American Journal of Transplantation 2017; 17: 2829–2840
Wiley Periodicals Inc.

doi: 10.1111/ajt.14323

Deciphering Tacrolimus-Induced Toxicity in Pancreatic β Cells

J. Triñanes[1,4,†], A. E. Rodriguez-Rodriguez[2,†], Y. Brito-Casillas[3], A. Wagner[3], A. P. J. De Vries[4], G. Cuesto[1], A. Acebes[1], E. Salido[1,5,6], A. Torres[1,2,7] and E. Porrini[1,*]

[1]Centre for Biomedical Research of the Canary Islands (CIBICAN), University of La Laguna, La Laguna, Tenerife, Spain
[2]Research Unit of the University Hospital of the Canary Islands, La Laguna, Tenerife, Spain
[3]Unit of Endocrinology and Nutrition, Complejo Hospitalario Universitario Insular Materno-Infantil de Gran Canaria, Instituto Universitario de Investigaciones Biomédicas y Sanitarias, University of Las Palmas de Gran Canaria, Las Palmas de Gran Canaria, Spain
[4]Division of Nephrology and Leiden Transplant Center, Leiden University Medical Center and Leiden University, Leiden, the Netherlands
[5]Pathology Department, Hospital Universitario de Canarias, La Laguna, Tenerife, Spain
[6]Centre for Biomedical Research on Rare Diseases (CIBERER)
[7]Nephrology Department, Hospital Universitario de Canarias, La Laguna, Tenerife, Spain
**Corresponding author: Esteban Porrini, estebanporrini72@hotmail.com*
[†]These authors contributed equally to this work.

β Cell transcription factors such as forkhead box protein O1 (FoxO1), v-maf musculoaponeurotic fibrosarcoma oncogene homolog A (MafA), pancreatic and duodenal homeobox 1, and neuronal differentiation 1, are dysfunctional in type 2 diabetes mellitus (T2DM). Posttransplant diabetes mellitus resembles T2DM and reflects interaction between pretransplant insulin resistance and immunosuppressants, mainly calcineurin inhibitors (CNIs). We evaluated the effect of tacrolimus (TAC), cyclosporine A (CsA), and metabolic stressors (glucose plus palmitate) on insulinoma β cells *in vitro* and in pancreata of obese and lean Zucker rats. Cells were cultured for 5 days with 100 μM palmitate and 22 mM glucose; CsA (250 ng/mL) or TAC (15 ng/mL) were added in the last 48 h. Glucose plus palmitate increased nuclear FoxO1 and decreased nuclear MafA. TAC in addition to glucose plus palmitate magnified these changes in nuclear factors, whereas CsA did not. In addition to glucose plus palmitate, both drugs reduced insulin content, and TAC also affected insulin secretion. TAC withdrawal or conversion to CsA restored these changes. Similar results were observed in pancreata of obese animals on CNIs. TAC and CsA, in addition to glucose plus palmitate, induced comparable inhibition of calcineurin and nuclear factor of activated T cells (NFAT); therefore, TAC potentiates glucolipotoxicity in β cells, possibly by sharing common pathways of β cell dysfunction. TAC-induced β cell dysfunction is potentially reversible. Inhibition of the calcineurin–NFAT pathway may contribute to the diabetogenic effect of CNIs but does not explain the stronger effect of TAC compared with CsA.

Abbreviations: C, control; CNI, calcineurin inhibitor; CsA, cyclosporin A; DAPI, 4′,6-diamidino-2-phenylindole; FoxO1, forkhead box protein O1; G, glucose; GSIS, glucose-stimulated insulin secretion; HisH3, histone H3; KRBBK, rebs-Ringer bicarbonate buffer; LZR, lean Zucker rat; MafA, v-maf musculoaponeurotic fibrosarcoma oncogene homolog A; NeuroD, neuronal differentiation 1; NFAT2, nuclear factor of activated T cells 2; OZR, obese Zucker rat; P, palmitate; PBS, phosphate-buffered saline; PDX-1, pancreatic and duodenal homeobox 1; PTDM, posttransplant diabetes mellitus; T2DM, type 2 diabetes mellitus; TAC, tacrolimus

Received 09 September 2016, revised 14 April 2017 and accepted for publication 14 April 2017

Introduction

Posttransplant diabetes mellitus (PTDM) affects up to 30% of renal transplant recipients, and this doubles the prevalence of type 2 diabetes mellitus (T2DM) in the general population (1–4). PTDM is associated with reduction in patient survival, high risk of infections and cardiovascular disease (3,5,6), lower quality of life, and increased health care expenditures, by $21 500 for every case (7).

PTDM is thought to result from β cell failure induced by immunosuppression, mainly tacrolimus (TAC) and cyclosporine A (CsA) (3). TAC is considered more diabetogenic than CsA (8,9), although this finding has not been replicated in recent studies (10–12). Our group hypothesized that the greater diabetogenic effect of TAC depends on the preexistence of insulin resistance. TAC was more diabetogenic than CsA only in patients with pretransplant hypertriglyceridemia, a marker of insulin resistance (12). In addition, TAC caused more diabetes than CsA in

insulin-resistant obese Zucker rats (OZRs; 100% vs. 40%), whereas neither TAC nor CsA caused diabetes in lean Zucker rats (LZRs; insulin sensitive) (13). TAC reduced β cell proliferation but did not increase cell apoptosis in animals with diabetes (13). Cessation of TAC and conversion to CsA led to partial recovery of β cell function and proliferation (13,14). Consequently, the toxic effect of TAC depends on the preexistence of the changes related to insulin resistance; however, the pathways involved in β cell dysfunction induced by TAC remain unknown.

β cell changes during the state of insulin resistance that precedes type 2 diabetes (T2DM) have been studied extensively (15–17). These include the active maintenance and regulation of β cell proliferation, insulin synthesis, and secretion. Many of these functions are regulated by β cell–enriched transcription factors that are essential for glucose sensing, insulin secretion, and cell proliferation, namely, pancreatic and duodenal homeobox 1 (PDX-1), v-maf musculoaponeurotic fibrosarcoma oncogene homolog A (MafA), neuronal differentiation 1 (NeuroD), and forkhead box protein O1 (FoxO1). FoxO1 mediates proliferation, and its nuclear exclusion is necessary for β cell expansion in the insulin resistance state (18,19). Insulin production is associated with the activation of PDX-1, MafA, and NeuroD (20). Mouse models of obesity and insulin resistance showed a drastic reduction in nuclear MafA (21,22). Transcriptionally inactive NeuroD, which fails to target promoters in pancreatic islets, leads to T2DM in humans (23). The loss of these factors during insulin resistance might limit the capacity of β cells to overcome the toxic effect of TAC. In addition, TAC might accelerate the dysfunction of the same pathways altered by insulin resistance. Finally, calcineurin and nuclear factor of activated T cells 2 (NFAT2), common targets of TAC and CsA, have a relevant role in β cell function and integrity (24), and the inhibition of this pathway may also promote β cell dysfunction.

We hypothesize that TAC (compared with CsA) accelerates β cell damage in pathways already altered by insulin resistance (i.e. PDX1, MafA, NeuroD, FoxO1, calcineurin, NFAT2). In this study, we evaluated (a) the changes in these transcription factors and in insulin content and secretion in β cells (INS-1 cell line) treated with TAC or CsA in normal and glucolipotoxic conditions and (b) the localization of these transcription factors in β cells of pancreata of OZRs and LZRs on TAC or CsA.

Research Design and Method

B Cells in culture

Cell line maintenance: Rat insulinoma INS-1 β cells were cultured in RPMI-1640 medium with 11 mM glucose, 10% (vol/vol) heat-inactivated fetal bovine serum, 1 mM pyruvate, 10 mM HEPES, 50 μM β-mercaptoethanol, 2 mM glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin in a humidified atmosphere with 5% CO_2. Media was renewed every 48 h, and maintenance culture was passaged at 80% confluence by gentle trypsin treatment. Cells were seeded in dishes of 55 cm^2, with 8 mL of complete medium, at a final density of 2×10^4 cells/cm^2.

Experimental design: **Glucolipotoxicity:** Cells were cultured in 24-well plates and treated for 5 days with glucose (22 mM), palmitate (100 μM), or both (Figure S1A). INS-1 cells live in a standard medium with 11 mM of glucose. To reproduce an insulin-resistant environment, we increased glucose up to 22 mM and added palmitate (100 μM). Although higher concentrations such as 33 mM (glucose) and 200 μM (palmitate) are used in models of glucolipotoxicity (25), we chose lower concentrations to mimic moderate insulin resistance and to avoid apoptosis induced by high concentrations of glucose or palmitate (25–27).

