycin, and 22.2 mM glucose, similarly to previously reported [15].

2.5. RNA extraction and RT-PCR

Total RNA was extracted using NucleoSpin RNA Columns (Macherey-Nagel, MN-740955.250) according to the manufacturer's protocol. Complementary DNA (cDNA) was synthesized using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Quantitative real-time PCR was performed on a CFX96 Real-Time System (Bio-Rad Laboratories, Madrid, Spain)

with Fast SYBR Green Master Mix (Applied Biosystems) in a final reaction volume of 10 μL. Relative gene expression levels were considered using the $2^{-\Delta\Delta CT}$ method and normalized to β-actin mRNA. Primer sequences are listed in Supplementary Table S1.

2.6. Small interfering RNA (siRNA)

Protocols were similar to those previously described [38]. Briefly, αTC1-9 cells were transfected with four different siRNAs (30 nM) for the S1PR2 receptor (S1pr2_1: AAGCTCTACGGCAGTGACAAA; S1pr2_2: CCCAAGGTACCACGGCCCTTAA; S1pr2_3: TACGTCCGAATCTACTTTGTA; S1pr2_4: CACCCCTTAACTCACTGCTCAA; Qiagen, 1027416), or with a non-targeting siRNA (Qiagen, 1027310) used as a negative control. Transfection was carried out overnight using Lipofectamine RNAiMAX reagent (Invitrogen). After a 48-hour recovery period, the cells were used for the subsequent glucagon secretion assay. All siRNAs were tested and validated by RT-PCR prior to the glucagon secretion experiments.

2.7. Ca^{2+} signaling

fresh isolated islets were left to recover for 2 h before incubation with 4 μM Fluo-4AM (Thermo Fisher, Waltham, MA, USA) for 1 h at RT. Then, islets were transferred to a thermostatically-regulated chamber (37°C) on the stage of a confocal microscope Zeiss LSM 510 (Carl Zeiss, Germany). Islets were perfused with Krebs-Bicarbonate medium (pH 7.4), containing 0.5 mM glucose in the presence or absence of 50 μM TUDCA or 5 μM adrenaline. Fluorescence changes were acquired from optical sections (8–10 μM) with a 40X objective by exciting samples at 488 nm. Analysis was performed using the confocal software (LSM Pascal 5, Carl Zeiss, Germany). Pancreatic α-cells were distinguished based on their distinctive pattern of spontaneous Ca^{2+} oscillations at low glucose concentrations and responsiveness to adrenaline [16,39]. In these conditions, pancreatic β-cells remain silent at 0.5 mM glucose and are inhibited by adrenaline [16,39]. Ca^{2+} signaling frequency was assessed in the final 5 min of each stimulus period to allow total equilibration of the perfusion media in the islet chamber, following established protocols [16].

2.8. Patch-clamp measurement of ion channel activity

The analysis of K_{ATP} channel activity was performed using isolated cells and standard patch-clamp procedures, as previously described [37]. Currents were measured with an Axopatch 200B patch-clamp amplifier (Axon Instruments, Union City, CA). Patch pipettes were obtained from borosilicate capillaries (Sutter Instrument, Novato, CA) using a flaming/brown micropipette puller P-97 (Sutter Instrument), achieving resistances between 3 and 5 MΩ with the pipette solutions described below. The bath solution consisted of 5 mM KCl, 135 mM NaCl, 2.5 mM $CaCl_2$, 10 mM HEPES, and 1.1 mM $MgCl_2$, with pH adjusted to 7.4, and supplemented with glucose as indicated. The internal solution of the pipette consisted of 140 mM KCl, 1 mM $MgCl_2$, 10 mM HEPES and 1 mM EGTA, with pH adjusted to 7.2. The pipette voltage potential was held constant at 0 mV throughout the measurements. K_{ATP} channel activity was quantified at room temperature by digitizing 60-seconds segments filtered at 1 kHz, and sampled at 10 kHz using a Digidata 1322A (Axon Instruments, Orleans Drive Sunnyvale, CA). The mean NPo was calculated during the sweep, where channel activity NPo is the product of N (number of functional channels) and Po (open state probability). Po was calculated as the ratio of the open state to the total sample time. NPo was normalized to baseline channel activity recorded before the application of test substances. As described in previous studies [40,41], pancreatic α-cells were recognized based on the presence of spontaneous electrical activity (manifested by the action currents) at 0.5 mM glucose, a stimulatory concentration for these cells.

2.9. Exocytosis measurement

Exocytosis was assessed in isolated cells by monitoring membrane capacitance using the standard whole-cell patch-clamp configuration and operated with the sine+DC mode of the Lock-In amplifier integrated in the Patchmaster software in an EPC-10 amplifier (HEKA) [42]. Currents were filtered using a 4-pole Bessel filter and digitized at 20 kHz. Recordings were conducted at 33–35°C. The extracellular solution consisted of 138 mM NaCl, 5.6 mM KCl, 2.6 mM $CaCl_2$, 1.2 mM $MgCl_2$, 5 mM HEPES, and 5 mM D-glucose, adjusted to pH 7.4 with NaOH. The electrodes, with an average resistance 6.3±0.2 MOΩ, contained an internal solution of 125 mM K-Glutamate, 10 mM KCl, 10 mM NaCl, 1 mM $MgCl_2$, 0.05 mM EGTA, 3 mM Mg-ATP, 0.1 mM cAMP and 5 mM HEPES, adjusted to pH 7.1 with KOH). To evoke exocytosis, trains of 20 depolarizing 500-ms pulses from (−70 mV to 0 mV) were employed to elicit maximal capacitance responses. Pancreatic α-cells were characterized based on electrical criteria; specifically, only cells smaller than 4 pF (2.70±0.05 pF) with the presence of significant Na^+ currents at −70 mV were measured [16,42,43].

