

# CXC chemokine ligand 12 $\alpha$ -mediated increase in insulin secretion and survival of mouse pancreatic islets in response to oxidative stress through modulation of calcium uptake

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**Abstract:** We examined whether CXCL12 $\alpha$  improves insulin secretion by influencing the Ca<sup>2+</sup> oscillation pattern and Ca<sup>2+</sup> influx ([Ca<sup>2+</sup>]<sub>i</sub>), thereby enhancing the viability of pancreatic islet cells in oxidative stress. The islets of Langerhans were isolated from male OF1 mice and pretreated with 40 ng/mL of CXCL12 $\alpha$  prior to exposure to 7.5  $\mu$ M hydrogen peroxide, which served to induce oxidative stress. Incubation of islets with CXCL12 $\alpha$  induced pancreatic  $\beta$ -cell proliferation and improved the ability of  $\beta$ -cells to withstand oxidative stress. Consecutive treatments of isolated islets with hydrogen peroxide caused a decline in  $\beta$ -cell functioning over time, while the CXCL12 $\alpha$  pretreatment of islets exhibited a physiological response to high glucose that was comparable to control islets. The attenuated response of islets to a high D-glucose challenge was observed as a partial to complete abolishment of [Ca<sup>2+</sup>]<sub>i</sub>. Treatments with increasing concentrations of CXCL12 $\alpha$  decreased the number of Ca<sup>2+</sup> oscillations that lasted longer, thus pointing to an overall increase in [Ca<sup>2+</sup>]<sub>i</sub>, which was followed by increased insulin secretion. In addition, treatment of islets with CXCL12 $\alpha$  enhanced the transcription rate for insulin and the CXCR4 gene, pointing to the importance of CXCL12/CXCR4 signaling in the regulation of Ca<sup>2+</sup> intake and insulin secretion in pancreatic islet cells. We propose that a potential treatment with CXCL12 $\alpha$  could help to remove preexisting glucotoxicity and associated temporary  $\beta$ -cell stunning that might be present at the time of diabetes diagnosis *in vivo*.

**Key words:** diabetes; calcium; CXC chemokine ligand 12 $\alpha$ ; insulin; pancreatic islet cells; voltage-gated calcium channels

## INTRODUCTION

Diabetes is characterized either by a near-absolute absence (type 1 diabetes; T1D) or depletion (type 2 diabetes; T2D) of pancreatic  $\beta$ -cells that is responsible for the condition that is described by insufficient or impaired insulin secretion. Therefore,  $\beta$ -cell mass regulation and improved  $\beta$ -cell viability and function through the stimulation of insulin secretion represent a focal point for prospective diabetes management.

Insulin secretion from the pancreatic  $\beta$ -cells induced primarily by glucose is mediated by the known mechanism that involves: raising the ratio of ATP/ADP, closing the ATP-sensitive K<sup>+</sup> (K<sub>ATP</sub>) channels leading to plasma membrane depolarization, voltage-gated calcium (Ca<sub>v</sub>) channels activation and increased Ca<sup>2+</sup> influx ([Ca<sup>2+</sup>]<sub>i</sub>) [1]. Finally, increased [Ca<sup>2+</sup>]<sub>i</sub> triggers the exocytosis of insulin-containing granules. There is extensive literature that examines the

relationship between  $\beta$ -cell electrical activity and intracellular  $\text{Ca}^{2+}$  concentration, both in the islets of Langerhans and in isolated islet cells [2-6]. Increased glucose concentration induces several types of cyclical spike activity and  $\text{Ca}^{2+}$  oscillations with different periods in insulin-secreting cells [7]. The  $\text{Ca}^{2+}$  oscillations are closely linked to multiple key aspects of  $\beta$ -cell functioning in physiological and pathological conditions. There are several  $\text{Ca}^{2+}$  oscillation types: “fast”, with a period from several seconds to one minute, “slow”, with a period ranging from one to several minutes and “mixed” (or compound) oscillations characterized by fast oscillations superimposed on slow oscillations [8]. Numerous hormones, small molecule agonists as well as toxins can change the  $\text{Ca}^{2+}$  oscillatory behavior of  $\beta$ -cells thereby influencing  $[\text{Ca}^{2+}]_i$  and insulin secretion.

Results obtained from  $\beta$ -cell research recently highlighted the important role of CXC chemokine ligand12 (CXCL12) in enhancing  $\beta$ -cell viability and proliferation. This positioned CXCL12 as a potentially important molecule for the treatment of diabetes. CXCL12 belongs to the CXC group of chemokines. It was first discovered as a pre-B cell growth-stimulating factor [9,10] that is also important for the proper formation of multiple organ systems. It has a significant role in tissue development, repair and regeneration [11-13]. CXCL12 is a ligand for two transmembrane receptors: CXCR4 and CXCR7 [14,15]. It was shown to stimulate  $\beta$ -cell survival by preventing apoptosis via activation of the prosurvival kinase Akt, which consequently upregulates the expression of antiapoptotic protein Bcl-2 and phosphorylates the proapoptotic protein Bad [16]. Transgenic mice overexpressing CXCL12 in their  $\beta$ -cells are resistant to  $\beta$ -cell apoptosis and diabetes. Our laboratory revealed a CXCL12-mediated improvement of  $\beta$ -cell viability, which is based on CXCL12 antinecrotic action through the modulation of PARP-1 activity [17]. Furthermore, CXCL12 is involved in the regulation of insulin secretion [18]. Since diabetes results from an insufficient number of  $\beta$ -cells or a lack of their functionality, the important role of CXCL12 in the process of increased  $\beta$ -cell viability and boosting of insulin secretion in the pancreas is of particular interest for the potential treatment of diabetes.

The present work sought to investigate if and by which mechanism CXCL12 signaling could confer insulinotropic effects under physiological and oxidative stress conditions in the intact islets of Langerhans. We hypothesized that the delivery of CXCL12 to intact mouse Langerhans islets would improve viability and increase insulin secretion, influencing the  $\text{Ca}^{2+}$  oscillation pattern,  $[\text{Ca}^{2+}]_i$  and overall concentration of the  $\text{Ca}^{2+}$  in the cytosol.

## **MATERIALS AND METHODS**

### **Animals**

All protocols followed the regulations approved by the Animal Care Committee of the Universidad Miguel Hernández according to national and European policies about ethics in animal research. Swiss albino OF1 mice (8-10 weeks old) were used.

### **Isolation of mouse pancreatic islets**

Swiss albino OF1 male mice were killed by cervical dislocation and pancreatic islets were isolated by collagenase (Sigma, Madrid, Spain) digestion as previously described [19]. Briefly, islet isolation was performed in Hank's balanced salt solution (HBSS; 115 mM NaCl, 10 mM  $\text{NaHCO}_3$ , 5 mM KCl, 1.2 mM  $\text{NaH}_2\text{PO}_4$ , 25 mM HEPES, 1.1 mM  $\text{MgCl}_2$ ) containing 1% (wt/vol) bovine serum albumin (BSA; fraction V) (Sigma, Madrid, Spain) and 5 mM glucose. The pancreas was cannulated by infusion of a 10-mL solution of 1.0 mg/mL collagenase in supplemented HBSS into the common bile duct. The distended pancreas was excised, transferred to a flask tube and incubated at 37°C with 1.0 mg/mL of collagenase in supplemented HBSS for 20 min. The digestion was stopped by adding cold supplemented HBSS solution. Using a dissection microscope and an external light source, the islets were handpicked (handpicking was repeated three times). The islets were cultured in groups in RPMI

1640 without phenol red at 37°C in a humidified atmosphere of 95% O<sub>2</sub> and 5% CO<sub>2</sub> for 48 h. The medium was supplemented with 10% fetal bovine serum (Thermo Scientific), 2 mM L-glutamine, 200 U/mL penicillin and 0.2 mg/mL streptomycin. To obtain single islet cell suspensions, the isolated islets were collected and washed in PBS before dispersal by mechanical shaking at 37°C for 3 min in 0.05% trypsin, 0.7 mM EDTA. The enzymatic reaction was stopped by the addition of RPMI 1640 culture medium, and after centrifugation at 400 x g for 10 min, the cells were washed in PBS and placed in the appropriate medium, depending on the experimental protocol.