Calcineurin inhibitors: CsA (250 ng/mL) or TAC (15 ng/mL) was added for 48 h to cells incubated on standard medium for 72 h (Figure S1A). These concentrations were derived from our previous experiments in rats, in which these levels induced diabetes in obese but not in lean animals (13). In a sensitivity analysis, we explored the effect of higher and lower concentrations: CsA at 25 and 500 ng/mL and TAC at 5 and 30 ng/mL.

Interaction between TAC or CsA and glucolipotoxicity: Cells were treated for 5 days with glucose (22 mM) and/or palmitate (100 μM), and TAC or CsA was added on the last 2 days (Figure S1A).

Reversibility of TAC-related changes: Two groups of INS-1 cells were treated for 10 days with glucose and palmitate, and TAC was added on days 4 and 5 and subsequently either withdrawn (reversibility group) or changed to CsA (conversion group) for 5 days (Figure S1A). All conditions were analyzed in triplicate, and experiments were performed three times.

Immunofluorescence analysis: In this analysis, 1×10^4 cells were seeded on sterilized 12-mm-diameter coverslips in 24-well plates and treated as explained above. After treatment, cells were washed with phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde for 10 min at room temperature. Slides were then permeabilized for 10 min with 0.1% Triton X-100 in PBS. Cells were blocked for 30 min with 3% bovine serum albumin, incubated at 4°C overnight with the primary antibody, and then washed and incubated for 40 min with the secondary antibodies. Images were taken with a Leica confocal microscope (Leica, Wetzlar, Germany) and analyzed using ImageJ software (National Institutes of Health, Bethesda, MD) and the JACoP plug-in to determine colocalization coefficients. The following primary antibodies were used: rabbit anti-FoxO1 (1:500;

Abcam, Cambridge, UK), rabbit anti-PDX-1 (1:500; Abcam), rabbit anti-MafA (1:200; Abcam), mouse anti-NeuroD (1:100; Abcam), and rabbit anti–active caspase 3 (1:100; Cell Signaling Technology, Danvers, MA) as a marker of apoptosis. Secondary antibodies were Alexa Fluor anti-mouse or antirabbit (1:600; Life Technologies, Carlsbad, CA). DNA was stained with DAPI (4′,6-diamidino-2-phenylindole). Results of nuclear localization are expressed as the proportion of DAPI colocalized with each transcription factor.

Insulin secretion and content: The secretion of insulin was analyzed according to Fu et al (28). In brief, we seeded 5×10^4 cells per well in a 24-well culture plate in 1 mL of RPMI medium. After the treatments indicated above (Figure S1A), cells were washed twice with Krebs-Ringer bicarbonate buffer (KRBB) and then sensitized with 2.8 mM glucose in KRBB for 30 min at 37°C (basal insulin). To measure glucose-stimulated insulin secretion (GSIS) and content, cells were incubated for 30 min at 37°C in 1 mL KRBB with 16.8 mM glucose in the presence of CsA or TAC. Insulin secretion was measured in supernatants. Cells were lysed overnight at 4°C with acidified ethanol (1 mL with 75% ethanol plus 0.5% HCl) to measure the remaining insulin content. Insulin was measured by ELISA (Mercodia, Uppsala, Sweden). All experiments were repeated seven times (n = 7) and the measurements were done in duplicate. Total insulin was calculated as insulin secretion after GSIS plus the insulin content, and the percentage of secretion was calculated as (insulin secretion / total insulin) × 100.

Activity of calcineurin and NFAT2 expression: Calcineurin activity was measured by the calcineurin phosphatase activity assay kit (ab139464; Abcam). After treatments, INS-1 cells were lysed with the supplied buffer, and the extracts were exposed to a desalting resin to remove the excess free phosphate and nucleotides. The extracts were then incubated with the calcineurin substrate RII phosphopeptide and EGTA buffers at 30°C for the 30 min to determine phosphatase activity, total and without calcineurin, respectively. Next, 100 μL of a green assay reagent were added to visualize the phosphate release. The color was allowed to develop at room temperature for 20 min before detection of the absorbance value at 620 nm on the microplate spectrophotometer. The absorbance value $A_{620,\ total} - A_{620,\ EGTA\ buffer}$ was converted to phosphate release using the standard curve line-fit that was prepared in each assay (n = 3). The phosphate release was adjusted to total protein concentration to obtain calcineurin activity (nmol/μg per microliter).

The expression of NFAT2 was measured by western blot in INS-1 cells (n = 3). We separated nuclear and cytoplasmic fractions, as reported by Kawamori et al (29). The concentration of protein was determined with the bicinchoninic acid method. We performed a western blot with 50 μg of total protein from nuclear fraction. The levels of NFAT2 were normalized to histone H3. Protein bands were detected by ECL western blotting reagent (Bio-Rad, Hercules, CA). To evaluate dose-response changes in the activity of calcineurin and the expression of NFAT2, the experiments were repeated using lower and higher doses of CsA (25 and 500 ng/mL) and TAC (5 and 30 ng/mL).

RNA extraction and retrotranscription: INS-1 cells were scraped and homogenized in ice-cold buffer D containing guanidinium thiocyanate for RNA isolation by the Chomczynski method (30). The purity and concentration of RNA were determined by NanoDrop 2000 (Thermo Fisher, Waltham, MA). Total RNA retrotranscription was performed with the ImProm-II reverse transcription kit (Promega, Madison, WI).

Real-time polymerase chain reaction: Insulin 1 (*Ins1*) and insulin 2 (*Ins2*) gene expression was evaluated using *Gapdh* as an internal control by real-time reverse transcriptase polymerase chain reaction. The oligonucleotides used for the *Ins1* gene were 5′-TCAGCAAGCAGGT CATTGTTC-3′ (forward primer) and 5′-GGGTCCTCCACTT CACGAC-3′ (reverse primer); oligonucleotides used for *Ins2* were 5′-TTCTCACTTGGTGGAAGCTCT-3′ (forward primer) and 5′-GTTGTGCCACTTGTGGGTCC-3′ (reverse primer). Gene expression is represented as $2^{-\Delta\Delta Ct}$, and the statistical comparisons were made between average $2^{-\Delta Ct}$ values of three experiments.

Studies in pancreata of OZRs and LZRs: We analyzed pancreata of insulin-resistant OZRs and insulin-sensitive LZRs from previous experiments (13,14). Animals (15 for OZR and 7 for LZR) were treated with daily intraperitoneal injections of vehicle (PBS), TAC (0.3 mg/kg per day), or CsA (2.5 mg/kg per day) during 11 days. In a subgroup of OZRs, TAC was withdrawn or replaced by CsA for 5 additional days (Figure S1B). Diabetes was diagnosed by repeated fasting glucose measurement and by intraperitoneal glucose tolerance tests performed at baseline and at the end of the experiment. All OZRs on TAC developed diabetes, whereas only 40% of those on CsA did. No LZRs treated with TAC or CsA developed diabetes. OZRs treated with TAC showed lower β cell proliferation and insulin gene expression than those treated with CsA. TAC withdrawal or conversion to CsA in animals with hyperglycemia reversed diabetes and increased β cell proliferation and insulin gene expression. β cell apoptosis was not increased in animals with diabetes induced by TAC or CsA. The ethics committee of the University of La Laguna approved all experiments.

Immunohistochemistry of pancreata from OZRs and LZRs: To corroborate the results of the experiments in

INS-1 cells, we performed immunohistochemistry analysis in sections of the pancreata from OZRs and LZRs to evaluate the nuclear localization of FoxO1, MafA, PDX1, NeuroD, and NFAT2. Sections of pancreata fixed during 24 h in 4% paraformaldehyde and paraffin embedded were cut at 3-μm thickness and mounted on separate slides for immunohistochemistry (n = 5 per treatment). We analyzed nuclear levels of FoxO1 (1:500; Abcam), MafA (1:250; Abcam), PDX-1 (1:500; Abcam), NeuroD1 (1:250; Abcam), and NFAT2 (1:500; Abcam) in pancreatic islets, using a secondary horseradish peroxidase–conjugated antibody with diaminobenzidine as chromogen. All images were captured with an Olympus DP72 camera (Olympus, Tokyo, Japan) fitted to an Olympus DX41 microscope (Olympus) and then analyzed using ImageJ software (National Institutes of Health) and the Wright Cell Imaging Facility plug-in from the Western Research Institute (Toronto, Canada).