2.10. Statistical analysis

Data were evaluated for normality using the Shapiro-Wilk test. Then, parametric data were assessed using one-way ANOVA followed by Tukey's post hoc test, while non-parametric data with the Kruskal-Wallis test. For comparisons between two groups, parametric data was evaluated using the Student's t-test, while the Mann-Whitney U-test for non-parametric data. Data are represented as mean±SEM. Analyses were carried out using GraphPad Prism, version 9.

3. Results

3.1. TUDCA inhibits glucagon secretion from isolated pancreatic islets and αTC1-9 cells

We have previously shown that TUDCA at 10–100 μM increases glucose-stimulated insulin release from pancreatic islets induced by glucose [29]. Under *in vitro* conditions, low glucose concentrations such as 0.5 mM result in maximal stimulation of α-cell glucagon release from pancreatic islets, whereas 11 mM glucose leads to maximal-to-submaximal inhibition [44]. To determine the effects of TUDCA on glucagon release, we exposed glucagon-secreting αTC1-9 cells and isolated mouse pancreatic islets to 5, 50, and 500 μM TUDCA for 1 h in the presence of low concentrations of glucose (0.5 mM). We also exposed them to high glucose levels (11 mM) as an inhibitory stimulus. As shown in Figure 1, while low glucose stimulated glucagon secretion, high glucose inhibited this process [3–5,44]. Remarkably, in the presence of low glucose, TUDCA reduced glucagon release from αTC1-9 cells at 5 and 50 μM, with no effect at 500 μM (Fig. 1A). In the case of pancreatic islets, only 50 and 500 μM TUDCA inhibited secretion (Fig. 1B). Accordingly, we decided to perform the following experiments at 50 μM TUDCA, which is consistent with our previous results in β-cells [29].

We also tested the effect of TUDCA in the presence of 11 mM glucose in a separate set of experiments (Fig. 2). Confirming the findings shown in Figure 1, TUDCA inhibited glucagon secretion at low glucose from αTC1-9 cells (Fig. 2A) and pancreatic islets (Fig. 2B). However, no significant effect was observed at 11 mM

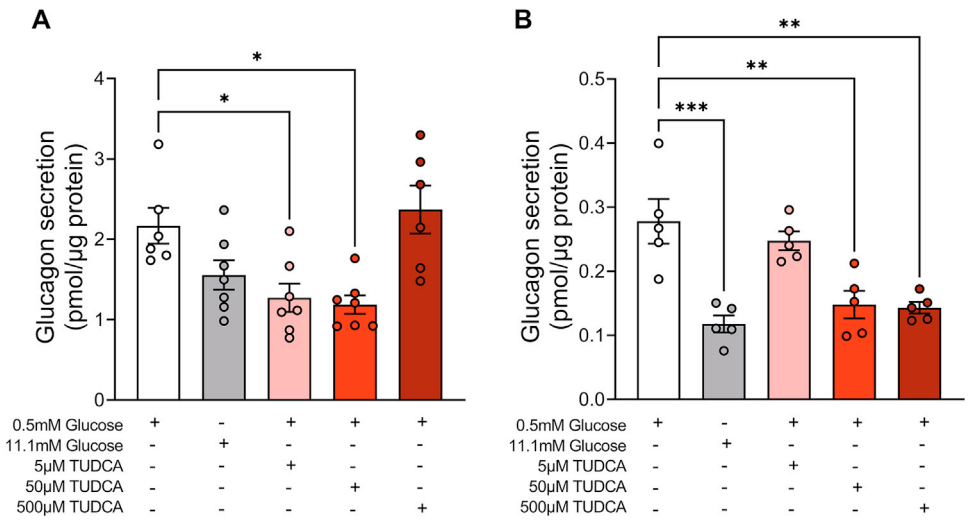

Fig. 1. Effects of TUDCA on glucagon secretion at different concentrations. Pancreatic αTC1-9 cells (A) and pancreatic islets (B) were exposed for 1 h to 0.5 or 11.1 mM glucose, in the absence or presence of 5, 50, or 500 µM TUDCA, as indicated in the graph. Glucagon secretion was measured using ELISA and normalized to protein content. Data are expressed as mean±SEM from 6 wells/condition, obtained from 6 different passages, or 5 wells/condition, obtained from 5 mice. Statistical significance was considered at: $^{*}P<.05$; $^{**}P<.01$; and $^{***}P<.001$.

