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**How to cite**

BARTLEY, Clarissa et al. Chronic fructose renders pancreatic beta-cells hyper-responsive to glucose-stimulated insulin secretion through extracellular ATP signaling. In: American Journal of Physiology. Endocrinology and Metabolism, 2019, vol. 317, n° 1, p. E25–E41. doi: 10.1152/ajpendo.00456.2018

This publication URL: https://archive-ouverte.unige.ch/unige:116616

Publication DOI: 10.1152/ajpendo.00456.2018

## Research Article

# Chronic fructose renders pancreatic beta-cells hyper-responsive to glucose-stimulated insulin secretion through extracellular ATP signaling

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**Running Title:** Chronic fructose and ATP signaling in pancreatic ß-cells

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**Keywords**: pancreatic islet; insulin secretion; fructose; AMPK; ATP; pannexin; purinergic receptor

## Abstract

Fructose is widely used as a sweetener in processed food and since then is associated with metabolic disorders, such as obesity. However, the underlying cellular mechanisms remain unclear, in particular regarding the pancreatic beta-cell. Here, we investigated the effects of chronic exposure to fructose on the function of insulinoma cells and isolated mouse and human pancreatic islets. Although fructose per se did not acutely stimulate insulin exocytosis, our data show that chronic fructose rendered rodent and human beta-cells hyper-responsive to intermediate physiological glucose concentrations. Fructose exposure reduced intracellular ATP levels, without affecting mitochondrial function, induced AMPK activation and favored ATP release from the beta-cells upon acute glucose stimulation. The resulting increase in extracellular ATP, mediated by pannexin1 channels, activated the calcium-mobilizer P2Y purinergic receptors. Immunodetection revealed the presence of both pannexin1 channels and P2Y1 receptors in beta-cells. Addition of an ectonucleotidase inhibitor or P2Y1 agonists to naïve beta-cells potentiated insulin secretion stimulated by intermediate glucose, mimicking the fructose treatment. Conversely, P2Y1 antagonist and pannexin1 inhibitor reversed the effects of fructose, as confirmed using pannexin1-null islets and by the clearance of extracellular ATP by apyrase. These results reveal an important function of ATP signalling in pancreatic beta-cells mediating fructose-induced hyper-responsiveness.

1

## Introduction

In the late 70's, the monosaccharide fructose was introduced in processed food as a sweetener and taste enhancer in the form of high-fructose corn syrup. While the evidence accumulates for a causal link between dietary fructose and metabolic dysfunctions (6, 29, 50, 53), such as hepatic steatosis and obesity, the associated cellular mechanisms remain obscure. Intriguingly, chronic effects of fructose on pancreatic ß-cell function have been poorly investigated to date. Energy homeostasis is maintained through the action of insulin on its target tissues, promoting glucose uptake and fat storage. The ß-cell is an energy sensor that couples glucose levels to insulin secretion *via* the elevation of intracellular ATP. Over-secretion of the anabolic hormone insulin favors lipogenesis, the development of obesity and then of insulin resistance. On such a pathophysiological background, changes in the ß-cell response going from hypersecretion to potential loss of normal glucose-stimulated insulin secretion (GSIS) represent crucial steps associated with insulin resistance and ultimately diabetes (30).

Although glucose is the chief nutrient triggering insulin release, other metabolites such as fatty acids act synergistically with glucose to potentiate insulin secretion. Fructose is not considered as a nutrient secretagogue. Indeed, the ß-cells do not express the fructose transporter GLUT5 (17) and acutely fructose induces a negligible secretory response, unless used at extremely high concentrations (80-240 mM) (17). However, a physiological concentration of fructose (10 mM) added with stimulatory glucose (10 mM) potentiates insulin secretion from rat islets, despite a drop in intracellular ATP (20). Regarding this apparent paradox of a sugar-induced decrease of cellular ATP, it is striking to note that, as opposed to glucose, central fructose lowers hypothalamic ATP levels and is associated with higher food intake (14). Accordingly, chronic central administration of fructose increases AMPK phosphorylation in the hypothalamus of treated rats (33), an effect also observed in cultured hypothalamic GT1-7 neurons (10). Given the close relationship between these two glucose sensors, *i.e*. the hypothalamus and the ß-cell

(57), one can hypothesise that similar mechanisms underlie the response of ß-cells to chronic exposure to fructose, favouring the reported weight gain associated with this sugar consumption.

In this context, another piece of information is intriguing. Indeed, fructose can potentiate GSIS when it binds to the sweet taste receptors T1R2/3 expressed not only in the taste buds of the tongue but also in ß-cells (34, 44). Although these studies have revealed the presence of these receptors in mouse and human ß-cells, the mechanisms underlying fructose-induced potentiation of GSIS remain yet unidentified (24, 40). The recently documented presence of additional cell-surface components in ß-cells highlights the receptor hypothesis, postulating the participation of specific ligands, i.e. metabolites (fructose, glutamate, fatty acids) or nucleotides (ATP, ADP, adenosine), as potent insulinotropic agents (24, 40). Regarding nucleotides, vallate receptor cells release ATP upon activation of their taste receptors (25). Such a mechanism relies on pannexin (Panx) channels for the release of cellular ATP (4, 15) and purinergic receptors for nucleotide signaling (1, 3, 32, 45). The opening of Panx1 channels is induced by phosphorylation of its Y308 residue, by membrane depolarization (18), as well as by the activation of purinergic receptors and the elevation of cytosolic $Ca^{2+}$; whereas their closure can be realized by some drugs, such as mefloquine or the peptide $^{10}$Panx1 (16, 43). Several P2Y and P2X purinergic receptors are found in ß-cells (1, 3, 22, 27, 32, 45). Accumulating evidence suggest that purinergic signaling stimulates $Ca^{2+}$ transients and regulates insulin exocytosis. Recently, the Panx1 channel and the P2X7 receptor were involved in glucose-induced autocrine regulation in INS-1E ß-cells and rodent islets (55). It is well documented that ß-cells release ATP through exocytosis of insulin granules upon glucose stimulation. ATP is also co-released with transmitters from nerve terminals. Extracellular ATP may activate the ATP- and ADP-sensitive P2Y purinergic receptors on the plasma membrane, thereby modulating intracellular $Ca^{2+}$ concentrations as shown in mouse and human islets (1, 3, 32, 45). Furthermore, the ectonucleotidase NTPDase3, a membrane-bound nucleotide-metabolizing enzyme that degrades extracellular ATP, is abundant in mouse and human ß-cells and its activity modulates insulin secretion by

48 controlling activation of purinergic receptors (52). Therefore, pannexins are key components in the
49 regulation of purinergic receptors during physiological and pathophysiological conditions (26). What
50 remains to be established is the putative link between these cell surface components

51 In order to investigate the effects of fructose on the signal transduction of pancreatic ß-cells, we have
52 chronically treated over days rodent and human ß-cells with 5.5 mM fructose before assessment of their
53 glucose response. Fructose reduced intracellular ATP levels and induced AMPK activation. The release of
54 ATP from the ß-cells through Panx1 channels resulted in the activation of the P2Y1 purinergic receptor
55 signaling pathway, ultimately enhancing the exocytosis of insulin.

56

## 57 Materials and Methods

58 D-glucose, D-fructose, aspartame, 2MeSADP, ADPbS, apyrase, KCl were obtained from Sigma-Aldrich (St.-
59 Louis, MO, USA); ARL156, MRS2179 from Tocris Bioscience (Bristol, UK); mefloquine from Bioblocks (San
60 Diego, CA). The doses of the various drugs were determined from the literature (3, 43, 52).