#### **Pancreatic islet cell viability assay**

Viability of islet cells was determined by simultaneously staining live and dead cells using a two-color fluorescence staining: acridine orange (AO) and propidium iodide (PI). Live cells stained green, while dead cells stained red. A working solution was prepared (0.6 µl AO from 5mg/mL and 10 µl PI from 1mg/mL stock solutions) in 1 mL PBS. Dye mix (25 µl) was added to the cell suspension and transferred to a microscope slide. Cells were visualized using Leica DMLB fluorescence microscope.

#### **5-bromo-2'-deoxyuridine (BrdU) incorporation**

The isolated islets were dispersed into single cells with trypsin/EDTA as described above. The cells were then centrifuged and resuspended in RPMI 1640 without phenol red (Cambrex, Belgium), which contained 10% fetal bovine serum (Thermo Scientific), 2 mM L-glutamine, 200 U/mL penicillin and 0.2 mg/mL streptomycin. Single cell suspensions were plated on glass covers and cultured for 48 h in the presence of 10 µM BrdU (Sigma, Madrid, Spain). The cells were fixed for 2 min with 4% PFA and washed with PBS. The cells were then treated with 2 N HCl for 20 min at 37°C and washed three times with 0.1 M Na<sub>2</sub>B<sub>4</sub>O<sub>7</sub> (pH 8.5). The cells were permeabilized with 1% Triton X-100 for 5 min. Non-specific interactions were blocked with PBS supplemented with 5% normal goat serum for 1 h. The cells were probed with mouse anti-BrdU (1:20, Dako, M0744) and rabbit anti-insulin (1:200 Abcam, ab181547) antibodies overnight at 4 °C. After 5 consecutive washing steps, islet cells were incubated with secondary antibodies: goat anti-mouse Alexa Fluor 488 was used at 1:500 and goat anti-Rabbit IgG Alexa Fluor 594 (A-11037) was used at 5 mg/mL for 1 h at room temperature. Nuclei were stained with 1 µM Ethidium Homidimer-1 for 15 min at room temperature. BrdU- or insulin-positive cells were represented as the percentage from a total number of 1000 cells (100%) per coverslip.

#### **Calcium imaging**

The isolated pancreatic islets were incubated in the presence of 5 µM fura-2-acetoxymethyl ester (Fura-2 AM; Molecular Probes) for at least 1 h at room temperature in a modified HBSS buffer (pH 7.35), containing 120 mM NaCl, 25 mM NaHCO<sub>3</sub>, 5 mM KCl, 1.1 mM MgCl<sub>2</sub> supplemented with 2.5 mM CaCl<sub>2</sub> and increasing concentrations (3, 8 or 11 mM) of D-glucose. Calcium records in the whole islets of Langerhans were obtained by imaging intracellular calcium under an inverted epifluorescence microscope (Zeiss, Axiovert 200). Images were acquired every ~3 s with an extended Hamamatsu Digital Camera C4742-95 (Hamamatsu Photonics, Barcelona, Spain), using a dual filter wheel (Sutter Instrument CO, CA, USA) equipped with 340 and 380 nm, 10 nm bandpass filters (Omega optics, Madrid, Spain). Data were acquired using ORCA software from Hamamatsu (Hamamatsu Photonics, Barcelona, Spain). The results were expressed as the ratios of 340 nm/380 nm wavelengths. The results were plotted and analyzed using commercially available software (Sigmaplot, Jandel Scientific).

#### **RT-qPCR analysis**

Isolated pancreatic islets of Langerhans served as control, nontreated or incubated in 40 ng/mL of CXCL12α for 30 min and 12 h. Total RNA from the control and CXCL12α-treated islets was extracted using the RNeasy Mini Kit (74106, Qiagen). For cDNA synthesis, 1 µg of the

total RNA was reverse-transcribed using the QuantiTect Rev. Transcription Kit (205311, Qiagen) and oligo(dT) primers. For RT-qPCR the Maxima SYBR Green/ROX qPCR Master Mix (Fermentas) was used. mRNA levels were quantitatively determined with an ABI Prism 7000 Sequence Detection system (Applied Biosystems). The fragments were amplified using the following primer sets: upstream 5'-GATTCTTTGAGAGCCATGTC-3' and downstream 5'-GTCTGTTGTTGCTTTTCAGC-3' for the rat *Cxcl12* gene; upstream 5'-CTGACTGGTACTTTGGGAAA-3' and downstream 5'-GGAACACCACCATCCACAGG-3' for the rat *Cxcr4* gene; upstream 5'-ATGTGCTGCTGTATACCCTC-3' and downstream 5'-GTGATGACGACCCACAGATC-3' for the rat *Cxcr7* gene; upstream 5'-CAAGCTGGAGAAGAATGAGG-3' and downstream 5'-TCACCTGTTTCCTCAAGGGAG-3' for the rat *Trpm2* gene; upstream 5'-AACGGCACCCCTTACCTTT-3' and downstream 5'-TGCTGACATAGGACCTGCTG-3' for the rat *Lc* type of calcium channel gene (*CACNA1c*); upstream 5'-AAGAGGACCTGGCAGATGAA-3' and downstream 5'-TTGAGCATTTTCCTCCCAAC-3' for the rat *Ld* type of calcium channel gene (*CACNA1d*); upstream 5'-AGATTACTGCCCTGGCTCCT-3' and downstream 5'-ACATCTGCTGGAAGGTGGAC-3' for the rat  $\beta$ -actin gene. The real-time PCR program for quantitative RT-PCR was as follows: an initial step at 50°C for 2 min, followed by an initial denaturation step at 95°C for 10 min and a subsequent two-step PCR program at 95°C for 15 s and at 60°C for 60 s for 40 cycles. Negative controls lacking the template were used in all RT-qPCR reactions. The expression levels of target genes were related to the averaged expression level of rat  $\beta$ -actin as the housekeeping gene.

#### **Measurement of insulin secretion**

For insulin secretion, the 48-h-cultured islets were washed for 2 h with a modified HBSS buffer (pH 7.35) when gassed with 95% O<sub>2</sub> and 5% CO<sub>2</sub>. Groups of 5 islets were then transferred to 400  $\mu$ l of a buffer solution containing (in mmol/L) 140 NaCl, 4.5 KCl, 2.5 CaCl<sub>2</sub>, 1 MgCl<sub>2</sub>, 20 HEPES and the corresponding concentration of glucose (3, 8 or 11 mmol/L), with a final pH of 7.4. Subsequently, 100  $\mu$ l of the buffer solution with the corresponding glucose concentration and 5% BSA was added, followed by incubation at room temperature for 3 min and a cool-down period of 15 min on ice. Then the medium was collected, and the insulin content was measured in duplicate samples via radioimmunoassay (RIA) using a Coat-a-Count kit (Siemens, Los Angeles, CA, USA). The protein concentration was measured using the Bradford dye method [20].

#### **Statistical analysis**

Data are expressed as the mean $\pm$ SEM. Pairwise comparisons were made using the Student's *t*-test, unless stated otherwise. A probability level of  $p < 0.05$  was considered statistically significant.