Statistical analyses

Data are expressed as mean plus or minus standard deviation. Statistical analyses were performed by ANOVA. Post hoc pairwise comparisons were performed with the Tukey test.

Results

B cells in culture: glucolipotoxicity, calcineurin inhibitors, and nuclear factors

Glucolipotoxicity: Glucose (22 mM), palmitate (100 μM), or both induced a comparable increase in nuclear FoxO1 (Figure 1A). Nuclear MafA and PDX-1 were significantly reduced only by glucose plus palmitate (Figure 1A). Metabolic interventions did not affect NeuroD (Figure 1A).

TAC and CsA alone: In cells on maintenance medium, neither TAC (15 ng/mL) nor CsA (250 ng/mL) affected FoxO1, MafA, or NeuroD, and both drugs reduced nuclear PDX-1 (Figure 2A).

The combined effect of glucolipotoxicity and calcineurin inhibitors: Glucose + palmitate and TAC increased nuclear FoxO1 and reduced nuclear MafA, whereas glucose + palmitate and CsA did not induce these changes (Figure 3A). Calcineurin inhibitors (CNIs) on top of metabolic stressors did not affect PDX-1. Glucose plus palmitate and CsA did not modify nuclear NeuroD, and the same combination with TAC reduced NeuroD compared with control (Figure 3A).

TAC withdrawal and conversion to CsA: TAC withdrawal and conversion to CsA restored nuclear FoxO1 and MafA (Figure 4A). We focused on FoxO1 and MafA, which showed major changes in the previous experiments.

B cells in culture: glucolipotoxicity, CNIs, and insulin secretion and content

Glucolipotoxicity: The combination of glucose and palmitate reduced insulin content but did not affect insulin secretion (Figure 1B).

TAC and CsA alone: In cells on maintenance medium (without glucose and palmitate), CNIs did not influence insulin secretion or content (Figure 2B).

The combined effect of glucolipotoxicity and CNIs: Glucose plus palmitate and TAC reduced insulin secretion, whereas glucose plus palmitate and CsA did not (Figure 3B). Glucose plus palmitate and TAC or CsA comparably reduced insulin content (Figure 3B).

TAC withdrawal and conversion to CsA: Compared with cells on glucose plus palmitate, insulin secretion and content improved after TAC withdrawal or conversion to CsA (Figure 4B).

Insulin gene expression: Glucose plus palmitate alone did not change the expression of *Ins1* and *Ins2* genes. Glucose plus palmitate plus TAC induced greater reduction of both genes than glucose plus palmitate and CsA (Figures S2A and B).

B cells in culture: activity of calcineurin and expression of NFAT2

Glucolipotoxicity: The combination of glucose and palmitate increased the activity of calcineurin (Figure 5A) and the expression of NFAT2 compared with control (Figure 5B).

TAC and CsA alone: No relevant changes were observed in this pathway in cells on maintenance medium that were treated with only TAC or CsA at the doses used in our study and at lower or higher doses (Figure S3).

The combined effect of glucolipotoxicity and CNIs: TAC and CsA diminished the increased activity of calcineurin and NFAT in cells growing on glucose plus palmitate. This was confirmed with lower, intermediate, and higher doses of TAC and CsA (Figure 5A and B). The reductions in calcineurin and NFAT were comparable for TAC and CsA.

Sensitivity analysis:

No changes in the nuclear localization of active caspase 3 were observed in the studied groups (Figure S4).

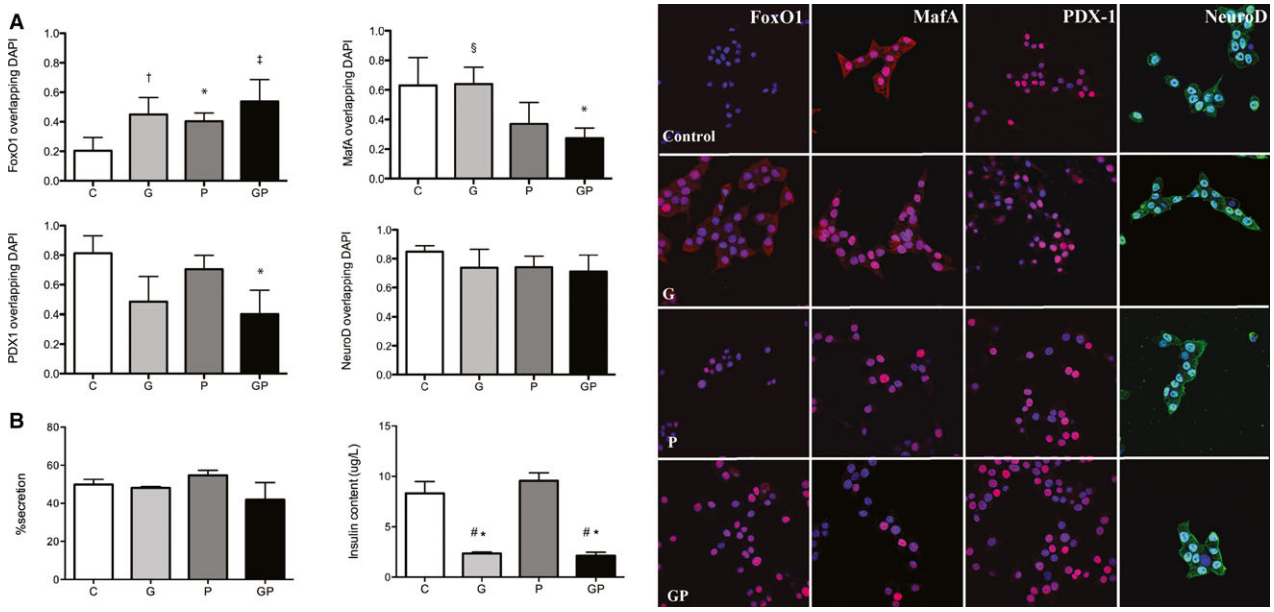

Figure 1: Effect of metabolic stressor in INS-1 cells. INS-1 cells grown in the presence of glucose (Glu) or/and palmitate (Pal) for 5 days. (A) Nuclear localization of forkhead box protein O1 (FoxO1), v-maf musculoaponeurotic fibrosarcoma oncogene homolog A (MafA), pancreatic and duodenal homeobox 1 (PDX-1), and neuronal differentiation 1 (NeuroD). All treatments were done in triplicate for each molecule, and three different pictures were taken in each replicate (n = 9). (B) Insulin content and percentage of insulin secreted by INS-1 cells after stimulation of insulin secretion. All measurements were done in duplicate (n = 7). Data are expressed as mean plus or minus standard deviation. Control group is common for the figure 1, 3 and 4. *$p < 0.05$ versus control; $^{\dagger}p < 0.01$ versus control; $^{\ddagger}p < 0.001$ versus control; $^{\S}p < 0.05$ versus GP; $^{\#}p < 0.001$ versus palmitate. C, control; G, glucose; P, palmitate.