### 61 *Cell culture and treatments*

62 INS-1E ß-cells (42), cloned from the parental rat INS-1 cell line, were grown in RPMI-1640 medium at
63 11.1 mM glucose supplemented with 10 mM HEPES, 5% (vol./vol.) heat-inactivated fetal calf serum
64 (FCS), 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 1 mM sodium pyruvate and 50
65 µM ß-mercaptoethanol. This medium is referred to as Control Medium (CM). Directly after
66 trypsinization, cells were maintained for 3 days in CM or CM supplemented with 5.5 mM fructose (CM+F)
67 in 24-well plates (Falcon, OmniLab, Mettmenstetten, Switzerland) or 10 $cm^2$ Petri dishes (Becton and
68 Dickinson AG, Allschwil, Switzerland). Medium was changed after 3 days and at day 4 the different assays

were performed as described. Human islets were isolated from pancreases of deceased multiorgan donors (n = 18), who had provided written informed consent (ECIT consortium). None of the donors were diagnosed for metabolic syndrome or diabetes (see clinical data in Supplementary Table 1). Donors had an average BMI of 25.2 ± 2.7 kg/$m^2$ and age of 51.7 ± 9.0 years. Islets were maintained for standard recovery period of time (1-3 days) in CMRL-1066 medium (Sigma-Aldrich, St.-Louis, MO, USA) at 5.6 mM glucose supplemented with 10% FCS and used for experiments straight away without shipping maneuver (isolation and experiment being performed in the same institution). Then, islets were hand-picked, washed and further cultured for 4 days in 10 $cm^2$ non-adherent culture dishes (Greiner, Huberlab SA, Aesch, Switzerland) in the presence of 10% FCS at either physiological 5.6 mM glucose (CMRL, control medium) or exposed to 5.5 mM fructose (CMRL+F).

*Animals and islet isolation*

Generation and analysis of pannexin1 knockout (*Panx1*-/-) mice have been described previously (2). Mice from in-house breeding were maintained on a C57BL/6J genetic background and handling was carried out in our local certified animal facility (CMU-zootechnie, Geneva) according to procedures that were approved by the animal care and experimentation authorities of the Canton of Geneva. Pancreatic islets were isolated from both male and female control WT or $Panx1^{-/-}$ mice matched for sex and age by collagenase digestion as described before (37). Immediately after isolation, islets were hand-picked, washed and further cultured for 4 days in control RPMI-1640 medium (CM) or supplemented with 5.5 mM fructose (CM+F) in the presence of 5% FCS.

*Cellular lipid stores*

Neutral lipids stored in INS-1E ß-cells were assessed by loading the cells with 1 µg/ml of 4,4-Difluoro-1,3,5,7,8-Pentamethyl-4-Bora-3a,4a-Diaza-s-Indacene dye (Bodipy 493/503) in Krebs-Ringer bicarbonate

buffer (KRBH) 2.5 mM glucose for 15 min at 37°C, 5% CO2. The dye fluorescent signal (Ex: 493 nm/Em: 503 nm) was quantified using the ImageXpress XL plate reader and the MetaXpress software (Molecular Devices, Sunnyvale, CA).

*Isolation of RNA and quantitative RT-PCR*

INS-1E cells were cultured in 6-well dishes as described above. Total RNA was extracted with the Trizol reagent (Invitrogen, Carlsbad, CA, USA) and 2 µg were converted into cDNA as described previously (9). Primers for Panx1 were obtained from ThermoFischer Scientific (#Rn01447976_m1). Primers were designed using the Primer Express Software (Applera Europe, Rotkreutz, Switzerland) (see list of primers in Supplementary Table 2). QT-RT-PCR was performed using an ABI 7000 Sequence Detection System (Applera, Norwalk, CT) and PCR products were quantified using the SYBR Green Master kit (Roche, Mannheim, Germany). Two distinct amplifications, derived from at least 3 independent experiments, were performed in duplicate for each transcript and mean values were normalized to those of the reference mRNA actin.

*Immunoblotting*

At the end of the culture period, protein extracts from total INS-1E ß-cells (4.0 x $10^6$ cells/dish) and human islets (200 islets/well) were harvested in lysis buffer as described (7, 9). Proteins from total cell extracts (10-20 µg/lane) were separated by 8-10% SDS-PAGE before transfer onto nitrocellulose membrane**.** For P2Y1 immunoblotting, INS-1E cells and human islet lysates were deglycosylated with PNGase F (Sigma-Aldrich) according to the product information. Briefly, cell extracts were incubated overnight in the presence of the enzyme at 37°C and 25 µg of the lysates were loaded on a 12% polyacrylamide gel. The membrane was then probed overnight at 4°C with: rabbit polyclonal antibodies against AMPK, p-AMPK (thr172) (1:1000 dilutions, Cell Signaling Technology, Danvers, MA), Panx1

(1:1000 dilution, #488100, ThermoFischer Scientific), P2Y1 (H-120) (1:500, Santa Cruz Biotechnology, Santa Cruz, CA); mouse monoclonal antibodies against ACTIN (1:5000, Chemicon-Millipore, Zug, Switzerland). After washing, the membranes were incubated for 1 h at RT with secondary horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG antibodies (1:10000, Amersham Biosciences, UK) according to primary antibodies. Proteins were revealed by chemiluminescence (ECL, Amersham), analyzed with the ChemiDoc XRS System (Bio-Rad, Hercules, CA) and bands were quantified with Scion Image software (Scion Corporation, Frederick, MD).

*Insulin secretion and perifusion assays*

For INS-1E ß-cells, after pre-incubation for 3 h in glucose-free medium, static insulin secretion was assayed over a 60 min stimulation period with basal G2.5 or stimulatory G8.3 (intermediate) and G15 (optimal) glucose concentrations as detailed previously (42). Mouse and human islets (10 islets/tube) were hand-picked, pre-incubated for 1 h in 2.8 mM glucose (G2.8), washed and challenged for insulin secretory response for 60 min at basal G2.8 and stimulatory G11.1 (mouse islets) or G8.3 and G16.7 (human islets) in the presence of the indicated compounds at 37°C as detailed previously (7, 9). For perifusion of human islets, after the chronic fructose exposure 100 hand-picked islets were put per chamber in 250 µl volume at 37°C (Brandel, Gaithersburg, MD). The flux was set at 0.5 ml/min and fractions were collected every min, first at basal G2.8 for 10 min followed by a 35 min stimulation at G8.3. Secreted and total cellular insulin contents were quantified by radioimmunoassay (Linco Research Inc., St. Charles, MO). Protein concentrations were determined by BCA assay kit (Thermo Scientific, Rockford, IL).

*Luminescence-based secretion assay*

Modified INS-1E ß-cells expressing a *Gaussia* luciferase in place of the insulin c-peptide (11) were used to assess kinetics of insulin secretion after the chronic fructose exposure. Cells were starved for 2 h in glucose-free RPMI-1640 medium, washed and incubated further for 40 min in KRBH buffer (containing in mM: 135 NaCl, 3.6 KCl, 10 HEPES, 5 $NaHCO_3$, 0.5 $NaH_2PO_4$, 0.5 $MgCl_2$, 1.5 $CaCl_2$, pH 7.4) at 2.5 mM glucose. Native coelenterazine (5 µM; Nanolight Technologies, Pinetop, AZ) was added to the wells before further pre-incubation of the cells for 20 min and then luminescence was monitored for 40 min using a thermostated plate-reader (Fluostar) as the cells were exposed to G2.5, G8.3 and G15, followed by the addition of KCl (30 mM final). The first phase was defined as the first 3.2 min of secretion (nadir after the first peak) and the second phase as the subsequent 6.5 min of secretion (nadir after the second sustained peak, *i.e*. 10 min after the initial stimulation). Over time, the oxidation of coelenterazine resulted in a downward drift of the luminescent signal. Therefore, for calculation of the cumulative secretion rates, relative luminescence units (RLU) were normalized to the signal of cells at G2.5 corresponding to the basal secretion rate (Supplementary Fig. 1*C* and *D*).

*Mitochondrial respiration assay*

The mitochondrial respiration was measured in a XF24 apparatus (Seahorse Biosciences, MA, USA). Briefly, $5 \times 10^4$ INS-1E ß-cells were seeded in polyornithine-treated plates and maintained for 4 days in either CM or CM+F. Next, cells were washed and pre-incubated without $CO_2$ at 37°C for 1 h in KRBH at 1 mM glucose. Basal respiration rate was measured for 10 min and then stimulated with 2.5, 8.3 or 15 mM glucose. After 21 min incubation, 1 µM oligomycin (Oligo), 2 µM carbonyl cyanide-4-trifluoromethoxyphenylhydrazone (FCCP), and 1 µM antimycin A plus 1 µM rotenone (Ant/Rot) were added by 3 successive injections in order to determine the uncoupled respiration, the maximal respiration, and non-mitochondrial respiration, respectively. ATP production and proton leak were calculated by subtracting oligomycin-sensitive oxygen consumption rates (OCR) to glucose-stimulated

157 OCR and OCR after Ant/Rot injection to OCR after oligomycin injection, respectively. For acute fructose
158 experiments, respiration and mitochondrial function were measured in INS-1E cells maintained 4 days in
159 control medium, then pre-incubated for 2 h with the indicated glucose concentrations and acutely
160 stimulated with 5.5 mM fructose.