## **RESULTS**

### **CXCL12 $\alpha$ exerts proliferative and prosurvival effects on pancreatic islet cells**

To examine the beneficial effect of CXCL12 $\alpha$  on pancreatic  $\beta$ -cells, the islets of Langerhans were isolated from male OF1 mice and pretreated with 40 ng/mL of CXCL12 $\alpha$  prior to exposure to 7.5  $\mu$ M hydrogen peroxide, which served to induce oxidative stress (Fig. 1). The proliferative potential of the pretreatment with CXCL12 $\alpha$  was validated by BrdU incorporation for 48 h, followed by double-staining with anti-BrdU and anti-insulin antibodies (Fig. 1A). Statistically significant increases in the number of double-stained BrdU and insulin positive cells after the pretreatment of islets with CXCL12 $\alpha$  as compared to control cells were observed (inset in Fig. 1A). Overnight incubation of the islets decreased cell viability to about 70%, whereas the treatment with hydrogen peroxide decreased cell viability further, which was observed as a 15% higher number of dead cells (Fig. 1B). However, when the islets were incubated overnight with 40 ng/mL of CXCL12 $\alpha$  and exposed to oxidative stress, their viability

remained at the control level. This result shows that CXCL12 $\alpha$  provided for an increased proliferative potential and improved the ability of  $\beta$ -cells to withstand oxidative stress.

### **CXCL12 $\alpha$ preserves islet cell functionality by normalizing their Ca<sup>2+</sup> response under oxidative stress**

To assess the functionality of  $\beta$ -cells under oxidative stress, we analyzed the rise in [Ca<sup>2+</sup>]<sub>i</sub> that primes cells for insulin release. [Ca<sup>2+</sup>]<sub>i</sub> served as a dynamic parameter of islet functionality in response to stimulatory glucose concentration. In our experimental setup, we first examined whether a 7.5- $\mu$ M concentration of hydrogen peroxide influences [Ca<sup>2+</sup>]<sub>i</sub>. Isolated islets were preloaded with Fura-2 AM and subjected to calcium imaging (Fig. 1C). Exposure to hydrogen peroxide completely abolished the influx of Ca<sup>2+</sup> so that the glucose-induced [Ca<sup>2+</sup>]<sub>i</sub> response to 11 mM of D-glucose was clearly decreased. As shown on Fig. 1C, the initial Ca<sup>2+</sup> spike observed after the first hydrogen peroxide injection and D-glucose stimulation was absent; however, some oscillations could still be detected. The second hydrogen peroxide treatment of the same islet and exposure to 11 mM of D-glucose did not provoke a rise in [Ca<sup>2+</sup>]<sub>i</sub>, indicating unresponsiveness of the islet cells to glucose stimulus.

To check whether CXCL12 $\alpha$  increases cell viability by influencing Ca<sup>2+</sup> influx, isolated islets were incubated overnight with 40 ng/mL of CXCL12 $\alpha$ , preloaded with Fura-2 AM and subjected to calcium imaging. Islet viability was challenged with 25 mM KCl, which depolarizes the cell membrane and impedes Ca<sup>2+</sup> influx. The control islets exhibited the usual response to a high glucose challenge, observed as an increase in intracellular Ca<sup>2+</sup> concentration (Fig. 2A). In contrast, islets treated with 7.5  $\mu$ M hydrogen for 1 h exhibited decreased responsiveness to the high D-glucose treatment (Fig. 2B), displayed as an irregular Ca<sup>2+</sup> spike followed by an oscillatory pattern. When the same experimental setup was applied to islets that were incubated overnight with 40 ng/mL of CXCL12 $\alpha$  prior to exposure to 7.5  $\mu$ M hydrogen peroxide for 1 h (Fig. 2C), the CXCL12 $\alpha$ -pretreated islets exhibited a physiological response to high glucose that was comparable to the response elicited in control islets. The protective capacity of CXCL12 $\alpha$  was further tested on islets that were exposed to a 10-fold higher concentration (75  $\mu$ M) of hydrogen peroxide. This concentration of hydrogen peroxide, which induced a high level of oxidative stress, initiated widespread cell death, so that the islet cells were unable to properly respond to the high D-glucose concentration (Fig. 2D). The pretreatment of islet cells with CXCL12 $\alpha$  did not preserve cell functionality at the level of oxidative stress induced by 75  $\mu$ M hydrogen peroxide (Fig. 2E).

### **CXCL12 $\alpha$ modulates Ca<sup>2+</sup> oscillations in islet cells in response to high glucose**

The deterioration of pancreatic  $\beta$ -cell function over time in T2D is the consequence of poor glycemic control and the continuous exposure of  $\beta$ -cells to the deleterious effects of reactive oxygen species (ROS). We examined whether CXCL12 $\alpha$  could improve the response of pancreatic islet cells in the hyperglycemic state. To that end, [Ca<sup>2+</sup>]<sub>i</sub> was monitored in isolated islets preloaded with Fura-2 AM (Fig. 3). As can be seen in Fig. 3A, when islets were treated with 3 mM of D-glucose supplemented with 40 ng/mL of CXCL12 $\alpha$ , their intracellular Ca<sup>2+</sup> concentration did not change, and islet functionality was preserved, which was confirmed by their response to the high, 11-mM D-glucose concentration. When islets were exposed to a moderate to high, 8-mM D-glucose concentration (Fig. 3B), the initial Ca<sup>2+</sup> spike was followed by continuous oscillations as a result of the repeated entry of Ca<sup>2+</sup>. A mix of 8 mM D-glucose and 40 ng/mL of CXCL12 $\alpha$  induced changes in the mode of Ca<sup>2+</sup> entry and overall [Ca<sup>2+</sup>]<sub>i</sub>, manifesting as an oscillatory pattern with longer-lasting oscillations with a lower frequency. Calculation of the overall Ca<sup>2+</sup> uptake revealed that CXCL12 $\alpha$  modulated Ca<sup>2+</sup> influx, resulting in increased [Ca<sup>2+</sup>]<sub>i</sub> (inset in Fig. 3B). The same result was obtained when islet cells were challenged with 11 mM of D-glucose (Fig. 3C). In this setup, the islets were probed with 11 mM D-glucose, as well as in combination with two CXCL12 $\alpha$  concentrations (20 and 40 ng/mL). Treatments with both CXCL12 $\alpha$  concentrations decreased the number of oscillations

that also lasted longer, which pointed to an overall increase in  $[Ca^{2+}]_i$  (inset in Fig. 3C), with the 40-ng/mL CXCL12 $\alpha$  concentration showing a more significant effect.

### **CXCL12 $\alpha$ signaling influences the expression of voltage-gated Ca<sup>2+</sup> channels in pancreatic islets**

The voltage-gated calcium (Ca<sub>v</sub>) channels in  $\beta$ -cells are molecular switches that play a central role in insulin secretion. The observation that CXCL12 $\alpha$  is capable of modifying Ca<sup>2+</sup> oscillations and increasing  $[Ca^{2+}]_i$  led us to assume that the CXCL12 $\alpha$ /receptor axis regulates the behavior of the Ca<sub>v</sub> channels. To examine this further, isolated islets were incubated either for 30 min or overnight with 40 ng/mL of CXCL12 $\alpha$ , and their mRNA was isolated (Fig. 4). CXCL12 acts via two receptors, CXCR4 and CXCR7. RT-qPCR experiments revealed that the mRNAs encoding for both receptors were significantly increased after the 30-min incubation of islets with CXCL12 $\alpha$  (Fig. 4). The overnight incubation with CXCL12 $\alpha$  induced a 2-fold increase in CXCR4 mRNA, while the level of CXCR7 mRNA was unchanged. This result revealed that CXCR7 gene transcription was considerably lower in islets as compared to CXCR4 gene transcription. Further, RT-qPCR experiments revealed that CXCL12 $\alpha$  also induced insulin mRNA synthesis in islet cells. The exposure of islets to CXCL12 $\alpha$  for 30 min induced a 4-fold increase in insulin mRNA synthesis, while overnight islet incubation with CXCL12 $\alpha$  did not cause a significant increase in insulin gene transcription.