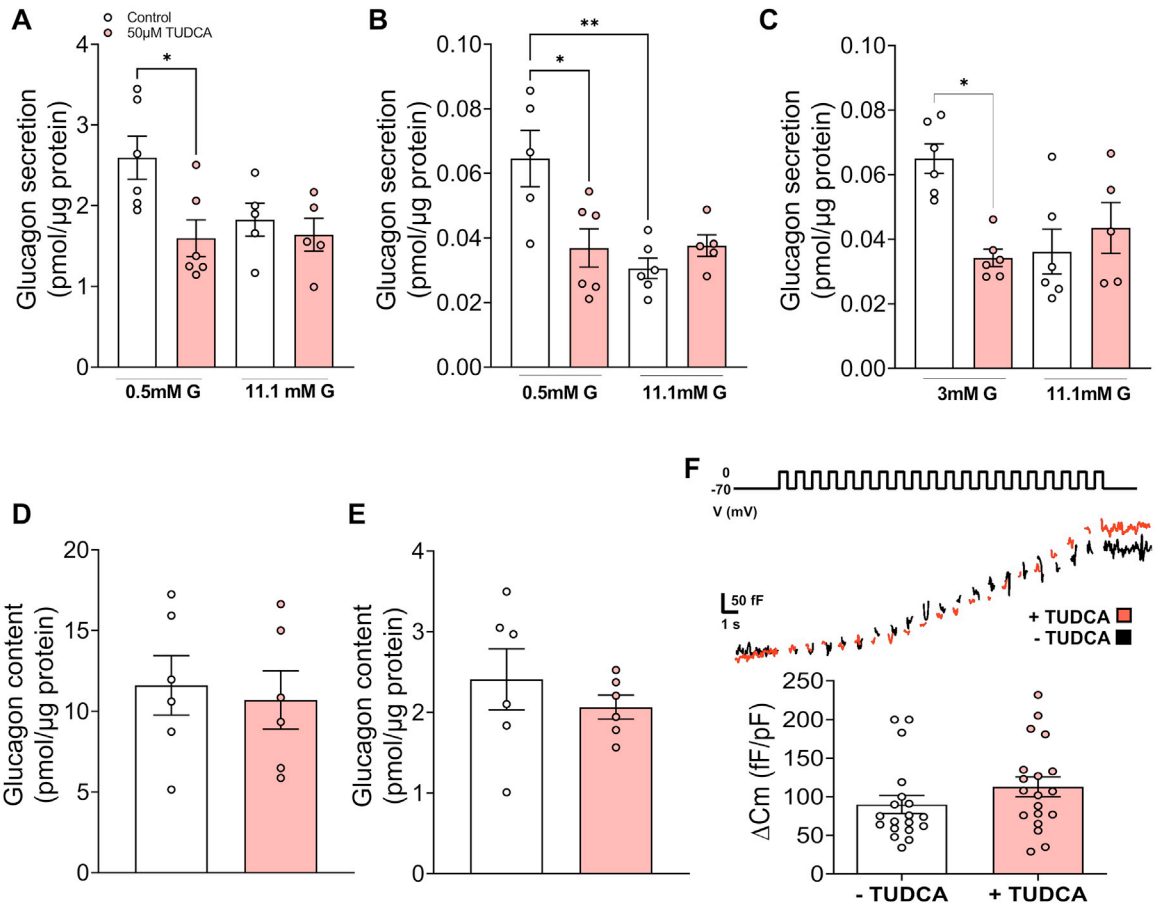

Fig. 2. TUDCA effects on glucose-modulated glucagon secretion. Pancreatic αTC1-9 cells (A) and pancreatic islets (B) were exposed for 1 h to 0.5 or 11.1 mM glucose, in the absence or presence of 50 µM TUDCA, as indicated in the graph. Glucagon secretion was measured using ELISA and normalized to protein content. Data are expressed as mean±SEM from 5 to 6 wells/condition obtained from 6 different cell line passages, or 5–6 wells/condition obtained from 6 mice. (C) Pancreatic islets were exposed for 1 h to 3 or 11.1 mM glucose, in the presence or absence of 50 µM TUDCA, as shown in the graph. Data are expressed as mean±SEM from 6 wells/condition obtained from 6 mice. (D, E) Total glucagon content quantification from experiments shown in A and B in αTC1-9 cells (D) and pancreatic islets (E). (F) Exocytosis was measured in isolated pancreatic α-cells in response to a stimulatory train of depolarizations (20×500 ms pulses from −70 mV to 0 mV) in the absence (−TUDCA) and presence (+TUDCA) of 50 µM TUDCA. The depolarization-evoked increase in capacitance was similar in both conditions. Quantification of exocytosis (capacitance difference normalized by cell size) in control cells and cells incubated with TUDCA is shown below capacitance records. Data are expressed as mean±SEM (n=19 cells in each group). Statistical significance was considered at $^{*}P<.05$ and $^{**}P<.01$. G: Glucose.