161 *ATP quantification assay*

162 For intracellular ATP measurements, 5 x $10^5$ INS-1E ß-cells were cultured in 6-well plates for 4 days with
163 CM or CM+F as described above. Then, the cells were washed in ice-cold PBS before addition of 100 µl of
164 lysis buffer (20mM Tris-HCl pH 8.0, 2mM EDTA and 0.2% Tween-20). Extracts were centrifuged and the
165 supernatants were collected and treated with perchloric acid (on ice for 20 min) before addition of
166 potassium carbonate and a final centrifugation (pH adjusted to pH 8.5-9). To measure cellular release of
167 ATP, INS-1E ß-cells were treated as for insulin secretion experiments (seeded at 4 x $10^5$ cells/ml in 24-
168 well plates). On the day of the assay, cells were washed and starved for 2 h with glucose-free RPMI-1640
169 medium at 37°C followed by pre-incubation for 1 h with glucose-free KRBH. Before stimulation, cells
170 were washed twice with the same buffer and then incubated for 1 h at 2.5 mM, 8.3 mM glucose and in
171 the presence of an inhibitor of ectonucleotidases (100 µM ARL67156). At the end of the incubation
172 period, the buffer was collected, centrifuged to remove contaminating cells and stored frozen before the
173 ATP assay. Finally, protein levels were quantified and the ATP levels (in cell extracts and cell
174 supernatants) were determined using the ATP Bioluminescent assay kit CLS II (Roche).

175 *YO-PRO-1 uptake*

176 Panx1 channel function was assessed by monitoring the uptake of the nucleic acid dye YO-PRO-1, as
177 described previously (47). INS-1E ß-cells were seeded on glass coverslips at a density of 5 x $10^5$ and
178 cultured for 4 days in CM or CM+F. Cells were pre-incubated at G0 for 3 h. Uptake was monitored by

imaging cells stimulated with either G2.5 or G8.3 in the presence of 5 µM of YO-PRO-1 (Invitrogen); without or with the Panx1 blocker mefloquine (Mfq, 10µM) added 10 min before stimulation. YO-PRO-1 has a molecular weight (629 g/mol) similar to ATP and does not permeate biological membranes. YO-PRO is not fluorescent in a solution but becomes fluorescent upon binding to nucleic acids. Fluorescent cells were visualized with a 40x objective mounted on an inverted TMD300 microscope (Nikon AG, Switzerland) equipped with a high sensitivity CoolSnap HQ2 camera (Visitron systems GmbH, Germany). Images were captured every 15 sec using the Visiview 3.1.01 software and fluorescence intensity was calculated from regions of interest defined on every cell in the field. Measured values were expressed as means of fluorescence intensity of Mfq-sensitive signal (total signal minus remaining signal in the presence of Mfq) at each indicated time point.

*Immunocytochemistry*

INS-1E ß-cells were cultured on polyornithine-treated coverslips with (CM+F) and without (CM) fructose for 4 days and then fixed with ice-cold 4% paraformaldehyde in PBS. Cells were permeabilized with 0.25% Triton X-100 in PBS, blocked in PBS/3% BSA for 30 min before incubation overnight with chicken antibody against mouse Panx1 (amino acids 414-425 (43)) (1:500 dilution produced at Aves Labs, Tigard, OR), polyclonal rabbit antibody against P2Y1 (H-120) (1:100 dilution, Santa Cruz Biotechnology), guinea pig antibody against insulin (1:400 dilution; DAKO, Carpinteria, CA), and monoclonal mouse antibodies against insulin or glucagon (1:200 dilution, Sigma-Aldrich). After PBS washes, samples were incubated with fluorescent dye-labeled secondary antibodies anti-goat-FITC, anti-chicken Alexa Fluor 488, anti-rabbit-FITC, anti–guinea pig Alexa Fluor 647 or anti-mouse-rhodamine (Molecular Probes) according to primary antibodies for 1 h at 1:500 dilution. Samples incubated without primary antibodies served as negative controls. The experiments were repeated at least 3 times. For Panx1 immunofluorescent staining, cryosections (5 µm) of pancreas from WT or Panx1$^{-/-}$ mice were fixed during 15 min with 4% PFA

and permeabilized 15 min with 0.3% Triton X-100. After blocking (30 min) in PBS/2% BSA chicken anti-mouse Panx1 (414–425) antibodies (1:500) were incubated overnight on the sections. Detection was performed using a goat anti-chicken DyLight488 secondary antibody (1:500; Jackson Laboratories) for 2 h at RT and subsequent Evans Blue and DAPI counterstainings. Images were captured on a Leica 2P-SP5 confocal laser microscope, equipped with a 63x/1.40-0.60 oil objective. Optical single sections were acquired with a scanning mode format of 512 × 512 pixels, sampling speed of 400 Hz, and 16 bits/pixel images. Images were processed using ImageJ and Imaris image analysis software.

*Statistical analysis*

Results are presented as means ± SEM. Statistical tests between values of fructose-treated and control cells were performed using one-way ANOVA analysis followed by least significant difference post hoc tests when multiple comparisons were made. All data were analyzed with the IBM SPSS Statistics 22.0 software (SPSS Inc., Chicago, IL). Where appropriate, a two-tailed paired *t* test using Prism software was performed. A *P* value lower than 0.05 was considered statistically significant.

## Results

*Chronic fructose treatment potentiates GSIS in INS-1E ß-cells and in human islets.* In order to validate the INS-1E ß-cell line for the effects of chronic fructose treatment, we first compared the responses of INS-1E ß-cells to those of human islets on GSIS. INS-1E ß-cells were exposed for 4 days to 5.5 mM fructose (CM+F) in standard RPMI-1640 medium containing 11.1 mM glucose, used for control groups (CM). At the end of the 4-day culture period, cells were washed (i.e. fructose withdrawal) and insulin secretion was tested over a 1 h period at G2.5 or G8.3 without fructose. Chronic fructose exposure potentiated acute GSIS at G8.3 (1.6-fold, $p<0.05$) compared to CM cells, while basal release at G2.5 was not affected (Fig. 1, *A* and *B*). Insulin content was slightly decreased in CM+F cells compared to

CM cells after 4 days of treatment (Fig. 1*C*). The responses to depolarizing concentrations of KCl were similar in CM and CM+F groups (Fig. 1*A*), indicating that the effects of fructose are not additive to robust depolarization-induced calcium elevation. Addition of an inhibitor of ectonucleotidases (ARL67156), in order to prevent the hydrolysis of extracellular ATP at the cell surface, increased GSIS in naïve CM cells up to the levels of CM+F cells, without additive effects on fructose-treated cells (Fig. 1*B*). Acute exposure of INS-1E ß-cells to 5.5 mM fructose (Fig. 1*B*) or to the artificial non-sugar sweetener aspartame (Supplementary Fig. 1*A*) had no effects on GSIS, confirming that sweeteners *per se* do not acutely stimulate insulin exocytosis (17). In contrast to fructose, chronic aspartame, while slightly increasing basal insulin release, did not change GSIS (Supplementary Fig. 1*B*). Of note, daily consumption of aspartame-containing beverages does not change insulin secretion in normal subjects (5). Therefore, the observed chronic effects of fructose in enhancing GSIS appear to be independent of sweet taste receptor activation. To get insights into the effects of chronic fructose on the kinetics of GSIS, the secretion was measured using INS-1E derived ß-cells expressing G-luciferase in place of the bio-inactive insulin c-peptide (11); allowing online monitoring of luciferase (by coelenterazine-mediated luminescence) co-secreted in a 1:1 ratio with insulin. Following the 4-day exposure to 5.5 mM fructose (CM+F) or standard medium (CM) for control cells, INS-1E ß-cells were then incubated with G2.5 (basal) for 8 min prior to stimulation with stimulatory glucose concentrations for 25 min. Upon glucose stimulation at G8.3 and G15, a first rapid phase of exocytosis was observed followed by a second slower phase of sustained secretion (Fig. 1, *D*-*F*). Basal secretion at G2.5 was not affected by fructose treatment (Supplementary Fig. 1*C*) and the responses to depolarizing concentrations of KCl were similar in CM and CM+F groups (Fig. 1, *D* and *E*). At intermediate 8.3-15 mM glucose concentrations, chronic fructose potentiated secretion rate over the period of glucose stimulation, during both the first phase (3.2 min post-stimulation) and the second phase (subsequent 6.5 min of secretion). Compared to control CM

247 cells, at G8.3 the fructose-treated group exhibited strong potentiation of the second phase, much less of
248 the first phase (Fig. 1*F*).