Next, we analyzed the expression of voltage-gated L-type Ca<sup>2+</sup> channels that belong to the high-threshold type of channels and are opened by large depolarizations, that contribute to the  $\beta$ -cell Ca<sup>2+</sup> current and insulin secretion. After the 30-min incubation with CXCL12 $\alpha$ , the mRNAs for both Lc and Ld subtypes were significantly increased (2.5- and 6-fold, respectively). The overnight incubation with CXCL12 $\alpha$  induced less pronounced but significant increases in Lc and Ld mRNAs (Fig. 4). We also analyzed the Ca<sup>2+</sup>-permeable, nonselective cation channel TRPM2, which is gated by intracellular ADP-ribose and has a key role in hydrogen peroxide-induced Ca<sup>2+</sup> transients and cell death [21]. In pancreatic islets under physiological conditions, TRPM2 mRNA was barely detectable. After the 30-min incubation with 40 ng/mL of CXCL12 $\alpha$ , a small, insignificant increase was observed (Fig. 4), and after the overnight incubation with CXCL12 $\alpha$ , a significant decrease in TRPM2 gene transcription was measured, pointing to the prosurvival effect of CXCL12 $\alpha$  in islet cells.

### **CXCL12 $\alpha$ stimulated insulin release in the hyperglycemic state**

An increase in blood glucose concentrations induces a series of cellular events leading to the depolarization of the  $\beta$ -cell plasma membrane that opens Ca<sub>v</sub> channels to mediate Ca<sup>2+</sup> influx and stimulate insulin secretion. In the next set of experiments we examined whether the increase in  $[Ca^{2+}]_i$ , observed after the incubation of pancreatic islets with CXCL12 $\alpha$ , was also linked with insulin secretion. The islets were incubated either for 30 min (Fig. 5A) or overnight (Fig. 5B) with 40 ng/mL of CXCL12 $\alpha$ , followed by treatment with 8 mM and 11 mM of D-glucose, and their insulin secretion was measured by RIA. Under physiological conditions, in the presence of 3 mM of D-glucose, CXCL12 $\alpha$  did not affect insulin secretion. Stimulatory glucose concentrations induced increased insulin secretion, further enhanced in the presence of CXCL12 $\alpha$  (Fig. 5). Incubation with CXCL12 $\alpha$  for 30 min induced significant insulin release after exposure to 8 mM and 11 mM of D-glucose (compared to matching controls, 2.18- and 1.53-fold increases, respectively; Fig. 5A). The overnight incubation of islets with CXCL12 $\alpha$  induced significant but slightly lower increases in insulin secretion after exposure to 8 mM and 11 mM of D-glucose (compared to matching controls, 1.59- and 1.35-fold increase, respectively) compared to the 30-min incubation with CXCL12 $\alpha$  (Fig. 5B). These findings underlined that the presence of CXCL12 $\alpha$  in the cell medium increases insulin release in islets by enhancing Ca<sup>2+</sup> uptake.

## DISCUSSION

Hyperglycemia contributes to the reduction of insulin secretion and to the progression from glucose intolerance to T2D [22], causing pancreatic  $\beta$ -cell dysfunction and decreasing the  $\beta$ -cell mass [23,24]. Therefore,  $\beta$ -cell protection remains the focus of strategies for the prevention and treatment of diabetes [25]. In general, the diabetogenic environment, including insulin resistance and low-grade systemic islet inflammation contribute toward the stunning of pancreatic  $\beta$ -cells, i.e. a state of temporary incapacity to properly respond to the presence of increased glucose concentration by secreting insulin [26]. The aim of the present study was to examine whether CXCL12 $\alpha$  exhibits a beneficial effect on pancreatic  $\beta$ -cells' ability to release insulin and whether it improves the response of pancreatic islets in the hyperglycemic state.

### **Antidiabetic action of CXCL12 $\alpha$ : normalizing Ca<sup>2+</sup> uptake in order to preserve the functionality of islets**

In overweight and obese individuals, pancreatic  $\beta$ -cells are exposed to metabolic changes and the consequences thereof, including oxidative stress resulting from increased ROS and RNS production. As a ligand of two receptors, CXCR4 and CXCR7, CXCL12 signaling is crucial for  $\beta$ -cell differentiation and pancreatic islet genesis [27]. Recent animal studies revealed the essential role of CXCL12 in duct cell survival, proliferation and migration during pancreatic regeneration, through its ability to activate Akt, Src and the extracellular signal-regulated protein kinase (ERK1/2) [28]. Our group also showed that the role of CXCL12 in diabetes attenuation is based on its ability to activate the antinecrotic/prosurvival pathway after hydrogen peroxide treatment [17]. In this study, we examined oxidative stress as a potential inducer of  $\beta$ -cell dysfunction [29]. Both effects, proliferative burst and increased survival rate of pancreatic  $\beta$ -cells, observed after islet incubation with CXCL12 $\alpha$  could be accomplished by the direct influence of CXCL12 on intracellular Ca<sup>2+</sup> influx.

It is widely accepted that exogenous ROS induces dynamic changes in Ca<sup>2+</sup> concentration in a variety of cell types [30-35]. This effect could be due to the mobilization of intracellular Ca<sup>2+</sup> stores and influx of extracellular Ca<sup>2+</sup>. An important feature of cross-regulation between ROS and Ca<sup>2+</sup> is that the ROS effect on Ca<sup>2+</sup> signaling can vary from stimulatory to repressive, depending on the type of oxidants, their concentrations and the duration of exposure. The effect of ROS on Ca<sup>2+</sup> signaling is also tissue-specific (reviewed in [36]). We found that CXCL12 $\alpha$  preserves mouse islet functionality by normalizing their Ca<sup>2+</sup> response under oxidative stress (hydrogen peroxide treatment). In contrast, islet cell treatment with a 10-times higher concentration of hydrogen peroxide (75  $\mu$ M) induced a constant entry of Ca<sup>2+</sup>, which indicated cell death as a consequence of exposure to the very high concentration of peroxide treatment. This finding is in accordance with previous studies showing that hydrogen peroxide causes an increase in intracellular Ca<sup>2+</sup> levels that leads to cell death in a variety of cell types [37-41]. The mechanism whereby hydrogen peroxide causes an increase in [Ca<sup>2+</sup>]<sub>i</sub> is still under debate, with several options proposed, including the involvement of voltage-gated Ca<sup>2+</sup> channels (Ca<sub>v</sub>) [41], nonspecific changes in membrane calcium permeability [42], alteration in Na<sup>+</sup>-Ca<sup>2+</sup> exchange [38], or changes in Ca<sup>2+</sup> release from intracellular stores [43]. Using the insulin-secreting cell line CRI-G1 and very high hydrogen peroxide concentrations (1-10 mM), Herson et al. [44] described the effects of hydrogen peroxide on [Ca<sup>2+</sup>]<sub>i</sub>. They clearly demonstrated that hydrogen peroxide disrupts calcium homeostasis. In contrast, it has also been reported that hydrogen peroxide exerts no significant effect on the L-type Ca<sup>2+</sup> current in pancreatic  $\beta$ -cells [45].