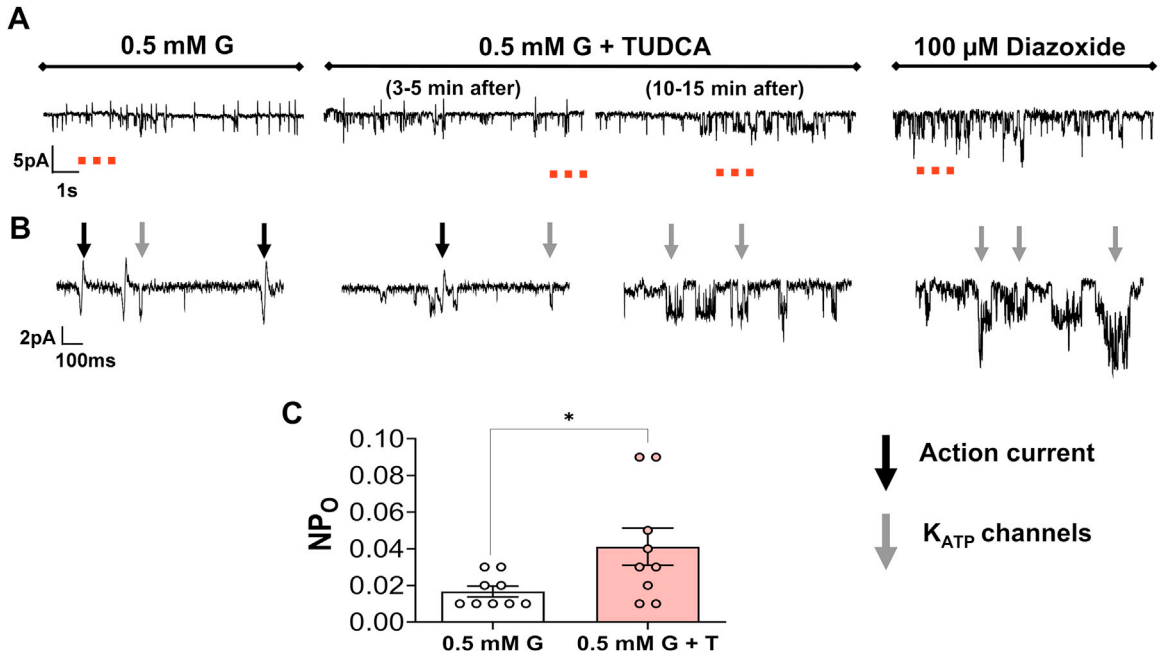

Fig. 3. TUDCA modulates K_{ATP} channel activity in pancreatic α cells. (A) Representative recording from an isolated α-cell illustrating K_{ATP} channel activity at 0.5 mM glucose (G), highlighting the presence of action currents, and 10–15 min after application of 50 µM TUDCA (T), where K_{ATP} channel activity increased. In the presence of TUDCA, action currents completely ceased in 5 of 9 cells. Diazoxide, a K_{ATP} channel opener, was used as a positive control. (B) Expanded traces from periods indicated with red dotted line. Black and grey arrows indicate action currents or K_{ATP} channel openings, respectively. (C) Open probability of K_{ATP} channel (NP_0) at 0.5 mM G and 10–15 min after application of TUDCA. Data are expressed as mean±SEM (n=9 cells from 5 mice). Statistical significance was considered at $^*P<.05$ (paired t-test).

G, when glucagon release is already inhibited [44]. At 3 mM glucose, which induces submaximal α-cell secretion [44], TUDCA also showed a glucagonstatic action (Fig. 2C). Acute exposure to TUDCA did not change the glucagon protein content in either αTC1-9 cells (Fig. 2D) or pancreatic islets (Fig. 2E), indicating that its inhibitory action on α-cell secretion was not due to effects on glucagon synthesis. Next, we aimed to analyze whether TUDCA might be affecting the α-cell exocytotic machinery. We assessed exocytosis by patch-clamp and capacitance measurements in response to a stimulatory train of depolarizations (20×500 ms pulses from −70 mV to 0 mV) in the presence or absence of TUDCA (Fig. 2F). The depolarization protocol induced exocytotic responses following a characteristic pattern [16,45], yet no differences were observed with TUDCA, suggesting that its effect on glucagon release was probably due to upstream steps in the secretory pathway. Therefore, in the next experiments, we explored preceding signaling events.

3.2. TUDCA modulates K_{ATP} channel activity and Ca^{2+} signals in the pancreatic α-cell

At low glucose concentrations, the α-cell membrane potential is within a range that allows the activation of voltage-dependent Na^+ and Ca^{2+} channels, generating action potentials, Ca^{2+} influx and secretion [3,4,9]. As in β-cells, the K_{ATP} channel plays an important role in α-cells modulating membrane potential and electrical activity, and its activation is coupled to hyperpolarization and suppression of glucagon secretion [4–7,41,46]. To analyze whether this channel was affected by TUDCA, we used the path-clamp technique in the cell-attached mode. At 0.5 mM glucose, α-cells exhibited low K_{ATP} channel activity together with action currents, indicating the firing of action potentials (Fig. 3A, B), as expected for this islet cell type [6,46]. TUDCA administration increased channel activity after few minutes, along with a reduction in the occurrence of action currents (Fig. 3A-C), which completely disappeared in 5 cells out of 9 recordings after 10–15 min of treatment. This effect intensified in the presence of the K_{ATP} channel opener diazoxide, which hyperpolarizes α and β-cells [5,6,46]. These findings suggest that TUDCA reduces action potential firing by increasing K_{ATP} channel activity.

Electrical activity governs α-cell Ca^{2+} signals, which in turn regulate the secretory process [47]. Decreased action potential firing in α-cells is coupled to inhibition of Ca^{2+} oscillations and α-cell exocytosis [47]. Consistent with the electrophysiological findings (Fig. 3), TUDCA application reduced the frequency of Ca^{2+} signals in pancreatic α-cells within isolated islets (Fig. 4).