249 Considering that acute fructose does not stimulate insulin exocytosis and that chronic fructose
250 potentiated the secretion at physiological G8.3 in INS-1E ß-cell lines, we next exposed human islets for 4
251 days to 5.5 mM fructose (CMRL+F) in standard CMRL-1066 culture medium containing 5.6 mM glucose,
252 used for control groups (CMRL). At the end of the 4-day treatment period, human islets from 3 different
253 donors (Supplementary Table 1) were perifused with G2.8 (basal) for 10 min prior to stimulation with
254 G8.3 for 35 min. In the 3 independent perifusion assays, basal secretion at G2.8 was similar in CMRL and
255 CMRL+F islets and chronic fructose treatment resulted in potentiated insulin secretion at G8.3 (Fig. 1, *G*
256 and *H* and *s*upplementary Fig. 1, *E-H*). According to the different characteristics of the human donors
257 (e.g. age and gender), the corresponding profiles of GSIS were not the same in the 3 independent
258 perifusion experiments. When results were expressed as the means of the 3 perifusion assays, chronic
259 fructose potentiated GSIS 1.6-fold ($p<0.005$) compared to CMRL islets over the period of stimulation (Fig.
260 1*I*). Overall, chronic exposure to fructose similarly potentiated insulin secretion in response to
261 physiological 8.3 mM glucose in both INS-1E ß-cells and human islets.

262 *Chronic exposure of INS-1E ß-cells to fructose activates AMPK, reduces intracellular ATP levels*
263 *and increases ATP release***.** In rat islets, acute fructose in combination with glucose potentiates insulin
264 secretion despite a significant decrease in cellular ATP and the ATP/ADP ratio (20). As metabolic stresses
265 may activate AMPK (23), by impairing ATP production or promoting its consumption, we investigated the
266 effects of chronic fructose treatment on AMPK activation. INS-1E ß-cells were cultured with 5.5 mM
267 fructose (CM+F) and at the end of the 4-day culture period cells were either analyzed straightaway or
268 starved for 3 h before incubation for 10 min at G2.5 or G8.3. Western blot analysis showed that chronic
269 exposure to fructose increased AMPK phosphorylation levels compared to control cells at any of the

incubation conditions. After 4 days exposure to 5.5 mM fructose (CM+F), AMPK phosphorylation was increased 1.6-fold ($p<0.05$) in INS-1E ß-cells (Fig. 2, *A* and *B* and supplementary Fig. 2*A*). Stimulation for 10 min with G8.3 caused 65% and 59% decreases in AMPK activation in control and fructose-treated cells, respectively, indicating that the acute AMPK response *per se* to glucose was preserved in INS-1E ß-cells. Consistent with INS-1E ß-cells, a 4-day exposure to fructose (CMRL+F) increased human islet AMPK phosphorylation levels following both a 1 h starving period (1 mM glucose, G1) and a further 10 min incubation time with G8.3 compared to control islets (CMRL) (Supplementary Fig. 2, *B* and *C*). Of note, the 10 min G8.3 incubation caused a relative decrease of AMPK activation in both control and fructose-treated human islets, indicating that the acute AMPK response to glucose was also preserved with chronic fructose, while being constitutively upregulated. In order to document putative acute effects of fructose on AMPK activation, naïve cells kept in CM were exposed for 10 min to G8.3 simultaneously with 5.5 mM fructose (acute fructose) and time course profiles of AMPK responses following G8.3 stimulation with and without acute fructose were compared to 4-day fructose exposure (CM+F). Whereas acute fructose increased phosphorylation levels of AMPK for about 1 min, chronic fructose exposure (CM+F) resulted in the sustained elevation of pAMPK versus control cells (CM) at all of the time points analyzed (Fig. 2, *C* and *D*). Consistent with Figure 2*A*, time course at G8.3 caused a relative decrease of pAMPK in both control and fructose-treated cells.

According to the observed changes in AMPK activation, the mitochondrial function was then assessed by measuring the oxygen consumption rate (OCR) in INS-1E ß-cells. Chronic fructose treatment modified neither basal (G2.5) nor glucose-induced (G8.3 and G15) OCR and did not change maximal respiration induced by the mitochondrial uncoupler FCCP (Fig. 2*E* and supplementary Fig. 2, *D-F*). Importantly, the ATP production (Fig. 2*F*), as well as the coupling efficiency of the mitochondria (Supplementary Fig. 2*G*), remained unchanged in CM+F cells. A potential acute effect of fructose on mitochondrial function was also tested by measuring OCR in INS-1E ß-cells acutely stimulated with 5.5 mM fructose at different

294 glucose concentrations (G2.5, G8.3 and G11.1). As expected, basal respiration was higher in G8.3 and
295 G11.1 cells compared to G2.5 cells. Fructose addition had no impact on OCR at any of the glucose
296 concentrations and did not alter the coupling efficiency of the mitochondria (Supplementary Fig. 2*H*).
297 The glucose-dependent ATP production was also fully preserved upon acute fructose exposure (data not
298 shown). Since AMPK activation depends on intracellular nucleotide levels and its ATP fraction (23), we
299 measured ATP levels in INS-1E cells at the end of the 4-day culture period, showing a 31% reduction
300 ($p<0.005$) in CM+F cells compared to CM cells (5.79 ± 3.3 *versus* 8.57 ± 4.9 nmoles/mg protein
301 respectively, Fig. 2*G*). Regarding the ATP/ADP ratio, this parameter was 31% lower at basal glucose in
302 CM+F cells versus CM cells at the end of the culture period, not significantly different at stimulatory
303 glucose concentrations (Supplementary Fig. 2*I*). Panx1 blockade by Mfq did not affect intracellular ATP,
304 suggesting that the contribution of Panx1 channels is not responsible for the intracellular ATP reduction
305 during the culture (data not shown). Accordingly, the fraction of ATP released by the cells directly at the
306 end of the culture period was not influenced by Mfq and similar between control and fructose-treated
307 cells, *i.e*. in the range of 0.25% per h of total intracellular ATP (Fig. 2*H*).

308 Following the 4-day culture period and pre-incubation in glucose-free medium, cells were incubated for
309 1 h at G2.5 and G8.3 before medium collection and ATP assay. Compared to naïve CM cells, the release
310 of ATP at G8.3 was higher from the cells previously cultured with fructose (Fig. 2*I*). The presence of an
311 inhibitor of ectonucleotidases (ARL67156) increased extracellular ATP in both control and fructose-
312 treated INS-1E ß-cells, uncovering the contribution of ectonucleotidases hydrolyzing ATP at the cell
313 surface (52). Overall, chronic exposure of INS-1E ß-cells to fructose reduced intracellular ATP levels,
314 increased AMPK phosphorylation and favored ATP release during acute glucose stimulation without
315 affecting mitochondrial function.

*Chronic fructose potentiates GSIS through extracellular ATP signaling mediated by pannexin1 channel and P2Y1 receptors.* The 4-day exposure to fructose altered neither the morphology nor density of INS-1E ß-cells compared to control CM cells (Fig. 3*A* and *B*). Accordingly, expression of the housekeeping genes actin, tubulin and cyclophilin, as well as the ß-cell specific transcription factor Pdx1, were all preserved (Supplementary Fig. 3*A* and *B*). As fructose has been linked to hepatic steatosis, we investigated its putative lipogenic effect in insulin-secreting cells. Whereas, as expected, high glucose culture (G25) increased cell density and promoted marked lipid synthesis in INS-1E cells (9), fructose treatment did not (Fig. 3*A* and *C*). In rodent hepatocytes, the lipogenic effect of fructose is mediated by the transcription factor SREBP1c (49). Chronic fructose did not change the *Srebp1c* transcript levels in INS-1E ß-cells (Fig. 3*D*).