On the other hand, a chronic exposure of pancreatic islets to nonphysiological high glucose concentrations caused adverse alterations in  $\beta$ -cell functioning in a phenomenon referred to as glucose toxicity, which may play a secondary pathogenic role in T2D. Several results suggested that one mechanism of glucose toxicity in  $\beta$ -cells may be linked to chronic exposure of cells to ROS (or chronic oxidative stress) [46-48]. Both low and high

concentrations of glucose have been shown to increase intracellular peroxide levels within the islets [49], which indicates that glucose metabolism is essential for the induction of glucose toxicity. This is in agreement with Ihara et al. [50], who reported elevated levels of oxidative stress markers (8-hydroxy-2'-deoxyguanosine and 4-hydroxy-2-nonenal-modified proteins) in the  $\beta$ -cells of Goto-Kakizaki rats. Our experimental setup is suitable for observing  $\beta$ -cell behavior in hyperglycemic conditions, since chronic hyperglycemia causes a progressive decline in  $\beta$ -cell functioning and their ultimately demise [26,51]. Electrical activity and stimulated secretion, coupled with intracellular  $\text{Ca}^{2+}$  dynamics that allow pancreatic  $\beta$ -cells to secrete insulin, first reported by Dean and Matthews in 1968 [52], are nowadays accepted [53-55]. Cells often respond to changes in stimulus intensity by varying the frequency of  $\text{Ca}^{2+}$  waves. To demonstrate the rescue potential of CXCL12 $\alpha$  in preventing  $\beta$ -cell dysfunction and exhaustion due to sustained hyperglycemia, mouse pancreatic islets were exposed to stimulatory glucose concentrations, both moderate to high (8 M) and high (11 M), for an average duration of 45 min [56,57]. In our study, the treatment of  $\beta$ -cells with CXCL12 $\alpha$  in hyperglycemic conditions decreased the number and duration of  $\text{Ca}^{2+}$  oscillations, with an overall increase in  $[\text{Ca}^{2+}]_i$ . It has been already shown that CXCL12 stimulates  $\text{Ca}^{2+}$  flux and transients in many cell types [58-60]. Similar to our finding, CXCL12 dose-dependently increased intracellular  $\text{Ca}^{2+}$  in adherent IEC-6 cells in the concentrations of 20 and 100 ng/mL [61]. Chemokine-induced  $\text{Ca}^{2+}$  mobilization was not solely a function of rat intestinal epithelial cells, since CXCL12 stimulated a rapid increase in  $\text{Ca}^{2+}$  within 40 s of ligand stimulation in the intestinal carcinoma cell line CaCo2 cells as well [61]. In human LX2 cells, treatment with CXCL12 led to a nearly two-fold increase in relative  $\text{Ca}^{2+}$  influx, whereas in murine JS1 cells CXCL12 did not promote  $\text{Ca}^{2+}$  movement [62]. The latter authors showed that the CXCL12-influenced hepatic stellate cell contraction is not  $\text{Ca}^{2+}$  dependent. In addition, the  $\text{Ca}^{2+}$ -influenced CXCL12 secretion has also been investigated. Schajnovitz et al. [63] reported that  $\text{Ca}^{2+}$  is transmitted via gap junctions in contacting cell cultures, and that  $\text{Ca}^{2+}$  signaling has a role in bone marrow stromal cell CXCL12 secretion.

### **CXCL12 $\alpha$ signaling influences the expression of voltage-gated $\text{Ca}^{2+}$ channels in pancreatic islets and stimulates insulin release in the hyperglycemic state**

In a high-glucose environment and/or in the presence of ROS, voltage-gated  $\text{Ca}^{2+}$  ( $\text{Ca}_v$ ) channels are activated, allowing extracellular  $\text{Ca}^{2+}$  to enter [64-67]. It is generally accepted that the subsequent increase in cytosolic  $\text{Ca}^{2+}$  triggers insulin release, even though there is some evidence for a  $[\text{Ca}^{2+}]_i$ -independent second phase of insulin release [68]. Thus, regulation of  $\text{Ca}_v$  activity and/or density may result in lower or higher insulin secretion [69-77]. Insulin release must be able to anticipate glucose increase and prevent persistent glucose elevations [26,78]. Insulin secretion analysis in rat insulinoma and primary islet cells revealed that N- and L-type channels are both involved in immediate glucose-induced insulin secretion. However, L-type  $\text{Ca}_v$  channel was preferentially coupled to secretion at a later phase. P/Q-type channels were not found to play any role in insulin secretion at any stage [79]. In our experimental setup, the exogenously added CXCL12 $\alpha$  influenced  $[\text{Ca}^{2+}]_i$  and insulin release, promoting less frequent oscillations of  $[\text{Ca}^{2+}]_i$  with longer duration, and allowing more  $\text{Ca}^{2+}$  to enter the islet. One explanation for this increase in  $[\text{Ca}^{2+}]_i$  could be linked to the ability of exogenously added CXCL12 $\alpha$  to enhance gene expression of the Lc ( $\text{Ca}_v1.2$ -subunit)/Ld ( $\text{Ca}_v1.3$ -subunit) types of  $\text{Ca}_v$ , shown in our experiments. The main subtype of the L-type of channels in mouse islet  $\beta$ -cells is the Ld subtype [77], although the Lc and Ld subtypes share almost a 70% homology [80]. In agreement with previous reports, herein we showed that CXCL12 $\alpha$  increases the transcription rate of  $\text{Ca}_v$  L-type channels, in particular Ld-type, which contains the  $\alpha1D$  subunit and represents the  $\text{Ca}_v1.3\alpha1$  channel subtype. We also observed an increase in N-type channels due to the increased presence of CXCL12. The increased transcriptional rate for the N-type of channels was significant but 7.2-fold less pronounced as compared to the increase in the



transcriptional rate for the L-type  $\text{Ca}_v$  channels (data not shown). In contrast, Barg et al. [81] found no differences in  $\text{Ca}^{2+}$ -current density and  $\text{Cav1.3}/\alpha1\text{D}$ -deficient  $\beta$ -cells, and concluded that  $\text{Cav1.2}$  is the principal L-type  $\text{Ca}^{2+}$  channel subtype in mouse  $\beta$ -cells. The functional consequences of the increase in  $\text{Ca}_v$  channel transcription rate may be relevant in diseases, such as T2D, since  $\beta$ -cells operate with very few  $\text{Ca}_v$  channels [55,82]. The observed CXCL12 $\alpha$ -related influence could provide  $\beta$ -cells with a mechanism to push  $\text{Ca}^{2+}$ -coupled regulation of insulin secretion in response to elevated glucose.

Since  $[\text{Ca}^{2+}]_i$  dynamics are also responsible for  $\beta$ -cell fate [55], we examined whether the  $\text{Trpm2}$   $\text{Ca}^{2+}$  channel is upregulated due to an excess of CXCL12 $\alpha$ .  $\text{Trpm2}$ , a calcium-permeable nonselective cation channel expressed in the plasma membrane is critically involved in ROS-induced processes and is implicated in cell death [83]. In contrast to  $\text{Ca}_v$  channels, CXCL12 $\alpha$  has no influence on the transcription rate of  $\text{Trpm2}$  after 30 min of incubation, in contrast to overnight incubation, which caused a lowering of the transcription of the  $\text{Trpm2}$  channel gene. The primary gating mechanism of  $\text{Trpm2}$  involves the binding of ADP-ribose polymers (ADPR) [84], but  $\text{Trpm2}$  currents can also be activated by reactive oxygen species (ROS) [85] through ROS-induced ADPR release from mitochondria [86]. Bearing in mind that the role of  $\text{Trpm2}$  in pancreatic  $\beta$ -cells involves situations in which  $\beta$ -cells are stressed [87,88], the observed downregulation of  $\text{Trpm2}$  mRNA in our experiments is not surprising. As a small chemokine, CXCL12 exerts its effect via coupling to its receptors, CXCR4 and CXCR7. The 30-min incubation with CXCL12 $\alpha$  was followed by an increase in the level of  $\text{Cxcr4}$  mRNA. The increased  $\text{Ca}^{2+}$  influx in islet cells was accompanied by increased transcription of the  $\text{Cxcr7}$  and  $\text{Ins}$ . Our RT-qPCR results showed significantly increased transcription of both receptor genes after islet incubation with CXCL12 $\alpha$ , targeting CXCR4 as the most important receptor responsible for signaling within the islet. This is in accordance with previous reports indicating that CXCL12 is involved in the regulation of insulin secretion [89].  $\beta$ -cell injury induces CXCL12 expression and the secreted CXCL12 causes dedifferentiation of adjacent  $\alpha$ -cells into pro- $\alpha$ -cells, which are further transformed into pancreatic  $\beta$ -cells [18, 90].

