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Trefoil factor 3 (TFF3) expression is regulated by insulin and glucose

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ABSTRACT

Introduction: Trefoil factors are effector molecules in gastrointestinal tract physiology. They are classified into three groups: the gastric peptides (TFF1), spasmolytic peptide (TFF2) and intestinal trefoil factor (TFF3). Previous studies have shown that trefoil factors are located and expressed in human endocrine pancreas suggesting that TFF3 play a role in: a) pancreatic cells migration, b) β-cell mitosis, and c) pancreatic cells regeneration. We speculated that the presence of TFF3 in pancreas, could be associated to a possible regulation mechanism by insulin and glucose. To date, there are not reports whether the unbalance in carbohydrate metabolism observed in diabetes could affect the production or expression of TFF3.

Methods: We determined the TFF3 levels and expression by immunoassay (ELISA) and semi-quantitative RT-PCR technique respectively, of intestinal epithelial cells (HT-29) treated with glucose and insulin. Also, Real Time-PCR (RTq-PCR) was done.

Results: Increasing concentrations of glucose improved TFF3 expression and these levels were further elevated after insulin treatment. Insulin treatment also led to the up-regulation of human sodium/glucose transporter 1 (hSGLT1), which further increases intracellular glucose levels. Finally, we investigated the TFF3 levels in serum of diabetes mellitus type 1 (T1DM) and healthy patients. Here we shown that serum TFF3 levels were down-regulated in T1DM and this levels were up-regulated after insulin treatment. Also, the TFF3 levels of healthy donors were up-regulated 2 h after breakfast.

Conclusion: Our findings suggest for the first time that insulin signaling is important for TFF3 optimal expression in serum and intestinal epithelial cells.

Keywords: Trefoil expression, Insulin, Glucose, Glucose transporter, Diabetes

INTRODUCTION

The trefoil family, are peptides of fewer than 80 amino acids that are present along the gastrointesti-

nal tract. The three main trefoil peptide families are the gastric peptides (TFF1), the spasmolytic peptide (TFF2) and the intestinal trefoil factor (TFF3). They play a significant role in the conservation of the surface integrity of oral mucosa and improve healing of the gastrointestinal tract by a process termed restitution (1). Trefoil peptides are widely distributed in intestinal epithelial cells, and are present in saliva, meconium, human breast milk, and serum

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(1- 3). Trefoil families are protease-resistant peptides that are amply secreted onto the intestinal mucosal surface by specific cells of the human gastrointestinal tract. The TFFs share an completely conserved distinctive motif of six cysteine residues which form three disulfide bonds and define the "trefoil" domain, which is also known as a 'P' domain (4). Previous studies have shown that trefoil factors are expressed in human endocrine pancreas playing important functions in the physiology of the pancreas (5). Jackerott *et al* (6) reported that trefoil factors are expressed in human and rat endocrine pancreas and TFF3 could be an important role in cells migration and regeneration. In the same way, Fueger *et al* (7) reported that TFF3 stimulates human and rodent pancreatic islet β-cell replication. Based in these results, we speculated that the presence of TFF3 in pancreas could be associated to a possible regulation mechanism by insulin and glucose.

Glucose is a crucial fuel in humans and a key metabolic substrate. It is obtained directly from the intake, and by synthesis from other substrates in the liver. Dietary glucose and glucose synthesized within the body is transported through transport proteins. These transporters are classified into two main groups, a) the Na^+-dependent glucose co-transporters (hSGLT), and b) the facilitative Na^+-independent sugar transporters (GLUT family) (8). The hSGLT group transport mainly glucose with different kinetics, through a secondary active transport mechanism. It is known that the Na^+/K^+ ATPase pump plays a key role in this transport glucose mediated by hSGLT. This form of glucose transport takes place through the luminal membrane of cells lining the small intestine and the proximal tubules of the kidneys (9). The first type of glucose transport protein was the SGLT1. It is known that glucose could produce intracellular signaling across its metabolism inside the cells. In β-cells isolated from pancreas, it has been demonstrated that glucose metabolism increases ATP production, closing the KATP channels, which results in membrane depolarization ($\Delta\psi$), thus opening of voltage-dependent calcium channels (VDCC) and allowing Ca^{2+} influx. The resultant rise in $[Ca^{2+}]i$ triggers insulin secretion and others intracellular signaling (10). To date, there are not evidences whether trefoil factors could be regulated by glucose and insulin and whether hyperglycemia and/or insulin deficiency are involved in TFF3 expression. In the present work, we evaluated the role of insulin and glucose in TFF3 expression. Diabetes disease produces a decrease in β-cell mass, mediated generally by autoimmune destruction of insulin-producing cells in type 1 diabetes and by increased rates of apoptosis secondary to metabolic stress in type 2 diabetes (11). Currently, there are not reports about the serum levels of TFF3 in diabetes mellitus type 1 (DMT1) nor diabetes mellitus type 2 (DMT2). Here we showed that growing concentrations of glucose and insulin treatment improved TFF3 expression in intestinal epithelial cells. Also, the Insulin treatment led to the up-regulation of human sodium/glucose transporter 1 (hSGLT1), which further increases intracellular glucose levels. Finally, we showed that TFF3 was down-regulated in DMT1 patients and these values were modified after insulin treatment. Thus, our findings suggest for the first time that insulin signaling is important for TFF3 optimal expression in intestinal epithelial cells by elevating intracellular glucose levels and by mediating gene expression.

METHODS

Patients

The patient group consisted of 26 patients with T1DM treated at the Especialidades Clínicas Laboratory from February 2012 to July 2012. We obtained blood samples from each patient before insulin treatment and 2 hours after insulin treatment. The control group consisted of 28 healthy male donors who received a health check by clinicians at Carabobo Central Hospital from February 2012 to June 2012. For the validation of the results, a second cohort of patients, consisting of 18 patients with T1DM treated at the same laboratory from August 2012 to November 2012, were analyzed for serum TFF3 levels. Serum samples were collected for research under an institutional review board–approved protocol. This study was approved by the Institutional Review Board of the Applied Biotechnology Laboratory. Written informed consent was obtained from each participants.