The increased ATP release observed from the 4-day fructose-treated cells (Fig. 2*H*) could be mediated by the cell membrane pannexin channels (4, 15, 55) and suggests a fructose-induced signaling role for such extracellular ATP. In this context, we analyzed the transcript levels of the sweet taste receptor subunit T1R3 and the components of the extracellular ATP signaling (Panx1 channel and purinergic P2Y1 receptor) in control and fructose-treated INS-1E ß-cells. Quantitative RT-PCR analysis revealed the expression of these components at the mRNA level, not altered by the 4-day fructose treatment (Fig. 3*D* and supplementary Fig. 3*B*). Immunoblot (Fig. 3*E*) and immunofluorescence (Fig. 3*G* and supplementary Fig. 3*C*, *E* and *F*) analyses substantiated the expression of the Panx1 channel at the protein level in INS-1E ß-cells (55). No significant changes regarding the levels or cellular localization of Panx1 protein were observed after 1 to 4 days of fructose exposure compared to CM control cells. Immunoblot analysis showed the P2Y1 protein, unchanged by chronic or acute fructose treatments (Fig. 3*F*). Of note, cell extracts were deglycosylated with PNGase F in order to detect P2Y1 at its native molecular weight of 45 kDa. Immunofluorescence images documented the localization of P2Y1 distributed throughout INS-1E ß-

339 cells (Fig. 3*H* and supplementary Fig. 3*D-F*). Fluorescence profiles showed Panx1 and P2Y1 distributions
340 across INS-1E ß-cells (Supplementary Fig. 3, *C* and *D*). The intracellular localization may suggest
341 association with insulin vesicles, as it is the case for dopamine receptors in rodent and human islets (48).

342 In order to determine the activation state of the Panx1 channels following fructose treatment, we
343 measured the Mfq-sensitive (pannexin-mediated) uptake of the nucleic acid dye YO-PRO-1 in INS-1E ß-
344 cells at G2.5 and G8.3 over a 30 min period. At G2.5, naïve CM cells rapidly became YO-PRO-1 positive, as
345 opposed to CM+F cells (Fig. 4*A*). At G8.3, the kinetics of YO-PRO-1 uptake were similar between CM and
346 CM+F cells, alike CM cells at G2.5. This shows that fructose treatment rendered INS-1E ß-cells sensitive
347 to acute glucose stimulation for the YO-PRO-1 uptake, in agreement with ATP release (Fig. 2*I*). Overall,
348 these data highlight Panx1 channel as an active component of the extracellular ATP signaling mediating
349 transport of nucleotides in INS-1E ß-cells.

It has been shown that the non-hydrolysable ADP analog 2-methyl-S-ADP (2MeSADP, P2Y1 purinergic receptor agonist) transiently increases intracellular calcium in single mouse islets at 5.5 mM glucose, an effect inhibited by a P2Y1 receptor antagonist (3, 45). Moreover, activation of these receptors in human islets induces membrane depolarization and phospholipase C-mediated calcium mobilization from endoplasmic reticulum stores, thereby amplifying the exocytosis-triggering calcium signal (32). In this context, cellular ATP release promoted by chronic fructose may activate the cell membrane P2Y1 purinergic receptors through autocrine or paracrine mechanisms. After the 4-day culture period, INS-1E ß-cells were then stimulated at G8.3 in the presence of the Panx1 blocker Mfq (10 µM), the P2Y1 agonist 2MeSADP (10 µM) or its antagonist MRS2179 (100 µM). 2MeSADP increased GSIS in naïve CM cells, mimicking the effects of chronic fructose. Conversely, Mfq and MRS2179 prevented the potentiation of the secretory response evoked by the fructose treatment at G8.3 (Fig. 4*B* and *C*). Cells were also stimulated with the higher glucose concentration G15 plus 100 µM of the ADP analogue ADPbS,

MRS2179 and the ATP scavenger apyrase used to hydrolyze the extracellular ATP. At maximal stimulatory G15, the secretory responses of CM and CM+F cells were similar, also in the presence of ADPbS (Fig. 4*D*). The P2Y1 antagonist MRS2179 added to CM+F cells, as well as the clearance of extracellular ATP and ADP by apyrase, reduced GSIS to the levels reached by control CM cells at the same G8.3. We also investigated the effects of extracellular ATP on the kinetics of the secretory responses following fructose treatment (Fig. 4*E*-*G*). INS-1E ß-cells were then incubated for 10 min with basal G2.5 in the presence of an inhibitor of ectonucleotidases (ARL67156) in combination with 2MeSADP in order to maintain extracellular ATP and activate P2Y1 purinergic receptor prior to stimulation with intermediate glucose concentrations for 30 min. The 4-day fructose treatment potentiated the secretion rate at G8.3 6.8-fold ($p<0.05$) compared to control CM cells (Fig. 4*F* and *H*). Combination of ARL67156 and 2MeSADP increased secretion in CM cells up to secretory response measured in the fructose-treated groups, both at G8.3 and G15 (Fig. 4*F*-*H*). The effects of extracellular ATP were neither effective at basal G2.5 nor additive to fructose-dependent secretion rate in CM+F cells (Fig. 4*E* and *H*). Thus, fructose exposure could activate P2Y1 purinergic receptors through extracellular ATP release in a similar manner as 2MeSADP did. These data indicate that the Panx1 channel and P2Y1 purinergic receptor can mediate the potentiation of insulin secretion at intermediate glucose upon chronic fructose through extracellular ATP signaling.

*Pannexin1 channels mediate the effects of fructose through extracellular ATP signaling on P2Y1 purinergic receptors in mouse islets.* In mouse islets, fructose may activate sweet taste receptors to amplify GSIS (34). Additionally, the selective stimulation of P2Y1 and P2Y6 receptor subtypes by 2MeSADP and UDP, respectively, increases insulin secretion along with intracellular calcium mobilization, whereas ADP acting on the P2Y13 receptor inhibits insulin release in mouse insulinoma cells (1, 3, 45). Our data on rat INS-1E ß-cells (Fig. 4) suggest that in fructose-treated cells Panx1 channels could mechanistically link these observations through extracellular ATP and activation of P2Y purinergic

receptors leading to the potentiation of GSIS. Paraffin sections of mouse pancreas revealed the expression of Panx1 channel and the P2Y1 purinergic receptor in insulin-containing ß-cells (Fig. 5, *A* and *C* and supplementary Fig. 4, *A*-C). Of note, fluorescence profiles across single cells showed that Panx1 was also expressed in glucagon-containing α-cells, whereas P2Y1 staining was specific to the ß-cells (Fig. 5, *B* and *D*). The specificity of Panx1 antibody was confirmed using paraffin sections of both wild type (WT) and $Panx1^{-/-}$ mouse pancreas (Supplementary Fig. 4*C*). Islets from WT or Panx1 knockout mice (2, 43) were then isolated and exposed for 4 days to fructose in complete medium (CM). Immunoblot revealed the presence of Panx1 in WT islets at the expected MW (52 kDa) and its residual expression in $Panx1^{-/-}$ mice (Supplementary Fig. 4*D* and *E*). In mouse islets, the 4-day exposure to fructose (CM+F) also increased insulin secretion in response to intermediate 11.1 mM glucose by 1.6-fold ($p<0.005$) compared to islets cultured in control medium (CM); GSIS versus basal was 5.8-fold and 16.2-fold ($p<0.005$) in CM versus CM+F islets, respectively (Fig. 5*E*). The P2Y1 agonist 2MeSADP increased GSIS in CM islets, mimicking the effects of chronic fructose exposure. Conversely, the Panx1 blocker Mfq reversed the potentiating effects of fructose at G11.1 in mouse islets (Fig. 5*F*), similarly to its effects in the INS-1E ß-cells (Fig. 4*B*). Insulin contents were similar between CM and CM+F islets (Supplementary Fig. 4*F*). The potentiation of the secretory response induced by fructose (1.5 fold; $p<0.05$) in WT CM+F islets was not operating in Panx1-KO CM+F islets, while GSIS was preserved in naïve Panx1-KO CM islets (Fig. 5*G*). The insulin content per islet was 41% lower ($p<0.05$) in CM islets isolated from $Panx1^{-/-}$ mice compared to CM islets from WT mice (Fig. 5*H*), possibly due to the observed smaller size of the KO islets. Of note, addition of 10 µM Mfq to the culture medium of INS-1E cells for 4 days did not significantly change their insulin contents versus standard culture conditions (1.28±0.26 versus 1.77±0.31 µg/well, respectively). Thus, mouse ß-cells express both Panx1 channel and P2Y1 purinergic receptors, which modulate in concert the secretory response to changes in extracellular nucleotide levels induced by chronic fructose.