3.3. The effect of TUDCA on glucagon secretion is mediated by activation of a S1PR2-AKT pathway

Insulin plays a significant role as a negative regulator of glucagon secretion, likely attributed to the activation of α-cell K_{ATP} channels [12]. Since bile acids, including TUDCA, have been shown to bind to the S1PR2 receptor in the liver, which in turn activates the insulin pathway [35,48,49], we explored whether this signaling route was involved in the TUDCA glucagonstatic actions. In the presence of either the PI3K inhibitor wortmanin (Fig. 5A) or the S1PR2 inhibitor JTE-013 (Fig. 5B), the effect of TUDCA on glucagon release was blunted in pancreatic islets. We further assessed the involvement of S1PR2 using siRNA protocols (Fig. 6; Supplementary Fig. S1). S1PR2 expression in αTC1-9 cells was validated by RT-PCR after transfection with a control siRNA and four different siRNAs specifically targeting S1PR2, selecting the most effective to downregulate the receptor (Supplementary Fig. S1). While TUDCA inhibited glucagon secretion in αTC1-9 cells under control conditions, this effect was abrogated after treatment with the S1PR2 siRNA (Fig. 6A). No effects were observed in glucagon content (Fig. 6B). Since the bile acid receptor TGR5 has also been reported to regulate the function of the endocrine pancreas [29,35], we evaluated its potential contribution to the inhibitory effect of TUDCA on glucagon secretion. Incubation of pancreatic islets with INT-777, a specific TGR5 agonist, led to stimulation of glucagon release (Supplementary Fig. S2), indicating that the inhibitory effects of TUDCA

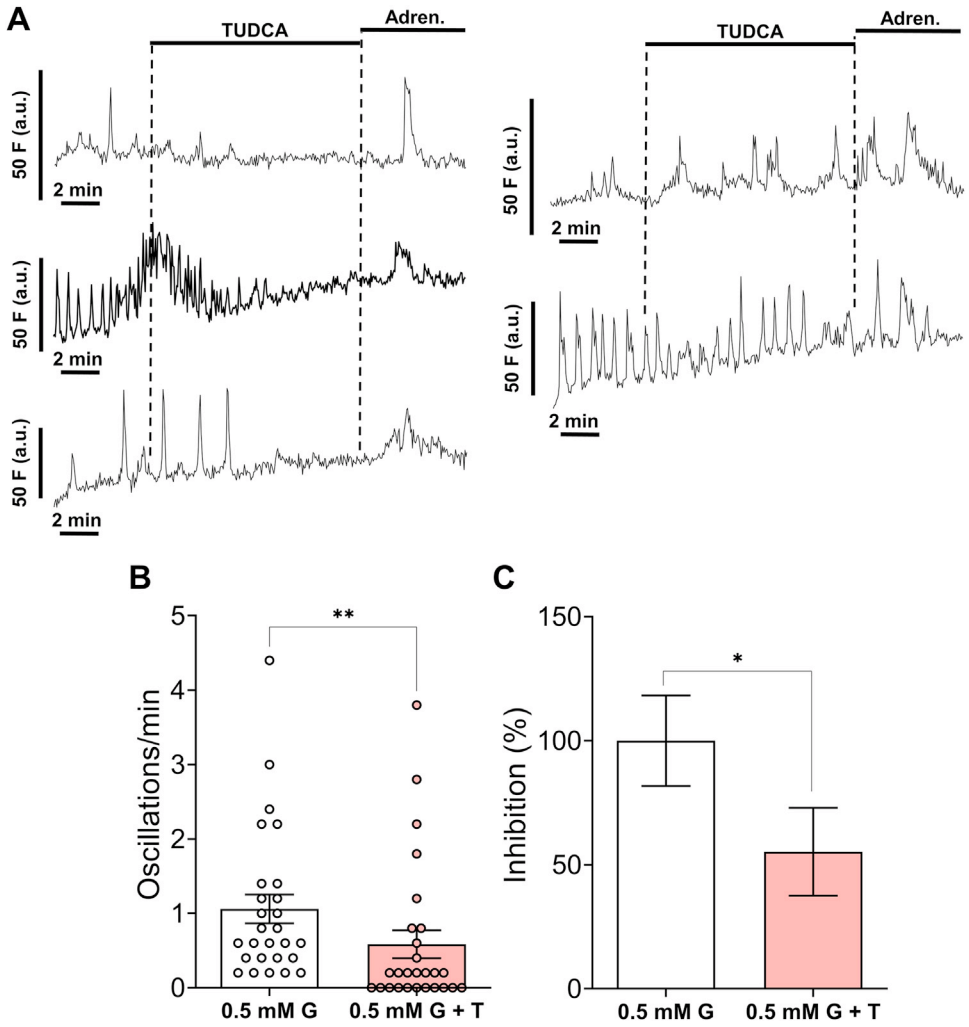

Fig. 4. TUDCA inhibits Ca^{2+} signaling in pancreatic α-cells within pancreatic islets. (A) Representative Ca^{2+} traces in response to 0.5 mM glucose (G) and 0.5 mM G+50 μM TUDCA. Changes in intracellular Ca^{2+} levels were monitored in single α-cells within intact mice islets by confocal microscopy. Pancreatic α-cells were distinguished by their typical pattern characterized by spontaneous Ca^{2+} oscillations at low glucose concentration (0.5 mM) and response to 5 μM adrenaline (Adren.). (B) Frequency of Ca^{2+} signals in oscillations per minute in the absence and presence of TUDCA. (C) Percentage of inhibition as frequency normalized to 100%. Data are expressed as mean±SEM of 27 cells recorded from 7 islets obtained from 4 mice. Statistical significance was considered at $^{*}P<.05$ and $^{**}P<.01$.