Cultured cells

The HT-29 colonic cell line (passages 10–20) was obtained from the American Type Culture Collection (ATCC, Manassas, USA) and cultured according to the supplier's instructions in Dulbecco's

Modified Eagle medium (DMEM) containing 1000 gr/L (5.5 mM) glucose, 2 mM glutamine, 50 IU/ml penicillin, 50 mg/ml streptomycin and 10% heat inactivated fetal bovine serum as standard medium at 37 ºC in a water-saturated atmosphere with 5% CO_2. The HT-29 cells were treated with different glucose concentrations (5.5 mM, 10 mM, 15 mM, and 50 mM) for 6 h. Also, the cells were treated with 100, 150 and 200 nM of insulin for 6 h.

SDS-PAGE and immunoblotting

HT-29 cell lysates treated with glucose and/or insulin were subjected to electrophoresis on 15% SDS-PAGE according to the method of Laemmli (12). After electrophoresis, the gels were either fixed and proteins were visualized with 0.1% Coomassie brilliant blue R250 (Sigma) in methanol:water:acetic acid (Merck) (1:8:1) or they were electro-blotted onto nitrocellulose for 4 h at 4 ºC (8–10 V/cm). The membranes were incubated 12 h. at 4 ºC with blocking solution (5% nonfat dried milk in PBS containing 0.1% Tween-20). After being blocked, the membranes were incubated for 2 h at room temperature with PBS containing 5% dried milk powder and a 1:1000 dilution of rabbit anti-human TTF3 (Santa Cruz Biotechnology, cat. Nº. sc-28927) or mouse ati-human β-Tubulin (Santa Cruz Biotechnology, cat. Nº. sc-55529). The membranes were washed five times in PBS-Tween and incubated with the peroxidase-coupled anti-rabbit secondary antibody (1:3000; Santa Cruz Biotechnology, cat. Nº. sc-2030, for TFF3) or anti-mouse secondary antibody (1:1000; Santa Cruz Biotechnology, cat. Nº sc-2005, for β-Tubulin) in PBS-Tween containing 5% nonfat dried milk, for 2 h at room temperature. The membranes were washed three times in PBS-Tween and specific bands were visualized by luminol reagent (Santa Cruz Biotechnology, cat. Nº. sc-2048).

ELISA

HT-29 cells were grown in 6-well plates at 50% confluence and serum-starved for 24 h. The cell number in wells was normalized by seeding equal quantity of HT-29 cells, previously counted and diluted at final concentration of $2x10^5$ cells/ml. Cells were then treated by 6 h with glucose and/or insulin at different concentrations, as described in the text. Afterwards, culture supernatants of HT-29 were collected and centrifuged at 1000×g for 15 min at 4ºC and

TFF3 was quantified. In some experiments, ELISA test was done from serum of healthy and diabetic donors. Briefly, the primary antibody used was: anti-human TFF3 (Santa Cruz Biotechnology, cat. Nº. sc-28927) at room temperature for 2 h (1:1000 diluted). Then, the plates were washed and incubated at room temperature with peroxidase-coupled anti-rabbit secondary antibody (Santa Cruz Biotechnology, cat. Nº. sc-2030) diluted to 1:2000 in PBS plus 1 mL/L Tween 20 for 30 min. Plates were washed and incubated with chromogenic substrate in the dark at room temperature for 10 min. Stop solution (100 μl, 0.5M H_2SO_4) was added to each well. Absorbance was measured at 405nm using a microtiter plate spectrophotometer Synergy HT (BioTek Instruments, Winooski, VT, USA).We quantified TFF3 by simultaneous ELISA runs using TFF3 purified as calibrators.

Reverse transcription polymerase chain reaction (RT-PCR)

RNA was extracted from HT-29 intestinal cells by Trizol™ (Invitrogen) according to the manufacturer's instructions. RNA concentration and purity were measured using a spectrophotometer Synergy HT (BioTek Instruments, Winooski, VT, USA). Total RNA (1 μg) was reverse transcribed into cDNA using a commercial kit (Invitrogen Thermo-Script™ RT-PCR System), according to the manufacturer's instructions. Control reactions to check for DNA contamination were run in parallel with samples processed without reverse transcriptase. The primer sequences were: β-actin sense, 5'-CACGC-CATCCTGCGTCGGAC-3'; β-actin antisense, 5'-CATGCCATCCTGCGTCTGGAC-3'; TFF3 sense, 5'-CCCGGCTGTGATTGCTGCCA-3'; and TFF3 antisense, 5'-TCCTGTGACGTGGGT-GCCAGT-3'; hSGLT1 sense, 5'-CACGCAC-CAGGAGAGGGGAACA-3' and hSGLT1 antisense, 5'-GGCATTGTCACCACCCCAGCC-3'. Aliquots (10 μl) of the polymerase chain reaction products were electrophoresed on 1.5% agarose gels and stained with SYBR Gold nucleic acid gel stain (Molecular Probes, Invitrogen™). Densitometric analyses were performed using the image analysis software Quantity One (Bio-Rad laboratories, Hercules, CA, USA). Briefly, the digital image was analyzed to determine the pixel intensity of each band. Relative quantities of TFF3 and hSGLT1 mRNA among different preparations were calculated as the

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ratio of the TFF3:β-actin and hSGLT1:β-actin pixel intensities from three independent RT-PCR experiments. Positive results were based on the presence of DNA bands of the expected size.