*In human islets, chronic fructose potentiates GSIS through extracellular ATP signaling to P2Y1 receptors.* In human islets, ß-cells express both the Panx1 channel (4) and the P2Y1 receptor (32); the latter modulating insulin secretion according to changes in the levels of extracellular nucleotides. Human islets isolated from different donors (Supplementary Table 1) were exposed to 5.5 mM fructose for 4 days in CMRL medium containing 5.6 mM glucose (CMRL+F) or in control CMRL medium (CMRL). Immunoblot detected the presence of Panx1 (MW: 48 kDa) and P2Y1 (MW: 60 and 48 kDa) in deglycosylated islet extracts (Fig. 6*A*). No significant changes at the protein level were observed in CMRL+F compared to CM islets right after the 4-day culture period or after a subsequent 1 h incubation at G2.8 (Fig. 6*A* and supplementary Fig. 5*A*). Of note, when islet extracts were not deglycosylated, P2Y1 appeared at the molecular weight of 72 kDa. At the end of the culture period, insulin contents were similar between CMRL and CMRL+F islets (Fig. 6*B*), as opposed to INS-1E cells having less insulin contents and being more sensitive to its release (Fig. 1*C*). Chronic 4-day exposure to fructose potentiated GSIS at G8.3 by 2.8-fold versus control CMRL islets ($p<0.005$) upon static incubation (Fig. 6*C*), i.e. similar to the effects observed in perifused human islets (Fig. 1*I*). This increase was reproduced in the different islet preparations, while there was no difference for basal release at G2.8 (Fig. 6, *D* and *E* and supplementary Fig. 5*C*). Inhibition of ectonucleotidases increased GSIS in control human islets, exhibiting similar secretory responses compared to fructose-treated CMRL+F islets. Conversely, clearance of extracellular ATP and ADP by apyrase inhibited fructose-potentiating effects, bringing back GSIS in CMRL+F islets to control levels (Fig. 6*C*). Next, human islets were stimulated for 1 h with G8.3 in the presence of the P2Y1 agonist 2MeSADP. There was no additive effects on the secretory responses of CMRL+F islets, while the difference with CMRL control islets was reduced (Fig. 6*D*). Consistent with data on rodent ß-cells, the effects of chronic fructose on GSIS in human islets were specific for intermediate physiological glucose concentrations and disappeared at the maximal stimulatory glucose concentration (Supplementary Fig. 5*B*).

Finally, we tested the role of extracellular ATP signaling on the fructose-induced potentiation of insulin secretion in human islets. Fructose-treated islets and their controls were stimulated for 1 h at G8.3 without or with (i) the P2Y1 agonist 2MeSADP in combination with ARL67156 in order to preserve extracellular ATP, (ii) the Panx1 blocker Mfq, and (iii) the P2Y1 antagonist MRS2179. The 4-day fructose treatment resulted in the potentiation of GSIS at G8.3 by 1.5-fold ($p<0.05$) compared to control CMRL islets (Fig. 6*E* and supplementary Fig. 5*C*). Combination of 2MeSADP plus ARL67156 potentiated GSIS in control CMRL islets up to secretory response measured in the fructose-treated group. Conversely, in CMRL+F islets Mfq and MRS2179 suppressed the fructose-potentiating effects on GSIS, restoring a secretory response similar to control CMRL levels. Overall, in human islets GSIS at intermediate physiological glucose concentrations was potentiated by chronic fructose, an effect mediated by Panx1 channel and extracellular ATP, thereby activating the P2Y1 purinergic receptors in a similar manner as in INS-1E ß-cells and mouse islets.

## Discussion

Because it is not synthesized in mammals, circulating blood fructose derives from exogenous dietary intake. Consequently, although almost undetectable in the fasting state, plasma fructose concentrations reach the mM range in postprandial conditions including soda consumption (31, 39) or following i.v. infusion (36). It is thought that most of circulating fructose is taken up by tissues expressing the sugar transporters GLUT5 and GLUT2 (53), such as pancreatic ß-cells for the latter. As opposed to glucose, the catabolism of fructose is essentially restricted to the liver, thanks to the expression of fructokinase, and its oxidation rate is not controlled by metabolic feedback. This promotes fatty acid synthesis along with important intracellular ATP consumption reflected by increased uric acid levels (19, 41). Thus, a high fructose diet increases *de novo* lipogenesis and triglyceride formation, favoring hepatic steatosis. In

addition, the associated elevation of circulating lipids may be lipotoxic to the pancreatic ß-cells (8, 46, 51).

Regarding pancreatic ß-cells, the present data on cultured islets demonstrate that chronic fructose renders ß-cells hyper-responsive through extracellular ATP signaling and uncover a novel aspect of ß-cell signal transduction. In this study, INS-1E ß-cells and islets were exposed to 5.5 mM fructose for 4 days prior to analyses. In human subjects, the ß-cell might be rarely exposed for extended periods of time to fructose concentrations greater than the 5 mM used in the present study. However, one has to take into consideration the time scale in the decade range when it comes to human pathophysiology. Glucose is the main stimulus for insulin exocytosis through the cascade of metabolism-secretion coupling (Fig. 7). Our data show that, at basal glucose concentration, fructose *per se* did neither acutely activate mitochondrial metabolism nor stimulate insulin secretion in INS-1E ß-cells, consistent with data on mouse and human islets. Although the ß-cell does not express the fructose transporter GLUT5, some fructose entry can be mediated by GLUT2 (53). It has been reported that the ß-cell can metabolize fructose, although much less efficiently than glucose (13, 21). Partial intracellular fructose metabolism could then participate to a futile pathway consuming ATP, contributing to the observed decrease in cytosolic ATP and the associated activation of AMPK. Of note, during glucose stimulations subsequent to the fructose treatment, the ATP/ADP ratio was preserved. Accordingly, the lowering of intracellular ATP was closely associated with the presence of fructose, vanishing after fructose treatment while witnessed for a few minutes by the elevated pAMPK. Extracellularly, recent evidence show that fructose binds the heterodimeric sweet taste receptors T1R2 and T1R3 expressed in ß-cells (34, 35, 44). Downstream of the taste receptors, chronic activation of ß-cell Gq signaling increases both mass and function of mouse ß-cells (28). However, both the extremely low mRNA levels of T1R2/T1R3 in INS-1E ß-cells (unpublished data) and the lack of effects of acute fructose and aspartame on GSIS argue against a mechanism of action of fructose through the sweet taste receptors for the present observations. This study introduces

449 extracellular ATP signaling in a model mediated by Panx1 channel and P2Y1 purinergic receptor (Fig. 7).
450 In line with these effects, ß-cells chronically exposed to fructose exhibited potentiation of insulin release
451 in response to glucose concentrations corresponding to the postprandial range. Indeed, the effect of
452 chronic fructose was uncovered at intermediate physiological glucose concentrations (G8.3-G11.1) and
453 the effect disappeared at higher glucose concentrations (G15-G16.7) usually used *in vitro* to yield optimal
454 secretory responses.

455 Nutrient-dependent signals, whether they are hormonal (*e.g*. leptin, insulin) or intracellular (*e.g*. AMPK,
456 $K^+_{ATP}$-channel), are critical for the maintenance of energy homeostasis. In the central nervous system, the
457 hypothalamus regulates appetite and food intake. In the mouse brain, dietary fructose inhibits the
458 lowering of AMPK activation normally mediated by leptin (56). Direct intracerebroventricular
459 administration of fructose into the hypothalamus activates hypothalamic AMPK/malonyl-CoA signaling
460 pathway and alters energy sensing mediated by AMPK activation, impairing the feeling of satiety.
461 Collectively, such central fructose-induced AMPK activation promotes a feeding behaviour and results in
462 weight gain in mice (10, 14). In rats, fructose-induced hypothalamic AMPK activation modulates
463 gluconeogenesis in the liver (33). Overall, one of the noticeable effects of fructose is the AMPK
464 activation, previously reported both in the liver and the hypothalamus (10, 14, 53), now in the ß-cell. It is
465 interesting to note that the same intracellular effect of fructose, independently in the hypothalamus and
466 the ß-cell, produces respectively increased food intake and potentiation of the secretion of the main
467 anabolic hormone insulin, synergistically favoring body weight gain.