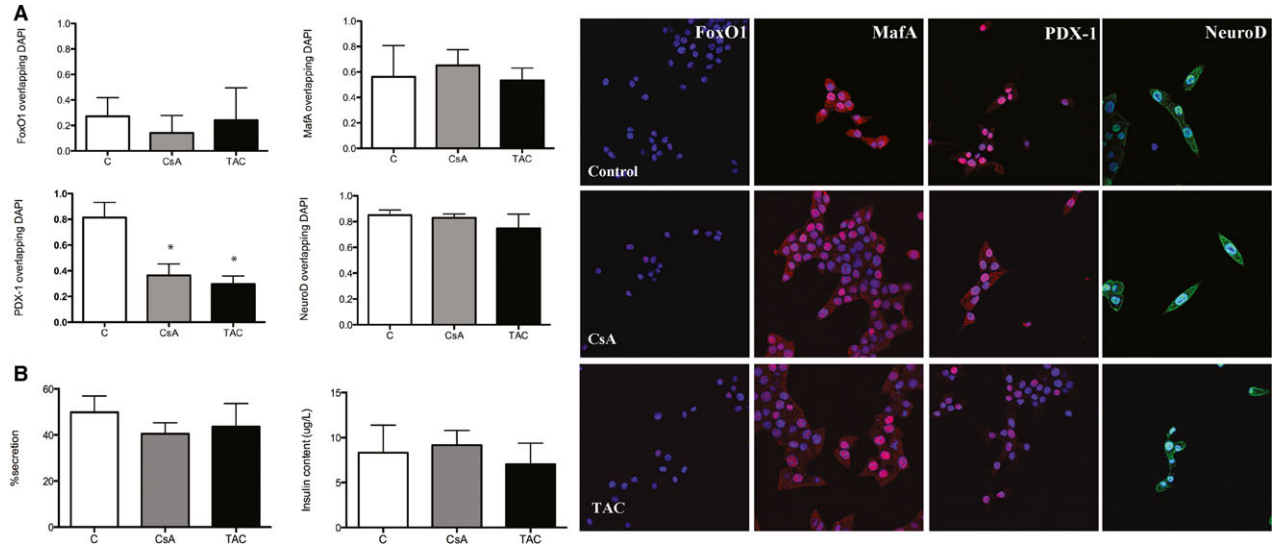

Figure 2: Effect of calcineurin inhibitors after 2 days of treatment in INS-1 cells. (A) Nuclear localization of forkhead box protein O1 (FoxO1), v-maf musculoaponeurotic fibrosarcoma oncogene homolog A (MafA), pancreatic and duodenal homeobox 1 (PDX-1), and neuronal differentiation 1 (NeuroD). All treatments were done in triplicate for each molecule, and three different pictures were taken in each replicate (n = 9). (B) Insulin content and percentage of insulin secreted by INS-1 cells after stimulation of insulin secretion. All experiments were done in triplicate (n = 3). Data are expressed as mean plus or minus standard deviation. *$p < 0.01$ versus control. C, control; CsA, cyclosporine A; TAC, tacrolimus.

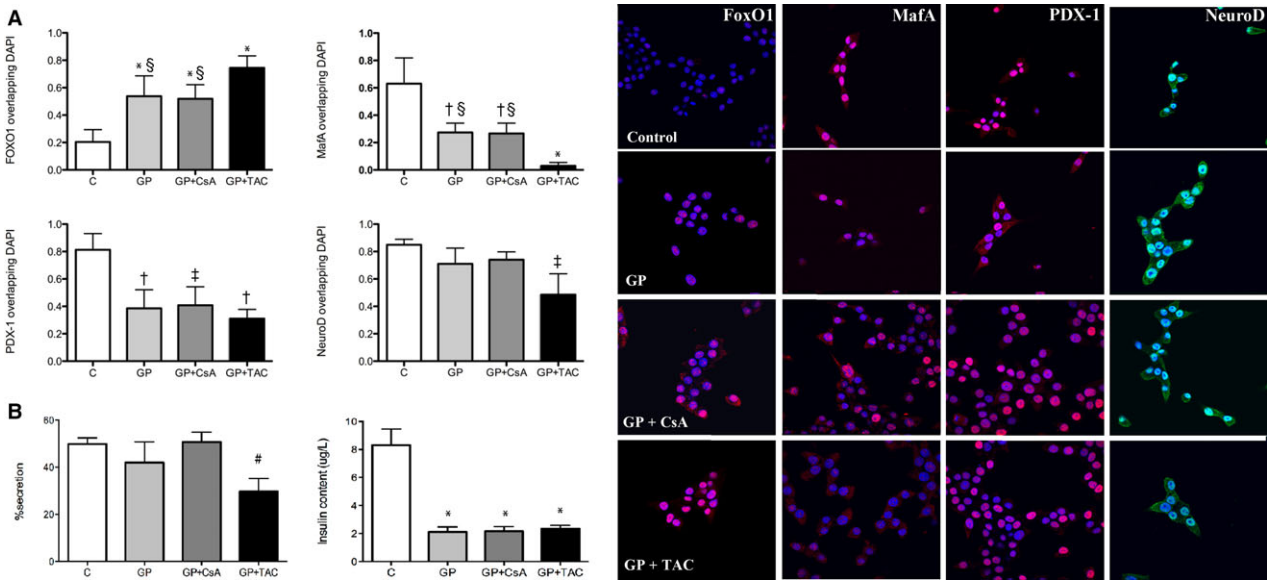

Figure 3: Combined effect of metabolic stressors and calcineurin inhibitors in INS-1 cells. (A) Nuclear localization of forkhead box protein O1 (FoxO1), v-maf musculoaponeurotic fibrosarcoma oncogene homolog A (MafA), pancreatic and duodenal homeobox 1 (PDX-1), and neuronal differentiation 1 (NeuroD). All treatments were done in triplicate for each molecule, and three different pictures were taken in each replicate (n = 9). (B) Insulin content and percentage of insulin secreted by INS-1 cells after stimulation of insulin secretion. All experiments were done in triplicate (n = 3). Representative images obtained for the different treatments and transcription factors in INS-1 cell line. Data are expressed as mean plus or minus standard deviation. Control group is common for the figures 1, 3 and 4. *$p < 0.001$ versus control; $^{\dagger}p < 0.01$ versus control; $^{\ddagger}p < 0.05$ versus control; $^{\S}p < 0.05$ versus GP-TAC; $^{\#}p = 0.02$ versus control, $p = 0.03$ versus GP-CsA. C, control; CsA, cyclosporine A; G, glucose; P, palmitate; TAC, tacrolimus.

Lower concentrations of TAC (5 ng/mL) in cells on glucose and palmitate induced lower nuclear FoxO1 and higher nuclear MafA compared with 15 ng/mL of TAC. A higher concentration of TAC (30 ng/mL) did not change the effect on FoxO1 and MafA of the previous dose (15 ng/mL) (Figure S5).

Pancreata of lean and obese rats: glucolipotoxicity, CNIs, and nuclear factors

Glucolipotoxicity: The nuclear localization of FoxO1 and MafA was comparable between OZRs and LZRs. Nuclear PDX-1 and NeuroD were increased in islets from OZRs compared with LZRs (Figure 6).

TAC and CsA: In LZRs, the nuclear localization of FoxO1, MafA, PDX-1, or NeuroD was similar in animals treated with TAC, CsA, or PBS (Figure 6).

The combined effect of glucolipotoxicity, CNIs, and nuclear factors: In islets from OZRs, TAC induced an increase in nuclear FoxO1 and a decrease in nuclear MafA compared with OZRs treated with CsA or PBS (Figure 6). Nuclear PDX-1 was reduced in OZRs treated with TAC compared with CsA or PBS. In all groups of OZRs, nuclear NeuroD was significantly higher compared with LZRs independent of the use of TAC or CsA (Figure 6).

TAC withdrawal and conversion to CsA: In OZRs, TAC withdrawal and conversion to CsA reduced nuclear FoxO1 and increased nuclear MafA compared with animals treated with TAC. Nuclear localization of PDX-1 increased with the conversion to CsA. No change was observed in nuclear NeuroD. Finally, the changes in nuclear factors were similar for TAC withdrawal and conversion to CsA (Figure 7).

Expression of NFAT2: The expression of NFAT2 was slightly reduced but comparable between obese animals treated with TAC or CsA for 11 days (Figure 5C).

Discussion

We observed that CNIs, TAC in particular, induced β cell dysfunction mainly in cells under metabolic stress. Interestingly, TAC potentiated the changes in transcription factors and insulin secretion and content already induced by glucolipotoxicity. In the presence of glucose and palmitate, TAC promoted (a) an increase in nuclear FoxO1 and a reduction in nuclear MafA compared with CsA and (b) reduction of insulin content with lower