Quantitative Real Time PCR (RTq-PCR)

Real time PCR was performed to precisely quantify the trefoil and sodium/glucose transporter 1 mRNA expression. Total RNA was isolated as described above. Total RNA (1 μg) was reverse transcribed into cDNA using a commercial kit (Invitrogen ThermoScript™ RT-PCR System), according to the manufacturer's instructions. PCRs amplification were performed using the Sybr Green kit (Applied Biosystems), GAPDH for normalizing the threshold cycle (Ct), while H_2O was used as negative control. All measurements were performed in triplicate. Primer sequences: TFF3 (sense: 5'- GAGGCTC-GGAGCACCCTTGC -3'; antisense: 5'- AAGC-GCTTGCCGGGAGCAAA-3'), hSGLT1 (sense: 5'-GGAGGCTTTGAATGGAATGC-3'; antisense: 5'- CAGCCAGCCCAGCACAAC-3'), GAP-DH (sense: 5'-CATGTTCGTCATGGGTGTGAA-3'; antisense: 5'-TGCAGGAGGCATTGCTGAT-3'). The most of these primers have been previously reported (13). The results were analyzed by using the comparative Ct method. This method is based on the Sample data were normalized to glyceraldehyde-3-phosphate dehydrogenase mRNA and are presented as -fold change relative to mRNA from untreated cells. Assumption that target and reference template DNA amplifies with the same efficiency. Only PCR experiments producing a single DNA fragment, analyzed by gel electrophoresis, were used for the statistical analysis.
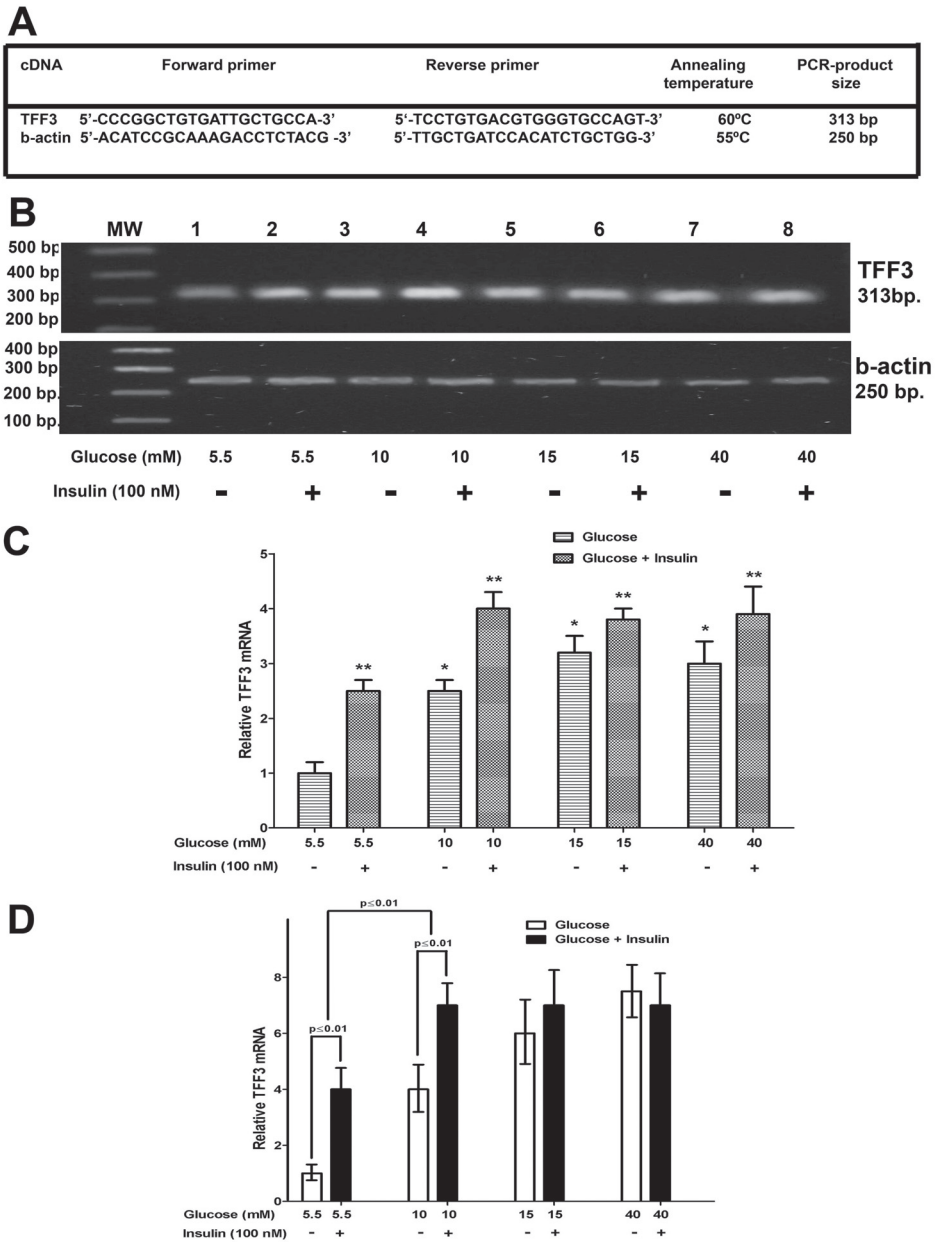
RESULTS