468 Following chronic fructose, the ATP released by INS-1E ß-cells upon acute glucose stimulation activates
469 the $Ca^{2+}$-mobilizer P2Y purinergic receptors present on the plasma membrane. Our study documents the
470 expression and localization of Panx1 channels and P2Y1 purinergic receptors in INS-1E ß-cells as well as
471 mouse and human islets. Addition of either an ectonucleotidase inhibitor or a P2Y1 agonist potentiated

insulin secretion stimulated by intermediate glucose, thereby mimicking chronic fructose treatment. Conversely, a Panx1 inhibitor and a P2Y1 antagonist reversed the potentiated secretory response induced by chronic fructose. Moreover, clearance of extracellular ATP and ADP by apyrase abrogated the fructose-induced potentiation of GSIS, again both in INS-1E ß-cells and human islets. Although the ATP concentration outside of the ß-cell may be lower *in vivo* with continuous clearance by the blood flow, our results uncover extracellular ATP signaling as an autocrine or paracrine pathway implicated in ß-cell function.

The observed contribution of Panx1 channel induced by fructose is apparently due to increased activity since there was no change at the mRNA and protein levels. The factors regulating its activity, such as phosphorylation state of its Y308 residue, membrane depolarization, and $Ca^{2+}$ or PKC pathway (15, 16, 38), should be clarified regarding ß-cells in future studies. Expression of P2Y purinergic receptors has been reported in mouse (1, 3) and human islets (32). P2Y receptors activate inositol trisphosphate and transiently increase the concentration of intracellular $Ca^{2+}$. The ectonucleotidase NTPDase3, a membrane-bound nucleotide-metabolizing enzyme that degrades extracellular ATP, is abundant in mouse and human ß-cells and its activity modulates insulin secretion by controlling activation of purinergic receptors (52). Consequently, the interplay between pannexin channels and purinergic receptors (12, 54), favored by chronic fructose, represents an unprecedented mechanistic link in metabolic diseases associated with fructose exposure.

In conclusion, chronic fructose treatment induced extracellular ATP signaling in the ß-cell, resulting in the potentiation of glucose-stimulated insulin secretion. This effect was mediated by the activation of the purinergic P2Y1 receptors and was associated with the release of cellular ATP through the Panx1 channel. Consequently, the interplay between pannexin channels and purinergic receptors could represent a novel target with substantial therapeutic implications in diabetes.

## Acknowledgements

We are grateful to Florian Visentin and Dominique Duhamel (University of Geneva) for excellent technical assistance and to Francesca Frigerio and Sachin Supale (University of Geneva) for pilot experiments at the early stage of the study.

## Grants

This work was supported by the State of Geneva and the Swiss National Science Foundation [#146984 and #166625 to P.M.]. Human islets were provided through the Juvenile Diabetes Research Foundation (JDRF) award 1-RSC-2014-100-I-X (ECIT Islet for Basic Research program).

## Disclosures

The authors have declared that no conflict of interest exists.

## Author contributions

C.B., T.B., and P.M. designed the research and analyzed the data; C.B. did most of the experiments; L.O. conducted secretion kinetics; M.G. performed Seahorse experiments; F.M. and M.C. conducted Panx1 immunodetection; B.K. and M.C. provided scientific inputs and mice for pannexin studies; the laboratory of D.B. provided human islets; P.M. supervised the research; and T.B. and P.M. wrote the manuscript with contribution from B.K. and M.C.

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**Fig. 1. Potentiation of GSIS in INS-1E ß-cells and human islets after chronic exposure to fructose**. *A-C*: INS-1E ß-cells were treated for 4 days with 5.5 mM fructose (F) in complete RPMI-1640 medium (CM+F). At the end of the 4-day culture period, cells were incubated for 1 h at basal 2.5 mM glucose (G2.5) or stimulatory 8.3 mM glucose (G8.3) with (*A*) 30 mM KCl or (*B*) 100 µM ARL67156. *B*: For acute fructose tests, control CM INS-1E ß-cells were stimulated for 1 h to G8.3 + 5.5mM fructose. Results are means ± SEM of 4 independent experiments. *C*: Insulin contents at the end of the 4-day culture period (n=8). *D-F*: INS-1E ß-cells, expressing luciferase as a real-time secretory reporter, were cultured with either 5.5 mM fructose (CM+F) or control medium only (CM) for 4 days and then challenged with G8.3 (*D*) of G15 (*E*) for

25 min followed by 30 mM KCl stimulation; representative native traces expressed as relative luminescence units (RLU, n=4 wells). *F*: Cumulative secretion rates from *D* and *E* over the first phase (*left panel*), the second phase (*middle panel*), and the overall secretion before KCl addition (*right panel*). Results are means ± SEM expressed as AUC per min of the luminescent signal over basal release at G2.5 (see Supplementary Fig. 1, *C* and *D*). $^{\#}$P< 0.05, $^{\#\#\#}$P< 0.005 CM+F cells versus CM cells; *P< 0.05, **P< 0.01, ***P<0.005 versus CM G2.5 control values; $^{\$}$P<0.05, $^{\$\$}$P<0.01, $^{\$\$\$}$P<0.005 versus corresponding basal cells. *G-I*: Isolated human islets from three donors were treated for 4 days with 5.5 mM fructose in complete CMRL-1066 medium (CMRL+F). *G*: Representative insulin secretory responses and (*H*) corresponding means of AUC of treated human islets from 1 out of 3 donors, perifused with solutions containing basal G2.8 and stimulatory G8.3 (dashed line) for the indicated time periods. Values are means ± SEM of 3 independent perifusion chambers. *I*: Means ± SEM of the AUC of the perifusion profiles of the 3 independent donors. Values CMRL; n=9 independent chambers; values CMRL+F; n=8 chambers. $^{\#\#\#}$P< 0.005 CMRL+F islets versus CMRL islets; ***P<0.005 versus basal control values.

**Fig. 2. AMPK activation, intracellular ATP levels, mitochondrial function and ATP release in INS-1E ß-cells after chronic fructose exposure**. Cells were cultured for 4 days in complete medium (CM) and CM supplemented with 5.5 mM fructose (CM+F) before analyses. *A*: Representative immunoblotting showing levels of pAMPK, AMPK and ACTIN in cells right after the 4-day period (G11.1) and after the additional pre-incubation and 10 min stimulation with G2.5 or G8.3. *B*: Quantitative analysis of pAMPK/AMPK band densities normalized to ACTIN (n=4). Results are expressed as protein levels normalized to CM G2.5 values. *C*: Time course of AMPK phosphorylation states at G8.3 following acute exposure to 5.5 mM fructose compared to CM or chronic exposure (CM+F) of INS-1E ß-cells. *D*: Quantitative analysis of relative band densities are presented as means ± SEM of at least 4 independent

experiments. *E* and *F*: Mitochondrial function was determined from 3 independent Seahorse measurements in treated cells further stimulated with G8.3 and G15. *E*: Representative oxygen consumption rate (OCR) profile from Seahorse measurements in 4-day treated cells stimulated with G8.3 followed by the sequential addition of oligomycin (Oligo), FCCP and antimycin A/rotenone (Ant/Rot). *F*: ATP production calculated from the OCR measurements and expressed as fold changes. *G*: Intracellular ATP levels were determined in CM+F and CM control cells right after the 4-day period (G11.1) (n=3). *H*: The ATP released in the medium for the final 1 h of the culture period and the intracellular contents were measured (expressed as % released by the cells). Where indicated, the Panx1 blocker Mfq (10 µM) was added to the culture medium for the last 1 h period (G11.1+Mfq). *I*: ATP released in the extracellular space was quantified from 4-day treated cells after further 1 h incubation at basal (G2.5) and stimulatory (G8.3) glucose concentrations (n=6). As control, addition of 100 µM ARL67156 increased extracellular ATP. $^{\#}$P< 0.05, $^{\#\#\#}$P< 0.005 CM+F cells versus CM cells; *P< 0.05, ***P< 0.005 versus CM G2.5 control values; $^{\$}$P< 0.05, $^{\$\$}$P< 0.01, $^{\$\$\$}$P< 0.005 versus CM+F G2.5 values.