$\beta$ -cell  $\text{Ca}_v$  channel activity can be modulated by changes in membrane potential and through a variety of signaling pathways, such as protein phosphorylation,  $\text{Ca}^{2+}$ -dependent inactivation and through interaction with G protein (reviewed in [91]). The observation that CXCL12 $\alpha$  is capable to modify  $\text{Ca}^{2+}$  oscillations and increase  $[\text{Ca}^{2+}]_i$  as well as influence the transcription of Lc and Ld  $\text{Ca}^{2+}$  channel genes, led us to assume that the CXCL12 $\alpha$ /receptor axis regulates the behavior of the  $\text{Ca}_v$  channels. Princen et al. [92] showed that a transient dose-dependent increase in intracellular  $\text{Ca}^{2+}$  concentration is an essential component of the signal transduction cascade activated upon CXCL12 binding to its receptor CXCR4. The authors showed that in all examined CXCR4 $^+$  cell lines, CXCL12 elicited a transient  $[\text{Ca}^{2+}]_i$  increase. For each cell line, the magnitude of the response was related to the level of CXCR4 expression. Published data suggest that CXCR4 signaling may not be limited to the G-protein-coupled receptor (GPCR)  $\text{G}\alpha_i$ , and that CXCR4 can couple to other  $\text{G}\alpha$  protein subunits such as  $\text{G}\alpha_q$ ,  $\text{G}\alpha_o$ , and  $\text{G}\alpha_s$  in order to stimulate  $\text{Ca}^{2+}$  flux [93]. However, it has been proven that  $\text{Ca}^{2+}$  flux is associated with the GPCR subunit  $\text{G}\alpha_q$  but not to  $\text{G}\alpha_i$  [94]. Quite the opposite, Hsu et al. [95] proposed that the L-type  $\text{Ca}_v$  channel in insulin-secreting cells is the major mediator of the somatostatin-induced inhibition of insulin secretion by the activation of G protein-coupled receptors. In the same line, Renstrom et al. [96] reported that stimulation of GPCRs in mouse islet  $\beta$ -cells does not influence the voltage activated  $\text{Ca}^{2+}$  influx. Our assumption of the CXCL12/receptor axis being a mediator in the elevated voltage activated  $\text{Ca}^{2+}$  influx in pancreatic  $\beta$  cells is supported by Roe et al. [97], who reported that decreased L-type channel activity and expression were associated with T2D.

Defects in the metabolic regulation of insulin secretion contribute to the development of T2D. Rescuing stunned  $\beta$ -cells is possibly a key therapeutic target for the treatment and

prevention of diabetes progression. It seems reasonable to propose that bringing any degree of hyperglycemia under tight control as early as possible can switch on stunned  $\beta$ -cells, in spite of the fact that glycemic memory and genetic/epigenetic programming may differ in every individual. Identification of the factors that regulate the overall  $\text{Ca}^{2+}$  intake, activity and expression of  $\text{Ca}_v$  channels and insulin release may provide a better understanding of pancreatic  $\beta$ -cell functioning in normal and diabetic states. CXCL12 displayed the potential to regulate  $[\text{Ca}^{2+}]_i$  and the transcription of  $\text{Ca}_v$  channel genes and thus may serve as a potential T2D attenuator.

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## Figure Legends

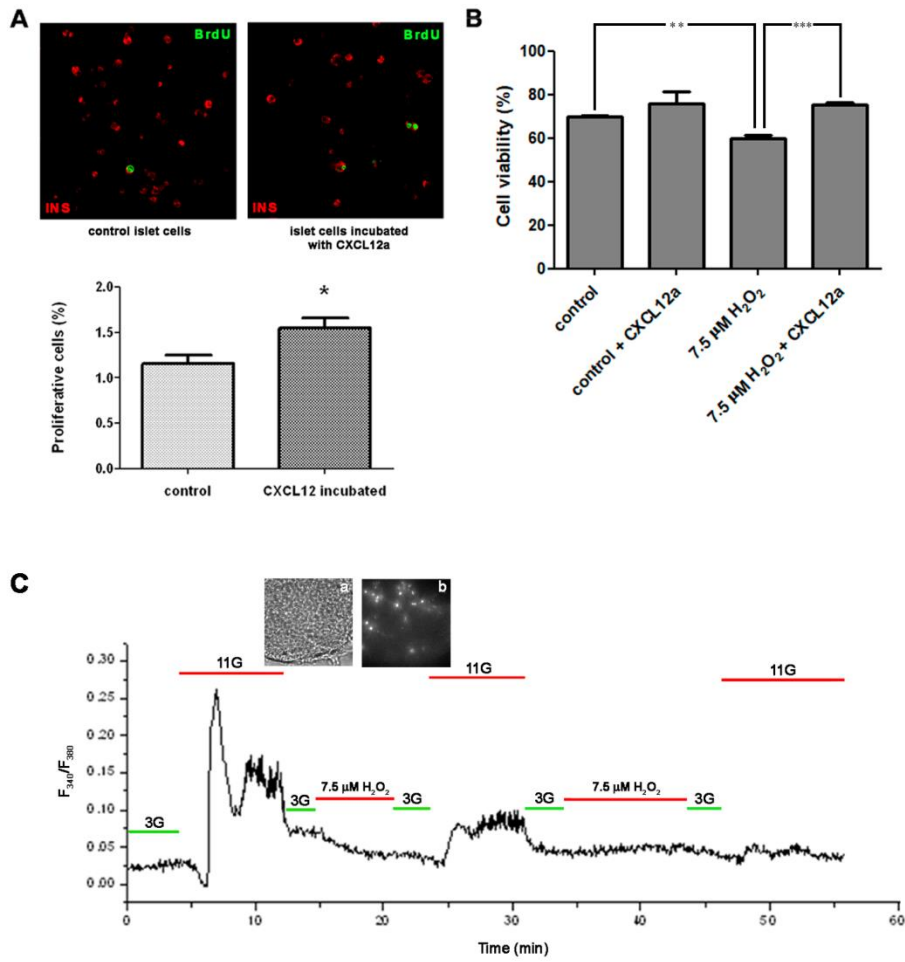
**Fig. 1.** CXCL12 $\alpha$  increases pancreatic beta-cell proliferation in basal conditions and protects islets from hydrogen peroxide-induced cell death. **A** – Proliferation assay of control and islet cells incubated with CXCL12 $\alpha$  (40 ng/mL) for 48 h. Cells were double-stained with anti-insulin (INS), displayed in red, and anti-BrdU (BrdU) displayed in green. **B** – Viability assay of control islet cells or pretreated with recombinant CXCL12 $\alpha$  (40 ng/mL) before and after treatment with a 7.5- $\mu$ M concentration of hydrogen peroxide. **C** – Alteration of [Ca<sup>2+</sup>]<sub>i</sub> of islet cells consecutively treated with 7.5  $\mu$ M of hydrogen peroxide. Isolated islets were preloaded with Fura-2AM and intracellular calcium-dependent fluorescence was measured. The physiological response was initiated with 11 mM of D-glucose (11G) and challenged with successive islet treatment with 7.5  $\mu$ M of hydrogen peroxide (3G-3 mM D-glucose). The insets represent one islet captured during the calcium imaging (a – phase contrast microscopy) stained with EtBr homodimer after the measurement (b). One representative result from three independent experiments is shown. \*p value from Student's t-test <0.05.