Effect of glucose and insulin treatment on TFF3 levels in HT-29 cells

ELISA was used to determine whether glucose and/or insulin treatment induce regulation of Trefoil factor 3 (TFF3) in cell culture supernatants from intestinal epithelial cells collected after glucose and insulin treatment. HT-29 cells were grown in 6-well plates at 50% confluence and serum-starved for 24 h. Cells were treated with different glucose concentrations (5.5 mM, 10 mM, 15 mM, and 40 mM) for 6 h. Afterwards, culture supernatants of HT-29 were collected and centrifuged at 1000×g for 15 min
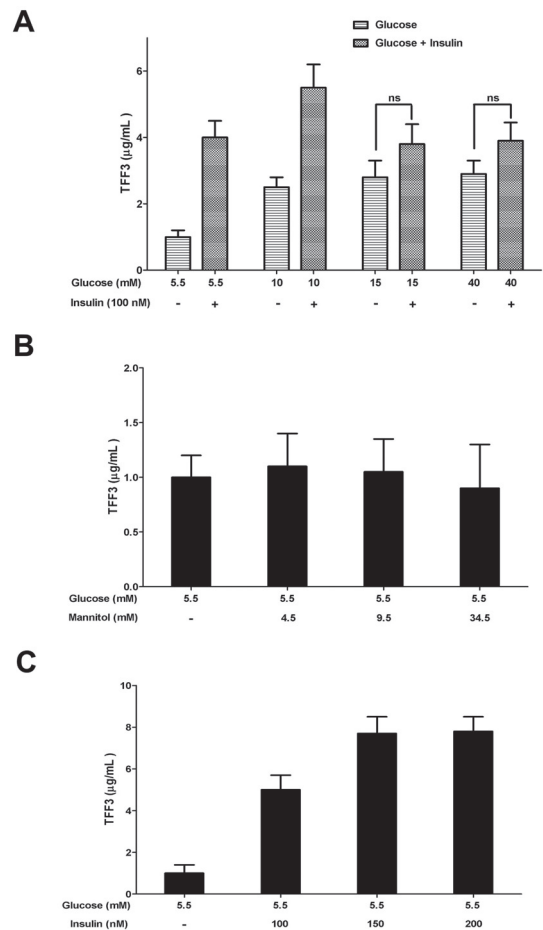
FIGURE 1. TFF3 levels in cell culture supernatants: HT-29 cells were grown in 6-well plates at 50% confluence and serum-starved for 24 h. Cells were treated with different glucose concentrations (5.5 mM, 10 mM, 15 mM, and 40 mM), for 6 h. In some experiments, HT-29 cells were treated with insulin ranging from 100 nM to 200 nM for 6 h. Afterwards, culture supernatants of HT-29 were collected and centrifuged at 1000×g for 15 min at 4°C, the proteins were precipitated by trichloroacetic acid (TCA) and TFF3 levels was measured by ELISA. (A) Intestinal HT-29 cells were treated with increasing concentrations of glucose in the absence or presence of 100 nM insulin. (B) HT-29 cells were treated with 5.5 mM glucose and increasing concentrations of mannitol for 6 h. (C) Intestinal cells were treated with 5.5 mM glucose and increasing concentrations of insulin for 6 h.

at 4 °C, the proteins were precipitated by trichloroacetic acid (TCA) and TFF3 levels was measured by ELISA. TCA precipitation permits concentrate proteins contained in cell culture supernatants and

A

cDNA	Forward primer	Reverse primer	Annealing temperature	PCR-product size
TFF3	5'-CCCGGCTGTGATTGCTGCCA-3'	5'-TCCTGTGACGTGGGTGCCAGT-3'	60ºC	313 bp
b-actin	5'-ACATCCGCAAAGACCTCTACG -3'	5'-TTGCTGATCCACATCTGCTGG-3'	55ºC	250 bp

B

| | MW | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |

TFF3 313bp.

b-actin 250 bp.

Glucose (mM)   5.5   5.5   10   10   15   15   40   40
Insulin (100 nM)   -   +   -   +   -   +   -   +

C

D

FIGURE 2. Quantification of differentially-expressed TFF3 mRNA by RT-PCR and RTq-PCR: (A) Specific primers and annealing temperatures employed in semi-quantitative PCR (RT-PCR). (B) RT-PCRs for TFF3 and β-actin were carried out from cell culture samples divided in eight groups: Cells treated with glucose 5.5 mM without and with insulin (100 nM) (lane 1 and 2), cells treated with glucose 10 mM without and with insulin (100 nM) (lane 3 and 4), cells treated with glucose 15 mM without and with insulin (100 nM) (lane 5 and 6), cells treated with glucose 40 mM without and with insulin (100 nM) (lane 7 and 8). The PCR-products were run onto 2% agarose gel electrophoresis. Control reactions without reverse transcriptase were carried out. PCR was performed in a final volume of 25μl containing 1μl of the reverse transcription reaction, 50μM of dNTPs, 1.5mM MgCl2, 50mM Tris–HCl (pH 8.0), 1 IU Taq polymerase and 0.2μM each of sense and antisense primers. Specific PCR for a constitutively expressed gene (β-actin) was carried out as a positive control. The relative amount of product was quantified by densitometric analysis of DNA bands (C). Trefoil-mRNA expression levels are shown normalized to β-actin. (D) Quantitative Real Time-PCR (RTq-PCR). Results are mean ± SEM of three independent experiments.