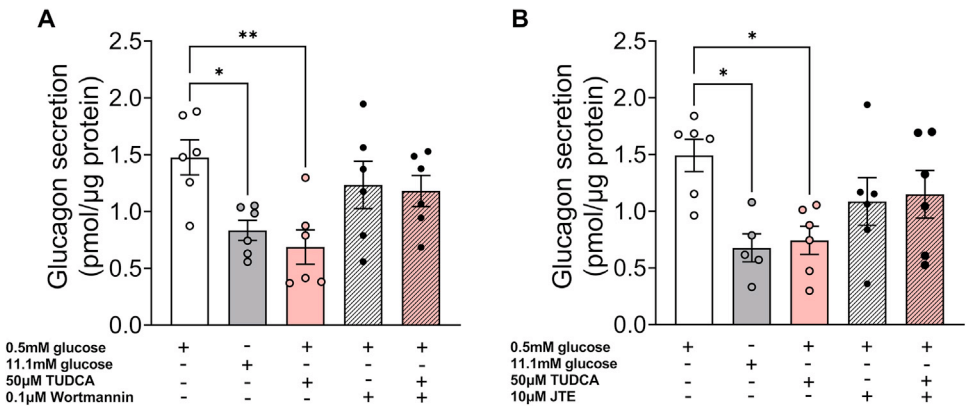

Fig. 5. TUDCA effects on glucagon secretion are blunted following pharmacological inhibition of AKT or the S1PR2 receptor. Pancreatic islets were exposed for 1 h to 0.5 or 11.1 mM glucose, in the presence or absence of 50 μM TUDCA or pharmacological inhibitors of AKT (A) and S1PR2 (B), as indicated in the graph. Glucagon secretion was measured using ELISA and normalized to protein content. Data are expressed as mean±SEM from 5 to 6 wells/condition obtained from 6 mice. Statistical significance was considered at $^{*}P<.05$ and $^{**}P<.01$.