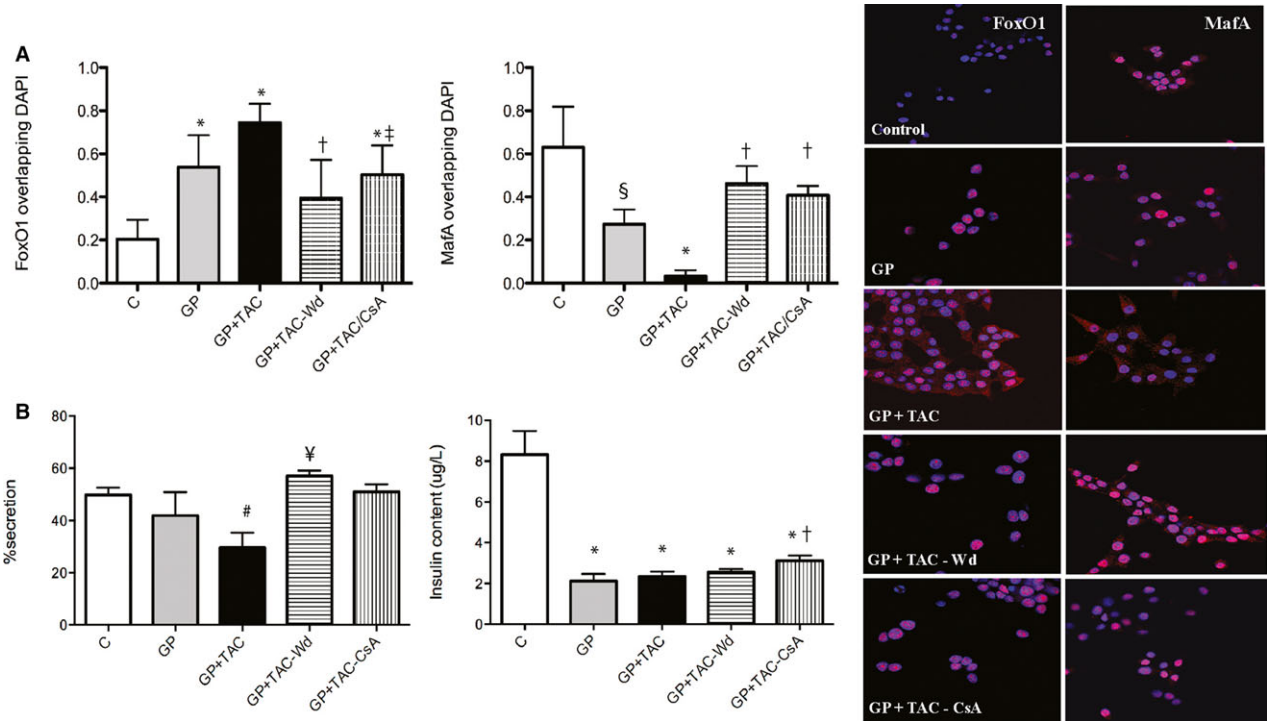

Figure 4: Effect of TAC withdrawal/conversion to CsA in INS-1 cells. (A) Nuclear localization of forkhead box protein O1 (FoxO1), v-maf musculoaponeurotic fibrosarcoma oncogene homolog A (MafA), pancreatic and duodenal homeobox 1 (PDX-1), and neuronal differentiation 1 (NeuroD). All treatments were done in triplicate for each molecule, and three different pictures were taken in each replicate (n = 9). (B) Insulin content and percentage of insulin secreted by INS-1 cells after sensitization and stimulation of insulin secretion. All experiments were done in triplicate (n = 3). Data are expressed as mean plus or minus standard deviation. Control group is common for the figures 1, 3 and 4. *$p < 0.001$ versus control; $^{\dagger}p < 0.01$ versus GP-TAC; $^{\ddagger}p < 0.05$ versus GP-TAC; $^{\S}p < 0.01$ versus control; $^{\#}p < 0.05$ versus control, $p < 0.002$ versus GP-TAC-W; $^{¥}p \leq 0.05$ versus control. C, control; CsA, cyclosporine A; G, glucose; P, palmitate; TAC, tacrolimus.

insulin secretion comparable to CsA. These changes were reversible after TAC withdrawal or conversion to CsA. In the absence of glucose and palmitate, neither CsA nor TAC induced significant changes in nuclear FoxO1, MafA, NeuroD, or insulin secretion and content. These findings were confirmed by immunohistochemistry analyses in pancreata of obese and lean animals receiving TAC or CsA. Obese animals treated with TAC showed higher nuclear localization of FoxO1 as well as lower nuclear MafA in pancreatic islets compared with those on CsA. Neither TAC nor CsA produced changes in the transcription factors studied in lean insulin-sensitive animals. TAC withdrawal or the conversion to CsA reverted these changes. Finally, glucose and palmitate increased the activity of calcineurin and the expression of NFAT. CNIs, acting on top of glucose and palmitate, reduced calcineurin and NFAT, and this reduction was comparable for TAC and CsA.

We observed that glucolipotoxicity induced changes in transcription factors like MafA and FoxO1 in β cells *in vitro* and in pancreases of OZRs. These results are in line with publications showing that free fatty acids and impaired glucose tolerance promote changes in MafA, PDX-1, NeuroD, and FoxO1 (19,31). These alterations may induce β cell dysfunction and mark progression toward diabetes. In addition, our findings suggest that we re-created a model of glucolipotoxicity *in vitro* to support the original findings in insulin-sensitive and -resistant animals treated with TAC or CsA.

The major finding of this study is that TAC exacerbated the same changes in nuclear transcription factors already promoted by glucolipotoxicity. Consequently, it is plausible that TAC and glucolipotoxicity share similar pathways in the induction of β cell dysfunction. The triumvirate formed by glucose, palmitate, and TAC increased nuclear FoxO1 and decreased nuclear MafA. In contrast, TAC did not promote these changes in the absence of glucolipotoxicity, and CsA did not add any effect to glucolipotoxicity alone. These results were confirmed in islets from OZRs and LZRs treated with TAC or CsA. An underlying insulin resistant milieu is crucial to induce β cell dysfunction with the use of TAC.

From a functional point of view, insulin content and secretion were altered by glucose, palmitate, and CNIs.

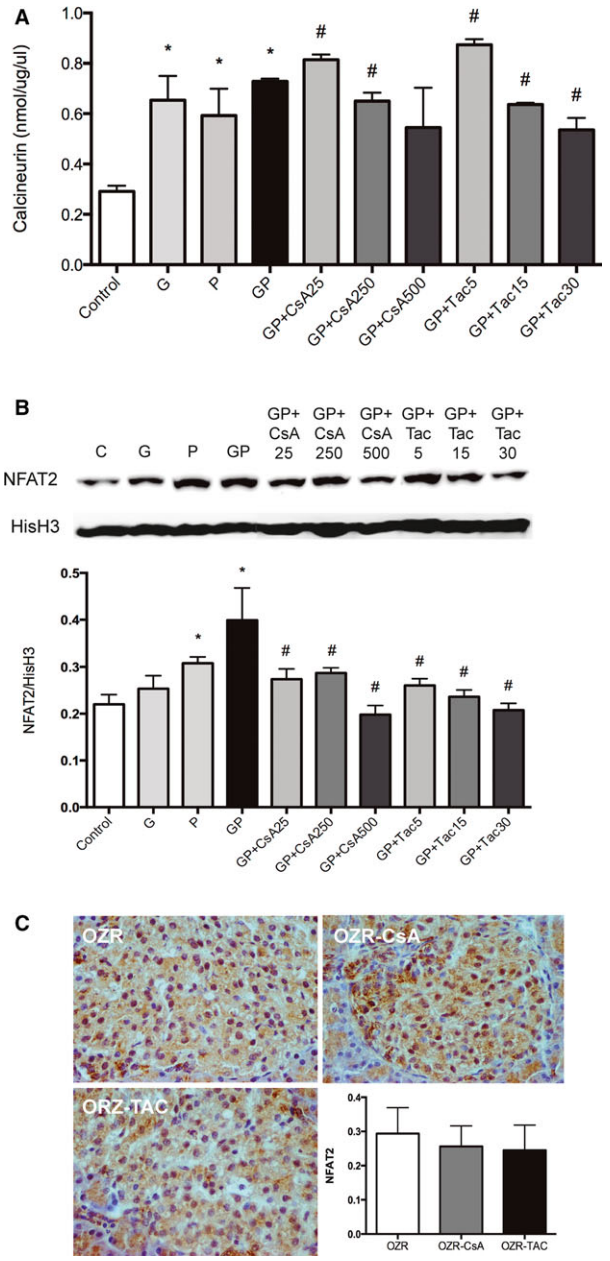

Figure 5: Calcineurin activity and expression of nuclear factor of activated T cells 2 (NFAT2). (A) Calcineurin phosphatase activity assay. Calcineurin activity was adjusted to total protein concentration (n = 3). *Versus control, $p \leq 0.01$; #versus GP, $p \leq 0.01$. (B) Western blot analysis of nuclear NFAT2 and histone H3 (HisH3) on INS-1 cells. Treatment: CsA 25, 250, and 500 ng/mL; TAC 5, 15, and 30 ng/mL; n = 3. *Versus control, $p \leq 0.001$; #versus GP, $p \leq 0.001$. (C) Representative images of treatment with calcineurin inhibitors on pancreata obese Zucker rats (OZRs). Histograms representing the proportion of hematoxylin/Diaminibenzidine staining for NFAT2, each group had five animals, and five islets per animal were analyzed (not significant). C, control; CsA, cyclosporine A; G, glucose; P, palmitate; TAC, tacrolimus.