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A

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B
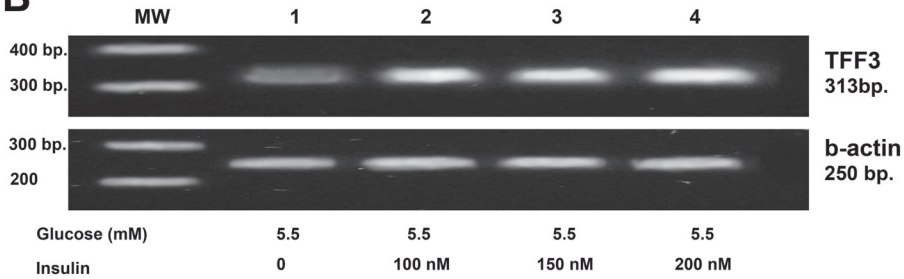
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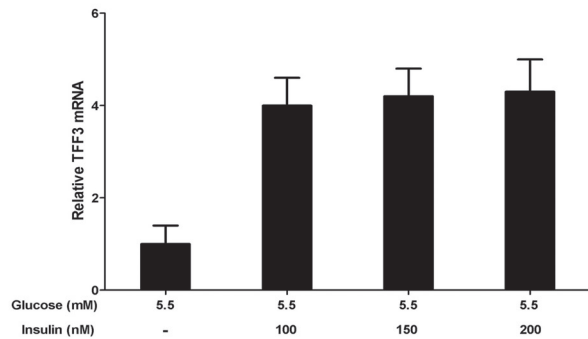
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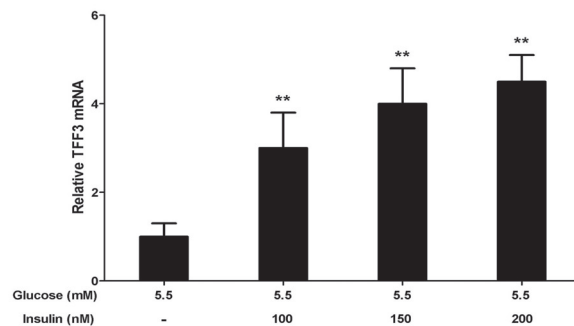
FIGURE 3. Quantification of differentially-expressed TFF3 mRNA by RT-PCR and RTq-PCR: (A) Specific primers and annealing temperatures employed in semi-quantitative PCR (RT-PCR). (B) RT-PCRs for TFF3 and β-actin were carried out from cell culture samples divided in four groups: Cells treated only with glucose 5.5 mM (lane 1), cells treated with glucose 5.5 mM plus insulin at 100 nM, 150nM and 200 nM (lanes 2, 3 and 4 respectively). The PCR-products were run onto 2% agarose gel electrophoresis. Control reactions without reverse transcriptase were carried out. PCR was performed in a final volume of 25µl containing 1µl of the reverse transcription reaction, 50µM of dNTPs, 1.5mM MgCl2, 50mM Tris–HCl (pH 8.0), 1 IU Taq polymerase and 0.2µM each of sense and antisense primers. Specific PCR for a constitutively expressed gene (β-actin) was carried out as a positive control. The relative amount of product was quantified by densitometric analysis of DNA bands (C). TFF3-mRNA expression levels are shown normalized to β-actin. (D) Quantitative Real Time-PCR. Results are mean ± SEM of three independent experiments.Results are mean ± SEM of three independent experiments.

A

B
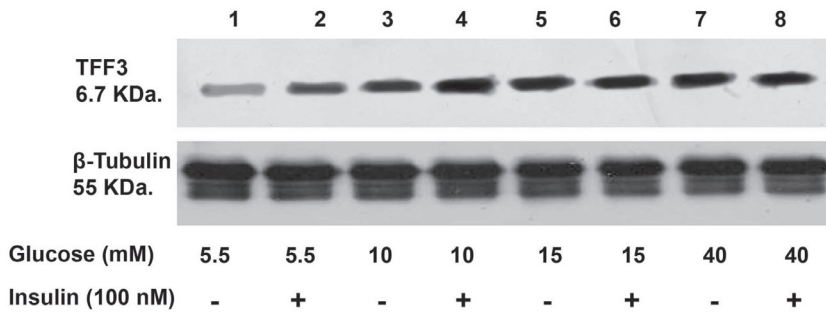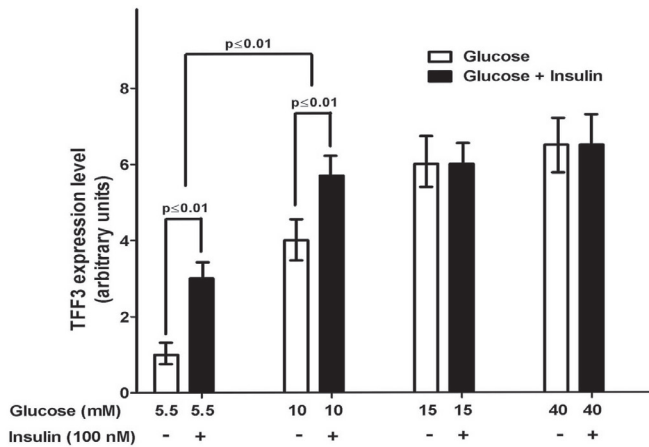
FIGURE 4. Inmunoblot for TFF3 and Tubulin. HT-29 cells were grown in 6-well plates at 50% confluence and serum-starved for 24 h. Cells were treated with different glucose concentrations (5.5 mM, 10 mM, 15 mM, and 40 mM), with or without insulin (100 nM) for 6 h. (A) Whole cell lysate (20 µg) from HT-29 cell monolayers which express TFF3, were run on non-denaturing 15% SDS-PAGE and electrotransferred to a nitrocellulose filter. Then, proteins in the membrane were denatured, renatured and blocked overnight at 4°C. TFF3 was detected using anti-TFF3 as primary antibody (upper panel). Then, membranes were stripped and reprobed using standard Immunoblotting to determine Tubulin on the lysate (lower panel). The relative amount of product was quantified by densitometric analysis of DNA bands (B). Results are mean ± SEM of three independent experiments.