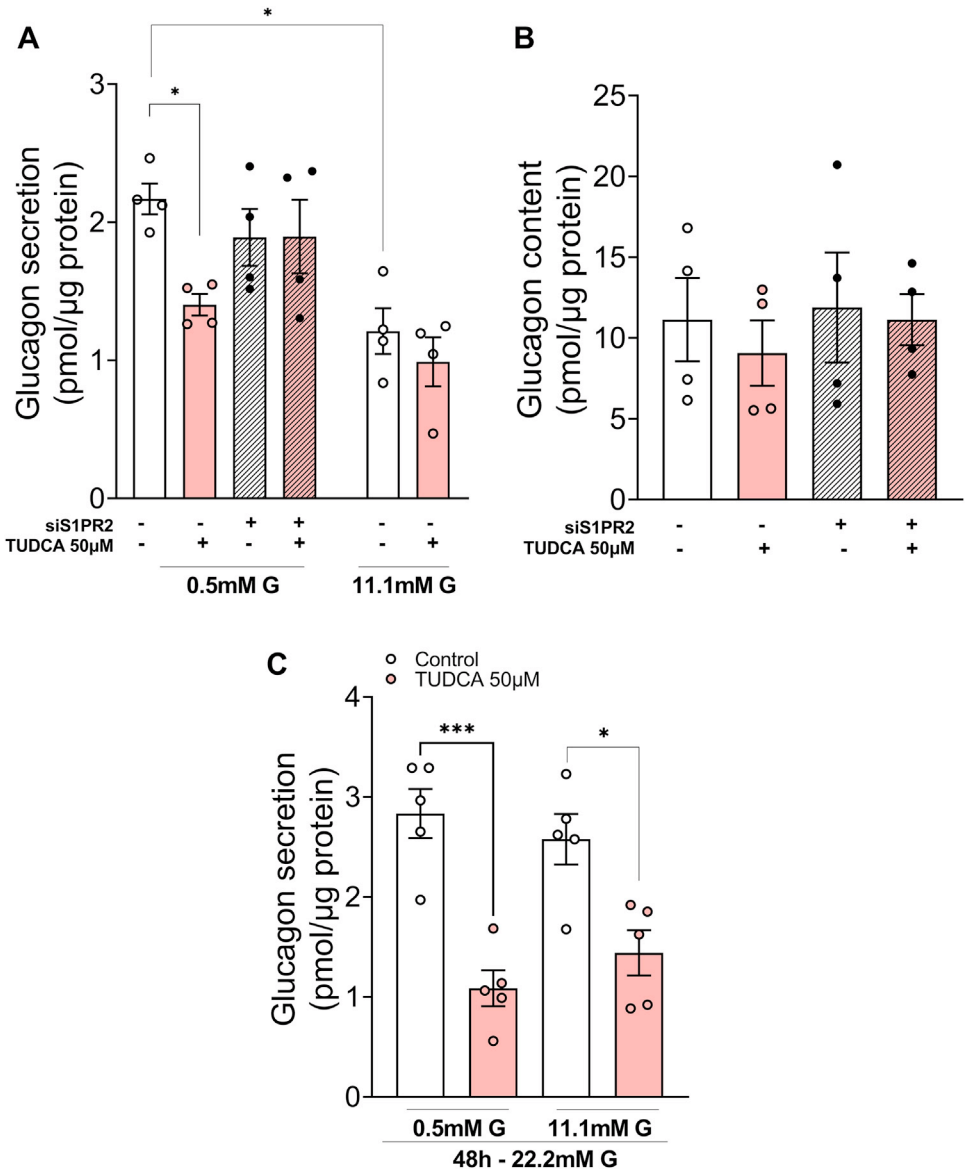

Fig. 6. S1PR2 receptor silencing abrogates TUDCA effects on glucagon secretion. (A) αTC1-9 cells were exposed for 1 h to 0.5 or 11.1 mM glucose, in the presence or absence of 50 µM TUDCA, 48 h after transfection with siS1PR2 (+siS1PR2) or control siRNA (-siS1PR2), as indicated in the graph. (B) Total glucagon content quantification in αTC1-9 cells in each condition. Glucagon secretion was measured using ELISA and normalized to protein content. Data are expressed as mean±SEM from 4 wells/condition, obtained from 4 different passages. (C) TUDCA reverses the impaired glucose-induced suppression of glucagon secretion under hyperglycemic conditions. After incubation with 22.2 mM glucose for 48 h to mimic a hyperglycemic environment, pancreatic islets were exposed for 1 h with 0.5 or 11.1 mM glucose in the presence or absence of 50 µM TUDCA, as indicated in the graph. Glucagon secretion was assessed by ELISA and normalized to protein content. Data are expressed as mean±SEM from 5 wells per condition obtained from 5 mice. Statistical significance was considered at $^{*}P<.05$ and $^{***}P<.001$.

would not be mediated by this receptor. Overall, these findings support the involvement of a S1PR2/PI3K pathway in the glucagonstatic action of TUDCA.

3.4. TUDCA reverses the lack of glucose-induced suppression of glucagon secretion under hyperglycemic conditions

Abnormal suppression of glucagon secretion at high glucose levels is a hallmark of T2D, which may further contribute to the altered glucose homeostasis in people with diabetes [4,15]. In this context, chronic hyperglycemia has been shown to progressively impair α-cell function [15]. Consistent with previous findings [15], the inhibitory effect of high glucose (11 mM) on glucagon secretion was lost after 48 h incubation of pancreatic islets under hyperglycemic conditions (22.2 mM) (Fig. 6C), echoing the alterations observed in T2D [41]. In this hyperglycemic setting, TUDCA was able to reverse the lack of glucagon suppression at 11 mM G to levels similar to those observed in Figures 1 and 2, further highlighting the therapeutic potential of this bile acid.

4. Discussion

Although T2D primarily involves impaired β-cell secretion in the context of insulin resistance [2], dysregulated α-cell function also contributes to the pathophysiology of this metabolic disease [14,18]. Extensive evidence shows that people with diabetes may present with relative or absolute hyperglucagonemia and compromised suppression of glucagon secretion at elevated glucose concentrations, which contributes to hyperglycemia by activating hepatic glucose output [14,18]. Several preclinical and clinical studies

have reported that blocking glucagon action or inhibiting its secretion offer therapeutic benefits in the control of hyperglycemia [18–20]. Therefore, this strategy could be effective as an adjuvant treatment combined with therapeutic tools aimed at stimulating insulin secretion.

TUDCA is a taurine-conjugated bile acid approved for the treatment of liver disorders [31,50]. Oral administration of TUDCA has also been associated with therapeutic advantages for the management of neurodegenerative diseases, obesity, and diabetes [31,50]. Indeed, TUDCA supplementation ameliorates insulin resistance and inflammation through various actions in peripheral tissues such as the liver, adipose tissue, and muscle [22,36,51]. Furthermore, this molecule increases β-cell survival and function by preventing ER stress, acting as a chaperone, and/or activating bile acid receptors and their down-stream signaling cascades [25,32]. Most of these actions may underlie the valuable TUDCA effects on glycemic homeostasis in patients with obesity and animal models of diabetes [22,24,31,33–35].

In the present study, we show that TUDCA also decreases glucagon secretion from pancreatic α-cells, highlighting its diversity of actions with potential metabolic benefits in disorders such as T2D. This effect was associated with increased K_{ATP} channel activity and reduced Ca^{2+} signals in α-cells, and the activation of a S1PR2/PI3K pathway. Bile acids act via several receptors such as TGR5, S1PR2, $\alpha5\beta1$ Integrin or farnesoid X receptor (FXR), among others, to exert their metabolic functions in the liver, intestine and other tissues [31,35,52]. Acute and chronic exposure of mouse and human pancreatic islets to bile acids, including TUDCA, increases glucose-stimulated insulin secretion, effects attributed to either FXR [30,32] or TGR5 [27–29] receptors. TUDCA and other taurine-conjugated bile acids exhibit a hydrophilic nature, which may confer higher affinity to membrane receptors such as TGR5 and S1PR2 [52,53]. Indeed, in addition to TGR5 [35], TUDCA has been shown to modulate hepatic metabolism by activation of S1PR2, similarly to other bile acids such as taurocholic acid [48,54]. S1PR2 is a G-protein coupled receptor, whose activation triggers several signaling cascades including PI3K/AKT. Moreover, TUDCA has been reported to stimulate the PI3K/AKT pathway in the liver and other tissues and cells such as cortical neurons [22,48,53–57]. Consistently, our results show the involvement of S1PR2 and PI3K in the glucagonstatic action of TUDCA. In any case, although the αTC1-9 cell line is widely recognized as a model of pancreatic α-cells and glucagon secretion, it does not exhibit the structural and functional complexity of this cell type within the islet. In this regard, and given the difficulties in analyzing α-cell-specific molecular pathways within the islet context, it would be interesting to conduct future studies using knockout mice with deletion of S1PR2 selectively in pancreatic α-cells.