Glucose and palmitate induced a relevant decrease in insulin content, whereas insulin secretion was maintained. This may reflect the known changes induced by high glucose in insulin metabolism in β cells (32). TAC in addition to glucose and palmitate reduced insulin secretion, whereas CsA did not. Compared with CsA, TAC may also affect insulin secretion in the context of glucolipotoxicity. Previous studies analyzed the impact of CNIs on insulin metabolism (13,14,33–35). Ozbay et al observed that TAC and CsA inhibited, to the same extent, glucose-stimulated insulin secretion in INS-1E cells (35); however, cells were cultured for 24 h with standard medium, whereas in our study, cells were cultured for 5 days with glucose and palmitate. This may explain the differences between studies. Finally, in cells growing on high glucose and palmitate, TAC induced stronger inhibition of insulin gene expression compared with CsA, which may contribute to the diabetogenic effect of TAC in the context of insulin resistance.

CsA and TAC bind to cyclophilin and FK506 binding protein, respectively, to form a complex that inhibits calcineurin and leads to dephosphorylation of NFAT and NFAT nuclear import. Glucose and palmitate increased the activity of calcineurin and expression of NFAT. Previous reports showed that calcineurin and NFAT are activated in the context of hyperglycemia, promoting insulin gene transcription and IRS-2 expression, which is required for maintenance of β cell mass and function (36,37). This is not unexpected because calcineurin and NFAT regulate multiple factors that control β cell functions and growth (24). In addition to overactivation of the pathway, both TAC and CsA inhibited calcineurin and reduced the expression of NFAT. Consequently, inhibition of calcineurin and NFAT in the context of glucolipotoxicity may play a role in the diabetogenic effect of TAC and CsA; however, inhibition of calcineurin and NFAT was comparable between TAC and CsA and was observed at lower and higher doses of both CNIs. Although TAC is supposed to be a more potent inhibitor of calcineurin and NFAT than CsA, this has been observed mainly in T cells (38,39). Few studies have analyzed inhibition of the calcineurin–NFAT pathway in β cells. Ozbay et al also observed comparable inhibition of calcineurin between CsA and TAC in β cells (INS-1E), even with doses higher than those used in our experiments. In this study, no effect on gene expression of NFAT was observed, which may be a consequence of a short-term (24 h) experiment (35). In the context of glucolipotoxicity, the inhibition of the calcineurin–NFAT pathway by CNIs may be diabetogenic but does not explain the greater effect of TAC compared with CsA. New studies are needed to elucidate other pathways involved in β cell damage induced by TAC.

We have observed that TAC is more diabetogenic than CsA only when given to patients with hypertriglyceridemia, a marker of insulin resistance (12), which

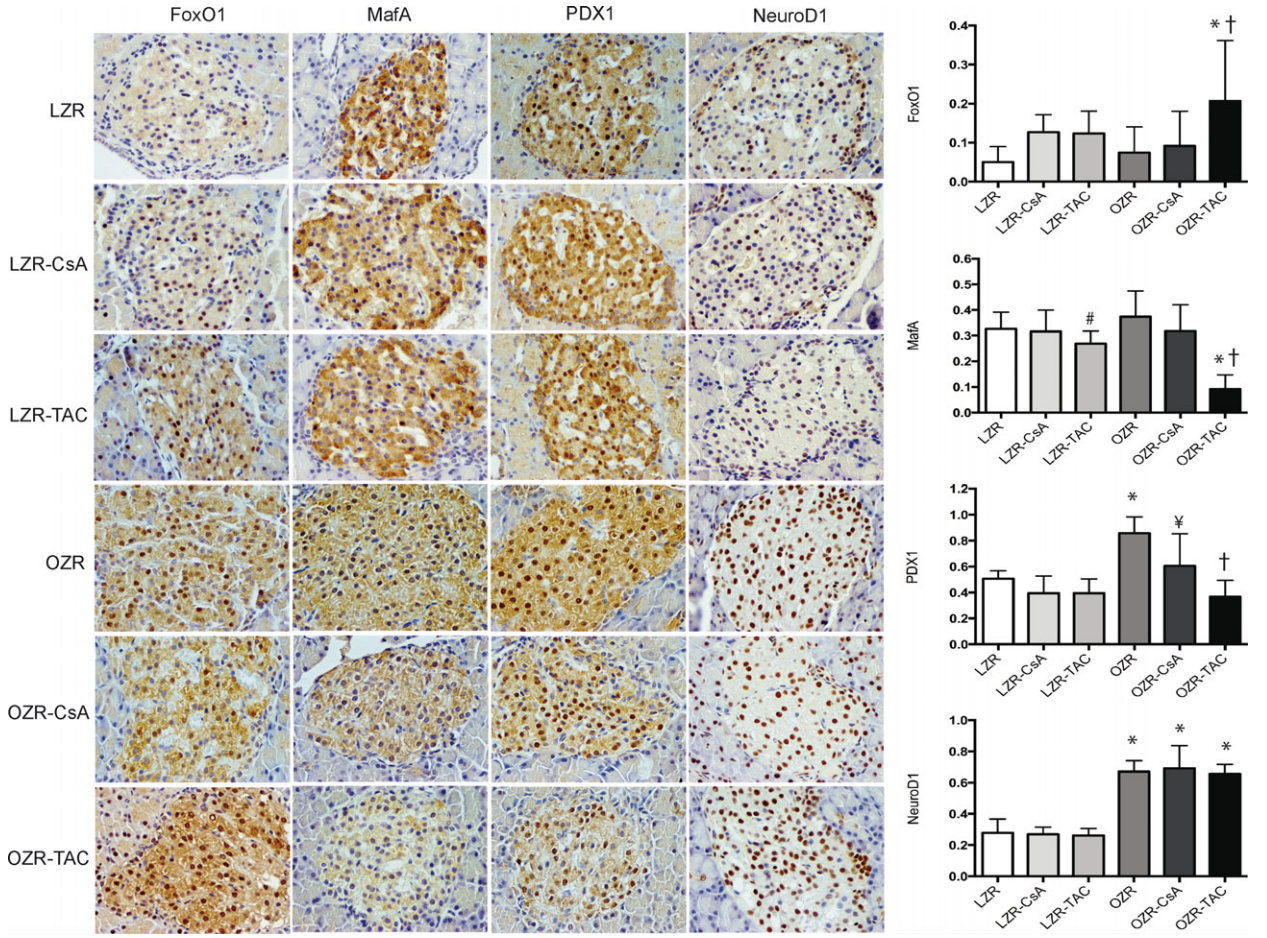

Figure 6: Effect of calcineurin inhibitor on pancreatic islets in lean Zucker rats (LZRs) and obese Zucker rats (OZRs). Representative images obtained for the different treatments and transcription factors in pancreata from animals. Histograms representing the proportion of hematoxylin/Diaminibenzidine staining for forkhead box protein O1 (FoxO1), v-maf musculoaponeurotic fibrosarcoma oncogene homolog A (MafA), pancreatic and duodenal homeobox 1 (PDX-1), and neuronal differentiation 1 (NeuroD). Each group had five animals, and five islets per animal were analyzed. Data are expressed as mean plus or minus standard deviation. *$p < 0.0001$ versus LZR, versus LZR-CsA, versus LZR-TAC. †$p < 0.001$ versus OZR, versus OZR-CsA. ¥$p < 0.01$ versus LZR-CsA, versus LZR-TAC. #$p < 0.01$ versus OZR.

indicated an interaction between insulin resistance and TAC. This hypothesis was further tested in OZRs and LZRs (13). Importantly, only obese animals developed diabetes with CNIs: all of those treated with TAC and only 40% of those with CsA. The fact that cells treated with TAC in a context of insulin resistance showed reduced insulin content and secretion, with important changes in relevant factors like FoxO1 and MafA, confirmed our previous results in rodents. Finally, the changes in FoxO1 and MafA induced by TAC in the context of glucolipotoxicity were dose dependent (Figure S5), which may indicate that lower doses can be less toxic to β cells. This may have important clinical implications.

We did not appreciate differences in apoptosis (caspase 3) between treatments in INS-1 cells; therefore, CNIs may not induce β cell death, which is in line with the reversibility of cell changes after TAC withdrawal or conversion to CsA observed in our study. At 5 days after drug withdrawal, nuclear levels of FoxO1 and MafA returned to their previous state. Interestingly, the conversion from TAC to CsA for 5 days produced similar recovery in nuclear FoxO1 and MafA. Similar results were observed in our previous study in animals (13). Consequently, β cells are resilient to damage induced by CNIs in both *in vitro* and *in vivo* studies. These results are in line with studies in humans in which PTDM was reduced to 42% after switching from TAC to CsA, even after 1 year of treatment (40,41). Finally, the reversibility observed after the conversion from TAC to CsA may indicate different mechanisms between both drugs besides the common calcineurin inhibitory capacity.