prevent non-specific reactions in the immunoassay (14). As shown in Figure 1, the levels of TFF (Fig. 1A) increased at 10 mM glucose compared with 5.0 mM, but higher glucose concentrations (15 mM and 40 mM) did not increase the expression above the level achieved with 10 mM glucose. To verify that the increased TFF3 levels in cell culture supernatants by elevated glucose concentrations is due to a specific intracellular signaling induced by glucose and not the osmotic property of glucose, mannitol was used instead of glucose. Mannitol did not lead to the up-regulation of TFF3 levels in cell culture

supernatants (Figure 1B) suggesting that glucose is needed for this effect. In the same way, insulin treatment (100 nM) led increase of TFF3 levels in all glucose concentration tested (from 5 mM to 40 mM), indeed, 100 nM insulin treatment for 6 h increased the levels of TFF3 at 5.0 mM and 10 mM glucose (Figure 1A). At higher glucose concentrations (15 mM and 40 mM), the insulin treatment did not further increased the levels of TFF3 (Figure 1A). As these levels were not statistically different than those with 5 mM and 10 mM glucose plus insulin treatment, these findings may suggest that higher glucose

concentrations could lead to some insulin resistance in this cell line. Finally, higher insulin concentrations further increased TFF3 levels in cell culture supernatants from HT-29 (Figure 1C). Thus, insulin and glucose are critically involved in mediating the up-regulation of TFF3.

Glucose and insulin produce up-regulation of TFF3 mRNA in HT-29 cells

The TFF3 levels increased in cells culture supernatants could be produced mainly by: 1) up-regulation in mRNA expression, or 2) increase in half-life time of TFF3 mRNA. To test which mechanism was involved in this process, semi-quantitative RT-PCR (Figure 2B) and Real Time-PCR (RTq-PCR, Figure 2D) were used to figure out. HT-29 cells were grown to 50% confluence in 6-well plates and serum-starved for 24 h. Cells were then treated with different glucose and insulin concentrations (as described above) and total RNA was extracted from HT-29 intestinal cells by Trizol™ (Invitrogen) according to the manufacturer's instructions. As shown in Figure 2, the levels of TFF3 mRNA increased at 10 mM glucose compared with 5.0 mM, but higher glucose concentrations did not increase the expression above the level achieved with 10 mM glucose. In the same way, 100 nM insulin treatment for 6 h increased the levels of TFF3 expression at 5.0 mM and 10 mM glucose. However, higher glucose concentrations (15 mM and 40 mM) plus insulin (100 nM) did not increase the expression of TFF3 above the level achieved with 10 mM glucose plus insulin. Also, we tested whether the increase showed in TFF3 levels of cell culture supernatants treated with different insulin concentration (from 100 nM to 200 nM) produced an increase in TFF3 mRNA. We found that higher insulin concentrations further increased mRNA TFF3 expression in HT-29 cells (Figure 3). However higher concentration of insulin (150 nM and 200 nM) did not further increased the levels achieved at 100 nM insulin. Finally, we did western blot from HT-29 whole cells lysate to confirm that glucose and/or insulin produce up-regulation of TFF3. We found that the levels of TFF3 (Figure 4) increased at 10 and 15 mM glucose compared with 5.0 mM, but higher glucose concentrations (40 mM) did not increase the level achieved with 15 mM glucose. In the same way, insulin treatment (100 nM) led increase of TFF3 levels only at 5.5 mM and 10 mM glucose concentration. Taken to-

gether, these results show that the TFF3 is up-regulated by glucose and insulin in HT-29 intestinal epithelial cells.

Effect of glucose and insulin treatment on sodium/glucose transporter 1(hSGLT1) expressed in HT-29 cells

To further evaluate the effect of glucose and insulin on TFF3 expression, we explored the epithelial cell transporter hSGLT1 expression in intestinal epithelial cells HT-29. In this work, we showed that glucose and insulin produced augment in TFF3 expression, to test whether hSGLT1 was involved in this process, semi-quantitative RT-PCR (figure 5B) and RTq-PCR (figure 5D) were done. HT-29 cells were grown to 50% confluence in 6-well plates and serum-starved for 24 h. Cells were then treated with different glucose and insulin concentrations (as described above) and total RNA was extracted from HT-29 intestinal cells by Trizol™ (Invitrogen) according to the manufacturer's instructions. As shown in Figure 5 glucose did modified the hSGLT1 mRNA levels in HT-29. Aditionally, 100 nM insulin treatment for 6 h increased the levels of hSGLT1 expression in all glucose concentration tested (from 5 mM to 40 mM). This result suggests that Insulin could facilitate glucose entry into the intestinal epithelial cell by increasing hSGLT1 expression.

Trefoil factor 3 expression is regulated by insulin and glucose in serum of Type 1 Diabetes Mellitus (T1DM) and healthy patients