Both insulin signaling and PI3K/AKT activation are associated with the inhibition of glucagon secretion [8,10,13]. This regulatory pathway has been related with the opening of K_{ATP} channels, which leads to membrane hyperpolarization, and subsequent attenuation of action potentials and Ca^{2+} entry into α-cells [9,11,12], playing an important role in the paracrine modulation of these cells [3]. Similar to the insulin/PI3K pathway, we observed that TUDCA increased K_{ATP} channel activity in the α-cell, and reduced the frequency of action currents and Ca^{2+} oscillations. Given the Ca^{2+}-dependent nature of glucagon secretion, reduced Ca^{2+} entry into α-cells is associated to decreased hormonal release [3,9]. TUDCA did not affect the exocytotic process, indicating that its glucagonstatic effect was primarily mediated by modulation of K_{ATP} channels and Ca^{2+} signals, key aspects in the regulation of α-cell function [4,5,7].

Unlike isolated islets, TUDCA had no effect on glucagon release from αTC1-9 cells at 500 μM, suggesting subtle differences between both cell models. Although αTC1-9 cells are a recognized model of pancreatic α-cells, they do not necessarily respond exactly in the same manner as primary islets nor do they exhibit identical expression levels of receptors and activity of the molecular pathways involved [58]. Additionally, bile acids can act on multiple receptors, potentially eliciting divergent effects depending on the concentration used [35]. At lower doses, TUDCA may selectively activate specific receptors and/or signaling pathways that mediate the inhibitory response. Conversely, higher concentrations could lead to receptor desensitization, activation of compensatory or opposing pathways, saturation of molecular targets, or even cellular toxicity [59]. Indeed, non-monotonic effects have been described for different receptor agonists on several endocrine cell types [60]. Furthermore, the role of TUDCA as a chemical chaperone at high concentrations, alleviating endoplasmic reticulum stress [22], could also influence the inhibitory effect on αTC1-9 cells. Therefore, further research is required to decipher the complexity of bile acid signaling in pancreatic α-cells.

Somatostatin paracrine signaling plays an important role in the modulation of islet function by inhibiting insulin and glucagon secretion [3]. Although we cannot completely rule out an indirect effect from δ-cells (or other islet cells types), our results indicate that the TUDCA effect on glucagon release is primarily mediated by direct actions on α-cells. This is supported by several pieces of evidence: first, TUDCA also inhibited glucagon secretion in αTC1-9 cells, a cellular model lacking paracrine regulation by somatostatin. Moreover, TUDCA exerted effects on K_{ATP} channel activity in isolated primary α-cells, which also lack paracrine signaling. Finally, TUDCA has been reported to stimulate insulin release from mouse islets at 11 mM glucose [29]. Given that somatostatin is a potent inhibitor of insulin release, it is unlikely that TUDCA could induce somatostatin release, since this would result in reduced insulin secretion rather than the stimulatory action of this bile acid observed in pancreatic β-cells [29].

Increasing evidence demonstrates the beneficial effects of TUDCA treatment in metabolic diseases such as obesity and T2D [22,24,33,34]. TUDCA supplementation has also been proposed to maintain a healthy microbiota with beneficial metabolic effects [61]. The properties of this bile acid in ameliorating glycemic disorders have been attributed to improved insulin sensitivity in peripheral tissues [31,35,36] and increased insulin secretion and survival in pancreatic β-cells [22–26,28–31]. In the present study, we report that TUDCA also exerts a glucagonstatic effect through direct actions on pancreatic α-cells, adding a novel effect to the constellation of its potential benefits in T2D treatment. In addition to β-cell dysfunction, people with T2D exhibit hyperglucagonemia and impaired suppression of glucagon secretion by glucose [41]. We used an *in vitro* approach to mimic a hyperglycemic scenario, which led to impaired glucose-induced α-cell inhibition, consistent with previous findings [15]. Interestingly, TUDCA was able to reduce glucagon release at 11 mM glucose, despite α-cell dysfunction. Therefore, these results further support the potential benefits of TUDCA in a pathological context.

Several strategies targeting either the pancreatic α-cell and/or glucagon signaling have emerged in recent years. While blocking glucagon action has been shown to ameliorate hyperglycemia in T2D, this approach carries some limiting side effects [18]. Given that TUDCA can exert both insulinotropic [27–30,32] and glucagonstatic actions, this combined effect could be interesting to improve its advantages as an adjuvant treatment in T2D. In addition to a therapeutic perspective, our findings also suggest a potential novel regulation of α-cells and glucagon release by bile acids. In the case of TUDCA, it is normally produced endogenously in humans in lower amounts than other bile acids [31,50]. Given that systemic physiological levels of TUDCA in humans and mice are in

the nanomolar range (approximately 2–40 nM) [62,63], the effects observed in the micromolar concentration in this study are likely operating in a supraphysiological context. This is similar to the situation reported for the actions of TUDCA on pancreatic islets and insulin secretion [29]. Nevertheless, the experimental design of the current study was aimed at analyzing the therapeutic potential of TUDCA on glucagon secretion based on previous studies from our laboratory and other research groups [29,35]. Therefore, further research is required to determine whether this signaling pathway may also play a physiological role.

Data availability

Data is available from the corresponding authors upon reasonable request.

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Declaration of competing interest

All contributing authors declare no conflicts of interest.

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Supplementary materials

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