**Fig. 2.** Treatment of pancreatic islets with CXCL12 $\alpha$  preserves islet's functionality by normalizing Ca<sup>2+</sup> uptake. Intracellular Ca<sup>2+</sup>-dependent fluorescence was measured in control islets (**A**) and islets treated with a 7.5- $\mu$ M hydrogen peroxide concentration (**B**, **D**) with and without overnight islet incubation with CXCL12 $\alpha$  (40 ng/mL) (**C**, **E**). The islet's membrane depolarization was induced with 25 mM KCl, while the islet's physiological response was initiated with 11 mM of glucose. One representative result from three independent experiments is shown.

**Fig. 3.** Direct stimulatory effect of CXCL12 $\alpha$  on intracellular calcium concentration. The CXCL12 $\alpha$  stimulates a dose-dependent increase in intracellular calcium. Islets were stimulated with 3 mM (**A**), 8 mM (**B**) or 11 mM of D-glucose (**C**) supplemented with or without CXCL12 $\alpha$ . Ca<sup>2+</sup> influx and number of oscillations were calculated in the same time interval (5 min). \*p value from the Student's t-test <0.05. One representative result from four independent experiments is shown.

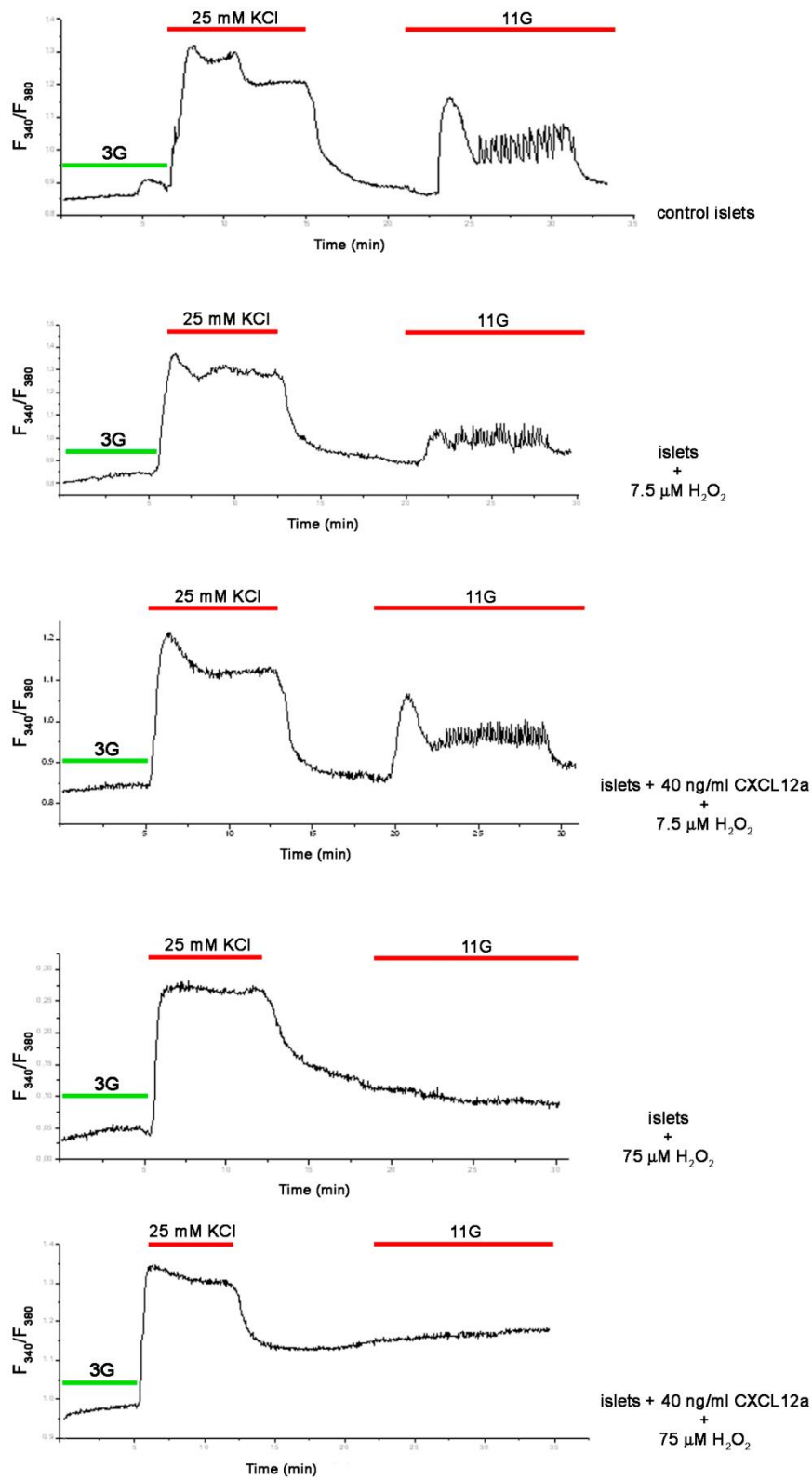
**Fig. 4.** CXCL12 $\alpha$  influence on gene expression for insulin, CXCR4, CXCR7, Lc/Ld and TRPM2 Ca<sup>2+</sup> channels. Transcription rate (mRNA levels) of Cxcl12 $\alpha$ , Cxcr4, Cxcr7, Lc/Ld types of calcium channels and Trpm2 in basal conditions and after increasing times of incubation with 40 ng/mL of CXCL12 $\alpha$  was estimated by RT-qPCR. Relative mRNA levels are presented as the ratios of each gene/ $\beta$ -actin. (\*) Mean values were significantly different from those of untreated control cells (p<0.05). Results are expressed as the means $\pm$ SEM from three separate experiments performed in triplicate.

**Fig. 5.** CXCL12 $\alpha$  stimulated glucose-induced insulin release. Insulin secretion in response to 3, 8 or 11 mM of glucose was measured in intact islets of Langerhans after 30 min (**A**) and overnight incubation (**B**) with CXCL12 $\alpha$  (40 ng/mL). \*p value from Student's t-test <0.05 \*\*p<0.01 value from Student's t-test.