In this work, we have shown that glucose and insulin play an important role in TFF3 expression. To date, there are not reports about the correlation between chronic disease related with carbohydrate metabolism such as diabetes and serum trefoil factors. In this sense, we investigated the serum levels of TFF3 in T1DM and healthy donors. Figure 6A shows the serum TFF3 levels of all patients divided in two main groups: 1) healthy donors, and 2) T1DM patients. All samples were taken at 7:00 a.m., fasting serum was collected and frozen until ELISA was done. In the control group (healthy patients), the serum TFF3 level was 7.11 ng/ml. This level was significantly higher than in T1DM group (Figure 6A). Next, we investigated if TFF3 levels could be modified by the breakfast, and a second sample was taken from every healthy donor 2 h after breakfast
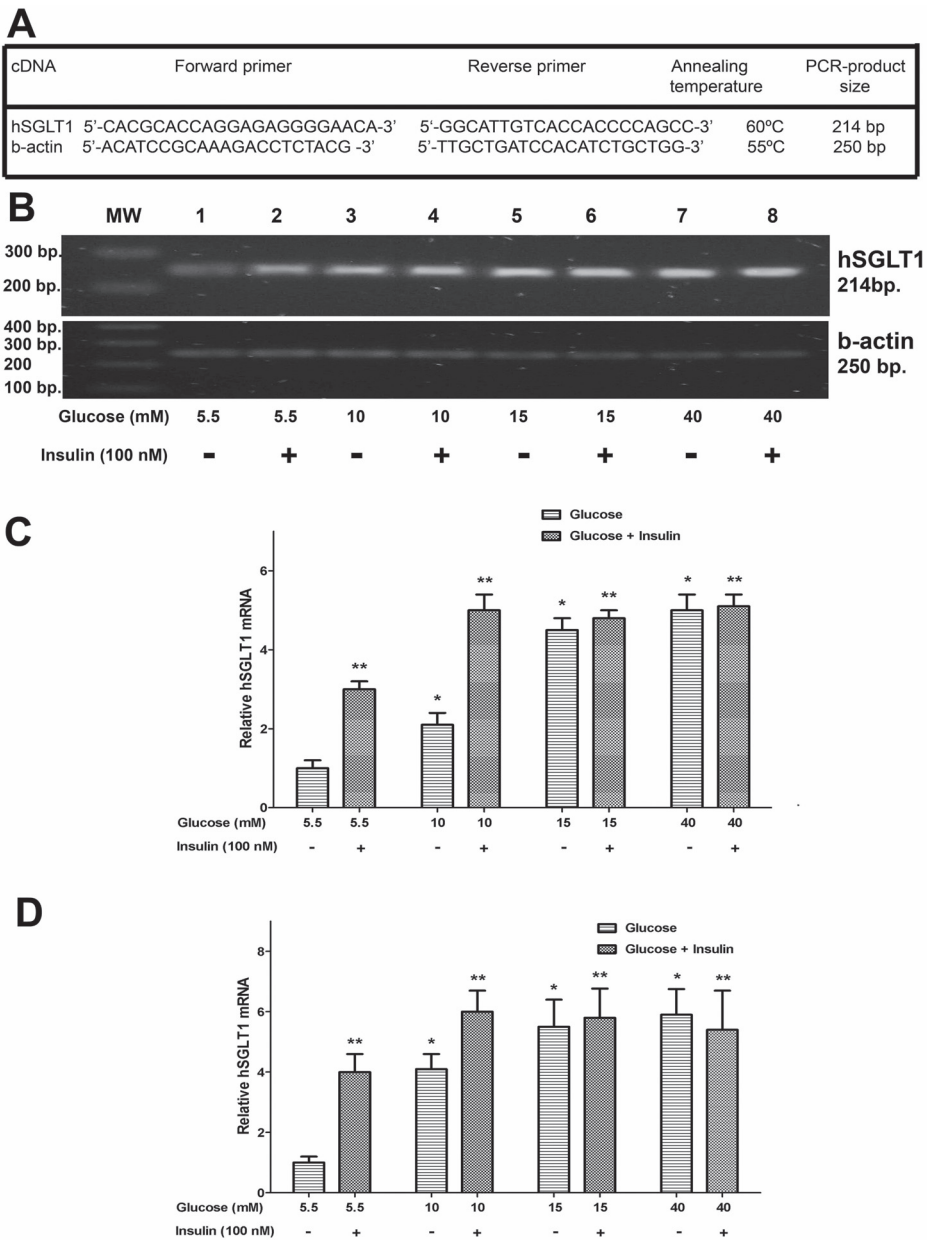
FIGURE 5. Effect of glucose and insulin on hSGLT1 expression in intestinal epithelial cells. (A) Specific primers and annealing temperatures employed in semi-quantitative PCR (RT-PCR). (B) Effect of increasing glucose concentrations and insulin treatment on hSGLT1 expression in HT-29 cells. HT-29 cells were treated with increasing concentrations of glucose in the absence or presence of 100 nM insulin for 6 h. The relative amount of product was quantified by densitometric analysis of DNA bands (C). RTq-PCR (D). Results are mean ± SEM of three independent experiments.

(9:00 am.). After 2-h postprandial period, the participants showed up-regulation of serum TFF3 levels (figure 6B). Here we hypothesized that the increase of glycemic and insulin followed by the meal consumption were the responsible of the serum TFF3 augment. These results are in concordance with our

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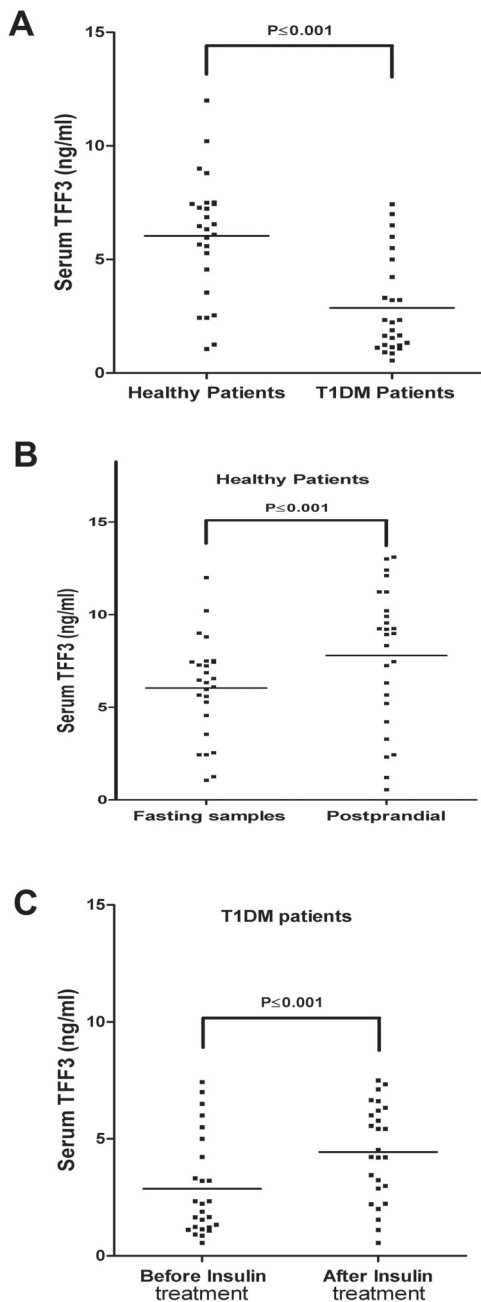
FIGURE 6. Serum TFF3 levels were measured by ELISA. (A) Fasting serum TFF3 levels in patients with type 1 Diabetes Mellitus were significantly lower than in the control group. (B) Serum TFF3 levels in the control group After 2-h postprandial period were also significantly higher than fasting serum. (C) Serum TFF3 levels in Type 1 Diabetes Mellitus Two hours after treatment with insulin were significantly higher than fasting serum.