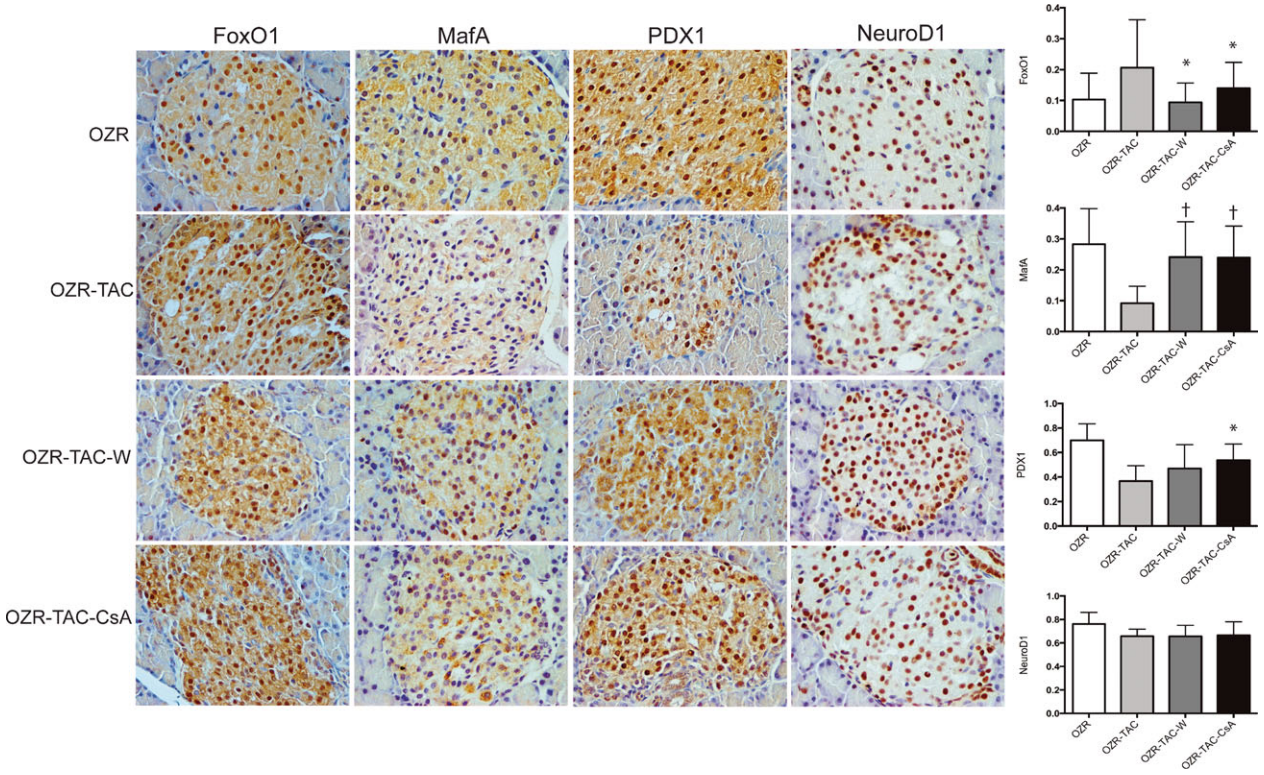

Figure 7: Reversibility and conversion in obese Zucker rats (OZR). Representative images obtained for the different treatments and transcription factors in pancreata from animals. Histograms representing the proportion of hematoxylin/Diaminibenzidine staining for forkhead box protein O1 (FoxO1), v-maf musculoaponeurotic fibrosarcoma oncogene homolog A (MafA), pancreatic and duodenal homeobox 1 (PDX-1), and neuronal differentiation 1 (NeuroD). Each group had five animals, and five islets per animal were analyzed. Data are expressed as mean plus or minus standard deviation. *$p < 0.05$ versus OZR-TAC; †$p \leq 0.0001$ versus OZR-TAC. CsA, cyclosporine A; TAC, tacrolimus.

The limitations of this study are basically those derived from the use of a cellular and animal model and the reliability of extrapolating its results to humans. The INS-1 β cell line was obtained from rats, and although our results with INS-1 and with Zucker rats are similar, caution is needed when trying to interpret these results in a human context.

In conclusion, TAC potentiates the damage of insulin resistance in β cells. This includes changes in transcription factors and in insulin secretion and content. CsA did not induce similar alterations. Consequently, the interaction between TAC and insulin resistance may explain the different diabetogenic effect between CNIs. The inhibition of calcineurin and NFAT was comparable for TAC and CsA, which may indicate a common pathway of β cell damage; however, the effect on calcineurin and NFAT may not explain the greater diabetogenic effect of TAC compared with CsA. TAC-specific pathways need further study, which may help prevent PTDM.

Acknowledgments

The authors would like to acknowledge the IMBRAIN (CIBICAN) project (FP7-RE6-POT- 2012-CT2012-31637-IMBRAIN) funded under the 7th Frameworks Programme (capacities); the Instituto de Salud Carlos III (ISCIII) for the following grants: PI13/00149, the REDINREN RD16/0009/0031 and PI16-02151; RyC-2014-16573), FEDER funds, and finally to Mads Hornum for a critical review or the manuscript.

Disclosure

The authors of this manuscript have no conflicts of interest to disclose as described by the *American Journal of Transplantation*.

References

1. Yates CJ, Fourlanos S, Hjelmesaeth J, Colman PG, Cohney SJ. New-onset diabetes after kidney transplantation changes and challenges. Am J Transplant 2012; 12: 820–828.