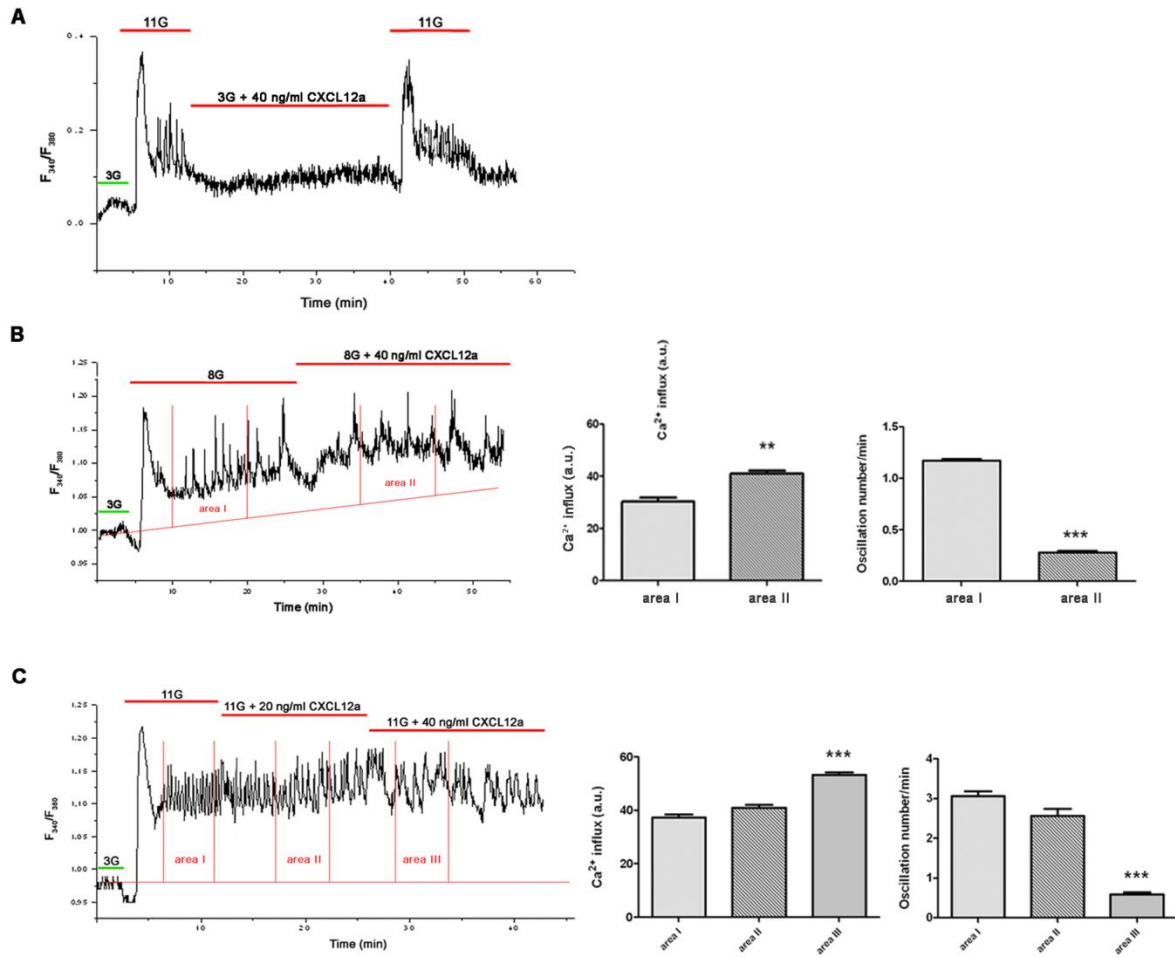


**Fig. 1.**

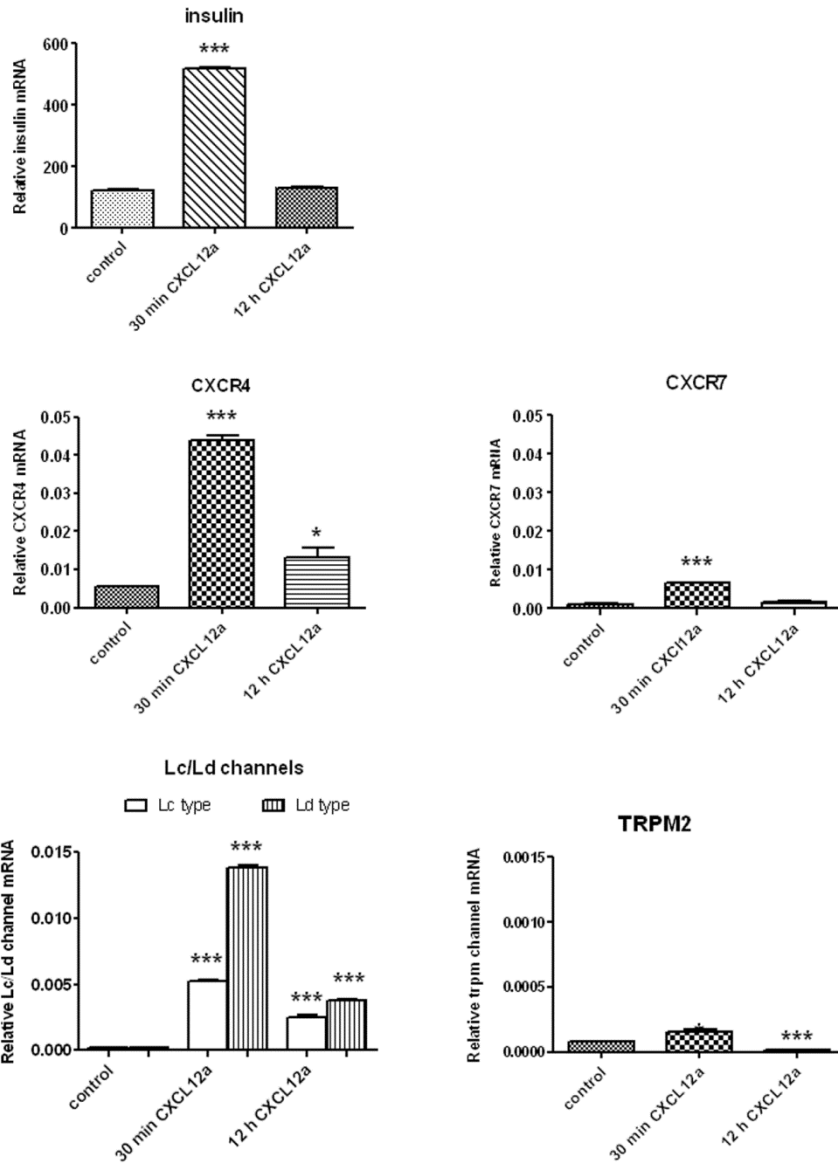




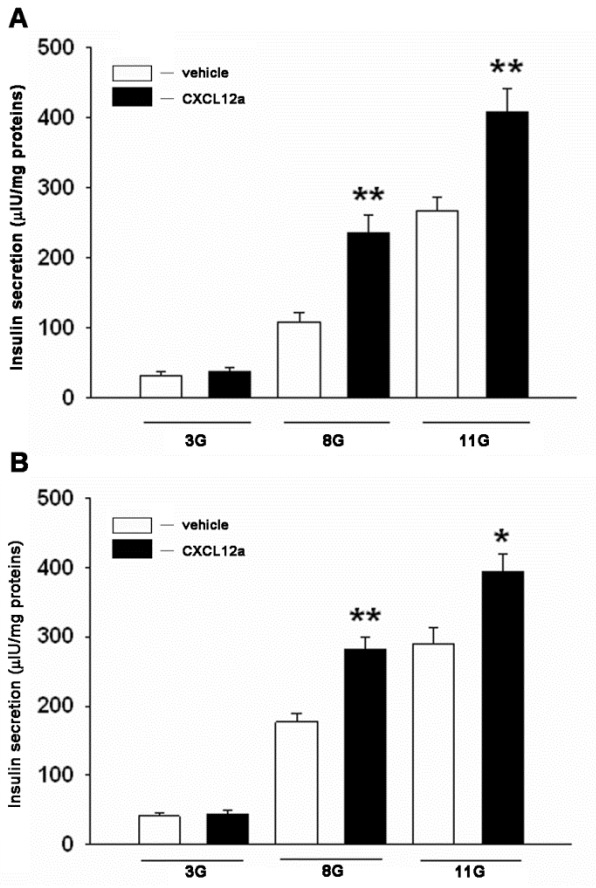
**Fig. 2.**



**Fig. 3.**



**Fig. 4.**



**Fig. 5.**