**Fig. 3. Expression of pannexin1 channels and P2Y1 purinergic receptors in INS-1E ß-cells after chronic fructose exposure**. Cells were cultured for 4 days in 11.1 mM glucose complete medium (CM) and CM supplemented with 5.5 mM fructose (CM+F). *A-C*: Neutral lipids were stained using Bodipy fluorescent probe. Cells treated with 25 mM glucose (G25) for 3 days served as control for cytosolic lipid accumulation from *de novo* synthesis. *A*: Light (top) and fluorescence (bottom) microscopy images of treated cells. *B*: Cell density is presented as means ± SEM (n>3) and expressed as ratio between total cell surface and background. *C*: Cytosolic lipids in treated cells (means ± SEM, n>3) expressed as Bodipy fluorescent signal normalized to cell density in B. *P< 0.05, ***P< 0.001, ****P< 0.001 versus CM control values. *D*: Transcript levels of the transcription factor Srebp1c, the Panx1 channel and the purinergic

P2y1 receptor were quantified in treated cells (n=3) as described in Materials and Methods. *E*: Immunoblotting showing levels of Panx1 from whole cell lysates following fructose exposure for the indicated culture periods. *F*: At the end of the 4-day culture period, cells were pre-incubated for 3 h in the absence of glucose and stimulated for 10 min with G8.3. Groups of CM cells were also exposed for 10 min to G8.3 + 5.5 mM fructose (acute fructose). Immunoblotting shows levels of P2Y1 in INS-1E ß-cells following deglycosylation of whole cell lysates. *G* and *H*: Confocal microcopy images (representative of more than 3 independent experiments) obtained from treated cells immune-labeled with antibodies against (*G*) Panx1 or (*H*) P2Y1 (green) and insulin (red). Immunofluorescence show the expression of Panx1 and P2Y1 in INS-1E ß-cells.

**Fig. 4. Chronic fructose exposure potentiates GSIS in INS-1E ß-cells through extracellular ATP signaling mediated by pannexin1 channel and P2Y1 purinergic receptors**. Cells were cultured for 4 days in CM and CM+F before a 3 h starving period and analyses. *A*: Time course of the uptake of the nucleic acid dye YO-PRO-1 at G2.5 and G8.3 without or with the pannexin inhibitor Mfq (10 µM) (. Fluorescence intensity (INT) was quantified and results expressed as means of Mfq-sensitive YO-PRO-1 uptake (see Methods) at the indicated time point (n>4). $^{\#\#}$P< 0.01 CM+F cells versus CM cells; *P< 0.05, **P< 0.01, ***P<0.005 versus corresponding CM basal values (0 min); $^{\$\$}$P<0.01, $^{\$\$\$}$P<0.005 versus corresponding CM+F basal values (0 min). *B-D* Treated naïve INS-1E ß-cells were exposed for 1 h to basal G2.5 and stimulatory G8.3 in the presence of (*B*) Mfq (*C*) the P2Y1 agonist 2MeSADP, the P2Y1 antagonist MRS2179. *D*: Alternatively, cells were stimulated with G15 with the ADP analogue ADPbS, MRS2179 and the ATP diphosphohydrolase apyrase. Results are expressed as means ± SEM of at least 3 independent experiments. *E-H:* Modified INS-1E ß-cells expressing *Gaussia* luciferase were cultured with either fructose (CM+F) or medium only (CM) for 4 days and then challenged online for 30 min in the presence

of ARL67156 and 2MeSADP with the indicated glucose concentrations followed by 30 mM KCl stimulation. *E-G*: Representative native traces of basal and GSIS in untreated and fructose-treated cells. Results are expressed as relative luminescence units (RLU) (n=8). *H*: Secretion rate are presented as means ± SEM of 3 independent experiments and expressed as AUC per min of the luminescent signal normalized to signal of CM cells at G2.5. $^{\#}$P< 0.05, $^{\#\#}$P< 0.01, $^{\#\#\#}$P< 0.005 CM+F cells versus CM cells; *P< 0.05, ***P<0.005 versus CM G2.5 control values; $^{\$}$P<0.05, $^{\$\$\$}$P<0.005 versus corresponding basal cells.

**Fig. 5. Chronic fructose exposure potentiates GSIS in mouse islets through extracellular ATP signaling mediated by pannexin1 channel**. *A* and *C*: Confocal microcopy images (n>3) obtained from mouse pancreas sections immunolabeled with antibodies against insulin (red) and glucagon (blue) combined with either (*A*) Panx1 or (*C*) P2Y1 (green) (n>3). Immunohistochemistry and (*B, D*) associated fluorescence profiles across single cells shows the localization and the intensity of Panx1 and P2Y1 in ß-cells. *E-H*: Freshly isolated islets from both male and female (*E*, *F*) WT or (*G*, *H*) Panx1$^{-/-}$ mice were cultured for 4 days in complete medium (CM) and CM supplemented with 5.5 mM fructose (CM+F). Islets were pre-incubated for 1 h at G2.8 and then stimulated for 1 h with G11.1 in the presence of 2MeSADP (10 µM) and (*F*) Mfq (10 µM). Results are expressed as means ± SEM of 4 (WT) and 6 (Panx1$^{-/-}$ mice) independent experiments done in triplicate. *H*: Insulin content at the end of the 4-day culture period (n>4). Data are expressed as means ± SEM. $^{\#}$P< 0.05, $^{\#\#\#}$P< 0.005 fructose-treated islets versus control islets; *P< 0.05, ***P<0.005 versus CM G2.8 control values; $^{\$\$\$}$P<0.005 versus corresponding basal islets.

**Fig. 6. Chronic fructose exposure potentiates GSIS in human islets through extracellular ATP signaling mediated by pannexin1 channels and P2Y1 receptors**. Isolated human islets from different donors were cultured for 4 days in complete medium (CMRL) or CMRL supplemented with 5.5 mM fructose (CMRL+F). Islets were pre-incubated for 1 h with G2.8 before subsequent stimulations. *A*: Immunoblotting from one donor showing the protein levels of PANX1 and P2Y1 in deglycosylated whole islet lysates. *B*: Insulin

content at the end of the 4-day culture period (n=4). *C*-*E*: After preincubation for 1 h at G2.8, islets were stimulated for 1 h with G8.3 (*C*, n=2 donors done in triplicate) in the presence of 100 µM ARL67156 or apyrase and (*D*, n=6 donors done in triplicate) with 10 µM of the P2Y1 agonist 2MeSADP. *E*: Isolated islets from the same donor as in Fig. 6*A* were also stimulated with G8.3, 2MeSADP and ARL67156, Mfq (10 µM) and the P2Y1 antagonist MRS2179. Stimulation was preceded by 1 h pretreatment with Mfq or the different antagonists. Data are expressed as means ± SD (n=3 islet batches per condition). $^{\#}$P< 0.05, $^{\#\#\#}$P< 0.005 fructose-treated islets versus control islets; *P< 0.05, **P<0.01, ***P<0.005 versus CM G2.5 control values; $^{\$}$P<0.05, $^{\$\$\$}$P<0.005 versus corresponding basal islets.

**Fig. 7. Proposed model for the potentiation of GSIS mediated by chronic fructose exposure in the ß-cell.** Chronic fructose exposure potentiated GSIS at sub-optimal glucose concentration in INS-1E ß-cells and islets. Fructose exposure reduced intracellular ATP levels and induced AMPK activation without affecting mitochondrial respiration. Fructose exposure activated Panx1 channels (putative effectors shown as black dotted arrows), leading to increased release of cellular ATP and paracrine activation of P2Y1 purinergic receptors on the plasma membrane. This cascade of events led to the potentiation of the secretory response. Of note, ß-cells release ATP through exocytosis of insulin granules upon glucose stimulation. Pharmacological activators (green) and inhibitors, as well as genetic manipulations (red) used in the present study supporting the model.