previous results that glucose and insulin produced increase of TFF3 levels and expression. Finally, we tested whether TFF3 levels are regulated by insulin treatment in each patient with T1DM (Figure 6C). Two hours after treatment with insulin (each patient received specific insulin dosage recommended previously by its specific clinician), we found that TFF3 levels were significantly increased after insulin treatment. Together, these results suggest that glucose and insulin regulate TFF3 expression.

DISCUSSION

Trefoil peptides are found along the gastrointestinal tract and play a central role in the physiology of the gut. TFF are located and expressed in many others organs and might be involved in several physiology process related with intracellular signaling, gene expression, and cell cycle regulation. Previous studies have shown that trefoil factors are expressed in human and rat endocrine pancreas suggesting that TFF3 might play an important role in: a) pancreatic cells migration, b) β-cell mitosis, and c) pancreatic cells regeneration. Based in these results, we speculated that the presence of TFF3 in pancreas, could be associated to a possible regulation mechanism by insulin and glucose. In this work, we showed that glucose and insulin play an important role in TFF3 expression. According to Jackerott *et al* (6), TFF3 are located with most insulin and some glucagon-expressing cells in the adult human pancreas, and this location correlates with the mRNA expression in isolated human islets. In this work, we showed that glucose and insulin produce augment in TFF3 levels. In this sense, previous studies have shown that others intestinal peptides like human beta defensins 1 (hBD-1) mRNA is directly up-regulated by glucose in cultured HEK-293 cells grown in 25 mM glucose for 4 days (15). Moreover, Barnea *et al* reported that glucose and insulin are needed for optimal defensin expression in human cell lines (13). According to Barnea et al, Increasing concentrations of glucose enhanced hBD-1 expression and these levels were further elevated after insulin treatment. Insulin treatment also led to the up-regulation of human sodium/glucose transporter 1(hSGLT1), which further increases intracellular glucose levels. In the same way, our results suggest that TFF3 is up-regulated by glucose and insulin.

To date, there are not reports about the correlation between TFF3 and glucose/insulin regulation in serum and/or intestinal epithelium. In this sense, we report for first time that TFF3 expression is regulated by glucose and insulin. It is known that 5.5 mM glucose is the normal glycemic level in humans, and these levels are controlled by insulin and glucagon mainly. We cultured Intestinal epithelial cells HT-29 in Dulbecco's Modified Eagle medium (DMEM) supplemented principally with 10% fetal serum bovine (FBS). Subsequently, in our cell system insulin is not present in the medium, glucose transport into each cell might be slow and the intestinal cells might need higher glucose concentrations for optimal TFF3 expression. Indeed, 100 nM insulin treatment for 6 h increased the levels of TFF3 expression at 5.0 mM and 10 mM glucose. This work suggest that TFF3 expression in intestinal epithelial cells HT-29 is mediated by glucose transport into the cells, for the reason that TFF3 higher expression levels were achieved with increasing glucose concentrations in the absence of insulin (Figures 1, 2 and 4). However, insulin was also found to be an important factor mediating TFF3 expression (Figures 1-4). The key role of insulin in TFF3 expression could be through a specific intracellular signaling pathway that leads to glucose transport into the cell via others glucose transporters like GLUT4 and the induction of hSGLT1 expression (Figures 5 and 6) . On the other hand, is thinkable that insulin mediates TFF3 up-regulation through the pathway leads regulation of the TFF3 promoter in intestinal cells. However, these suggestions deserve further investigation. To date, there are not reports about the correlation between chronic inflammatory disease, such as diabetes mellitus and TFF3 regulation in serum and/or intestinal epithelium, and there is not evidence whether the unbalance in carbohydrate metabolism observed in diabetes could affect the production or expression of TFF3 in intestinal epithelial cells. In this work, we reported that in DMT1 serum, the TFF3 levels are lower than control people (healthy donors), however it is not well known the origin of TFF3 in serum. On the other hand, Serum Levels of Trefoil Factor Family Proteins have been associated with others diseases as Gastric Cancer (16). In this sense, it is known that Foveolar hyperplasia, spasmolytic polypeptide (TFF2)-expressing metaplasia, and intestinal metaplasia are histologic changes observed in patients with atrophic gastritis; they express TFF1, TFF2, and TFF3, respectively (16, 17, 18). Finally, here we report for first time that intestinal TFF3 expression and TFF3 serum levels are regulated by glucose and/or insulin.

CONCLUSION

The glucose and insulin treatment led increase TFF3 levels in cell culture supernatants and up-regulation of TFF3 mRNA in HT-29 cells, suggesting that insulin and glucose are critically involved in mediating the up-regulation of TFF3. Also, glucose and insulin treatment did modified the hSGLT1 mRNA levels in HT-29, suggesting that Insulin could facilitate glucose entry into the intestinal epithelial cell by increasing hSGLT1 expression. Finally, the serum TFF3 levels was significantly higher in T1DM group than control group (healthy patients), suggesting that glucose and insulin regulate TFF3 expression.

CONFLICT OF INTEREST

The authors declare no competing interests.

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