2. Porrini EL, Díaz JM, Moreso F, et al. Clinical evolution of post-transplant diabetes mellitus. Nephrol Dial Transplant 2016; 31: 495–505. https://doi.org/10.1093/ndt/gfv368. Epub 2015 Nov 3.
3. Davidson J, Wilkinson A, Dantal J, et al. New-onset diabetes after transplantation: 2003 International consensus guidelines. Proceedings of an international expert panel meeting. Barcelona, Spain, 19 February 2003. Transplantation 2003; 75(10 Suppl): SS3–SS24.
4. Sharif A, Hecking M, de Vries AP, et al. Proceedings from an international consensus meeting on posttransplantation diabetes mellitus: Recommendations and future directions. Am J Transplant 2014; 14: 1992–2000.
5. Cosio FG, Pesavento TE, Kim S, Osei K, Henry M, Ferguson RM. Patient survival after renal transplantation: IV. Impact of post-transplant diabetes. Kidney Int 2002; 62: 1440–1446.
6. Valderhaug TG, Hjelmesæth J, Hartmann A, et al. The association of early post-transplant glucose levels with long-term mortality. Diabetologia 2011; 54: 1341–1349.
7. Woodward RS, Schnitzler MA, Baty J, et al. Incidence and cost of new onset diabetes mellitus among U.S. wait-listed and transplanted renal allograft recipients. Am J Transplant 2003; 3: 590–598.
8. Kasiske BL, Snyder JJ, Gilbertson D, Matas AJ. Diabetes mellitus after kidney transplantation in the United States. Am J Transplant 2003; 3: 178–185.
9. Van Hooff JP, Christiaans MH, van Duijnhoven EM. Tacrolimus and posttransplant diabetes mellitus in renal transplantation. Transplantation 2005; 79: 1465–1469.
10. Sharif A, Shabir S, Chand S, Cockwell P, Ball S, Borrows R. Meta-analysis of calcineurin-inhibitor-sparing regimens in kidney transplantation. J Am Soc Nephrol 2011; 22: 2107–2118. https://doi.org/10.1681/ASN.2010111160.
11. Sharif A, Baboolal K. Risk factors for new-onset diabetes after kidney transplantation. Nat Rev Nephrol 2010; 6: 415–423. https://doi.org/10.1038/nrneph.2010.66.
12. Porrini E, Delgado P, Alvarez A, et al. The combined effect of pre-transplant triglyceride levels and the type of calcineurin inhibitor in predicting the risk of new onset diabetes after renal transplantation. Nephrol Dial Transplant 2008; 23: 1436–1441.
13. Rodriguez-Rodriguez AE, Triñanes J, Velazquez-Garcia S, et al. The higher diabetogenic risk of tacrolimus depends on pre-existing insulin resistance. A study in obese and lean Zucker rats. Am J Transplant 2013; 13: 1665–1675.
14. Rodriguez-Rodriguez AE, Triñanes J, Porrini E, et al. Glucose homeostasis changes and pancreatic β-cell proliferation after switching to cyclosporin in tacrolimus-induced diabetes mellitus. Nefrologia 2015; 35: 264–272.
15. Kasuga M. Insulin resistance and pancreatic beta-cell failure. J Clin Invest 2006; 116: 1756–1760.
16. Cerf ME. Beta Cell Dysfunction and Insulin Resistance. Front Endocrinol (Lausanne) 2013; 4: 37. https://doi.org/10.3389/fendo.2013.00037.
17. Alejandro EU, Gregg B, Blandino-Rosano M, Cras-Méneur C, Bernal-Mizrachi E. Natural history of β-cell adaptation and failure in type 2 diabetes. Mol Aspects Med 2015; 42: 19–41. https://doi.org/10.1016/j.mam.2014.12.002.
18. Okamoto H, Hribal ML, Lin HV, Bennett WR, Ward A, Accili D. Role of the forkhead protein FoxO1 in beta cell compensation to insulin resistance. J Clin Invest 2006; 116: 775–782.
19. Buteau J, Accili D. Regulation of pancreatic β-cell function by the forkhead protein FoxO1. Diabetes Obes Metab 2007; 9 (Suppl 2): 140–146.
20. Kaneto H, Matsuoka TA, Katakami N, Matsuhisa M. Combination of MafA, PDX-1 and NeuroD is a useful tool to efficiently induce insulin-producing surrogate β-cell s. Curr Med Chem 2009; 16: 3144–3151.
21. Guo S, Dai C, Guo M, et al. Inactivation of specific β cell transcription factors in type 2 diabetes. J Clin Invest 2013; 123: 3305–3316. https://doi.org/10.1172/JCI65390.
22. Matsuoka TA, Kaneto H, Kawashima S, Miyatsuka T, Tochino Y, Yoshikawa A. Preserving Mafa expression in diabetic islet β-cells improves glycemic control *in vivo*. J Biol Chem 2015; 290: 7647–7657. https://doi.org/10.1074/jbc.M114.595579.
23. Malecki MT, Jhala US, Antonellis A, et al. Mutations in NeuroD1 are associated with the development of type 2 diabetes mellitus. Nat Genet 1999; 23: 323–328.
24. Heit JJ, Apelqvist AA, Gu X, et al. Calcineurin/NFAT signalling regulates pancreatic beta-cell growth and function. Nature 2006; 443: 345–349.
25. Zhou L, Cai X, Han X, Ji L. P38 plays an important role in glucolipotoxicity-induced apoptosis in INS-1 cells. J Diabetes Res 2014; 2014: 834528.
26. Sargsyan E, Bergsten P. Lipotoxicity is glucose-dependent in INS-1E cells but not in human islets and MIN6 cells. Lipids Health Dis 2011; 10: 115.
27. Higa M, Shimabukuro M, Shimajiri Y, Takasu N, Shinjyo T, Inaba T. Protein kinase B/Akt signalling is required for palmitate-induced β-cell lipotoxicity. Diabetes Obes Metab 2006; 8: 228–233.
28. Fu Y, Tian W, Pratt EB, et al. Down-Regulation of ZnT8 expression in INS-1 rat pancreatic beta cells Reduces insulin content and glucose-inducible insulin secretion. PLoS ONE 2009; 4: e5679.
29. Kawamori D, Kaneto H, Nakatani Y, et al. The forkhead transcription factor Foxo1 bridges the JNK pathway and the transcription factor PDX-1 through its intracellular translocation. J Biol Chem 2006; 281: 1091–1098.
30. Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. Anal Biochem 1987; 162: 156–159.
31. Prentki M, Nolan CJ. Islet beta cell failure in type 2 diabetes. J Clin Invest 2006; 116: 1802–1812.
32. Andrali SS, Sampley ML, Vanderford NL, Ozcan S. Glucose regulation of insulin gene expression in pancreatic beta-cells. Biochem J 2008; 415: 1–10. https://doi.org/10.1042/BJ20081029.
33. Chakkera HA, Kudva Y, Kaplan B. Calcineurin inhibitors: Pharmacologic mechanisms impacting both insulin resistance and insulin secretion leading to glucose dysregulation and diabetes mellitus. Clin Pharmacol Ther 2017; 101: 114–120.
34. Chakkera HA, Mandarino LJ. Calcineurin inhibition and new-onset diabetes mellitus after transplantation. Transplantation 2013; 95: 647–652. https://doi.org/10.1097/TP.0b013e31826e592e.
35. Øzbay LA, Smidt K, Mortensen DM, Carstens J, Jørgensen KA, Rungby J. Cyclosporin and tacrolimus impair insulin secretion and transcriptional regulation in INS-1E beta-cells. Br J Pharmacol 2011; 162: 136–146. https://doi.org/10.1111/j.1476-5381.2010.01018.x.
36. Lawrence MC, Bhatt HS, Easom RA. NFAT regulates insulin gene promoter activity in response to synergistic pathways induced by glucose and glucagon-like peptide-1. Diabetes 2002; 51: 691–698.
37. Demozay D, Tsunekawa S, Briaud I, Shah R, Rhodes CJ. Specific glucose-induced control of insulin receptor substrate-2 expression is mediated via Ca2 + -dependent calcineurin/NFAT

signaling in primary pancreatic islet β-cells. Diabetes 2011; 60: 2892–2902.
38. Sigal NH, Lin CS, Siekierka JJ. Inhibition of human T-cell activation by FK 506, rapamycin, and cyclosporine A. Transplant Proc 1991; 23(2 Suppl 2): 1–5.
39. Mattila PS. The actions of cyclosporin A and FK506 on T-lymphocyte activation. Biochem Soc Trans 1996; 24: 45–49.
40. Ghisdal L, Bouchta NB, Broeders N, et al. Conversion from tacrolimus to cyclosporine A for new-onset diabetes after transplantation: A single-centre experience in renal transplanted patients and review of the literature. Transpl Int 2008; 21: 146–151.
41. Wissing K, Kuypers D, Abramowicz D, et al. Conversion from tacrolimus to cyclosporine improves glucose metabolism in patients with new-onset diabetes after transplantation: Interim analysis of a prospective and randomized study. Abstract, ESOT-Vienna 2013, ref. 2314754.

Supporting Information

Additional Supporting Information may be found in the online version of this article.

Figure S1: Experimental design and timeline. (A) β Cells in culture. Metabolic stressors: cells growing for 5 days with glucose or/and palmitate. Calcineurin inhibitors (CNIs) alone: cyclosporine A (CsA) or tacrolimus (TAC) added for 2 days to cells growing in maintenance medium. Metabolic stressors and CNIs: Cells growing for 3 days with high glucose and/or palmitate, and then CsA or TAC was added for another 2 days. TAC withdrawal or conversion to CsA: Cells growing for 3 days with high glucose and palmitate, TAC was added for 2 more days and then withdrawn or converted to CsA. (B) Pancreata of obese and lean Zucker rats: Animals were treated for 11 days with an intraperitoneal dose of vehicle (phosphate-buffered saline), CsA (2.5 mg/kg), or TAC (0.3 mg/kg)s and pancreata were extracted at day 12. TAC withdrawal and conversion to CsA: At day 12, two subgroups were changed to vehicle or CsA, respectively, for 5 more days, and pancreata were extracted at day 17.

Figure S2: Effect of the metabolic treatments and calcineurin inhibitors on the expression of specific *Ins1* gene (A) and *Ins2* gene (B) (n = 3). Data are expressed as mean plus or minus standard deviation. *Versus C, $p \leq 0.0001$; versus GP, $p = 0.043$; versus GPCsA, $p \leq 0.05$. #Versus C, $p = 0.008$. ∫Versus C, $p \leq 0.0001$. †Versus C, $p \leq 0.0001$; versus GPCsA, $p = 0.012$. C, control; CsA, cyclosporine A; G, glucose; P, palmitate.

Figure S3: Calcineurin activity and expression of nuclear factor of activated T cells 2 (NFAT2) in cells on tacrolimus (TAC) or cyclosporine A (CsA). (A) Calcineurin phosphatase activity assay. Calcineurin activity was adjusted to total protein concentration (n = 3). *Versus control (C), $p \leq 0.05$; #Versus TAC 15 and TAC 30, $p \leq 0.05$. (B) Western blot analysis of nuclear NFAT2 and histone H3 on INS-1 cells. Treatment: C; CsA 25, 250, and 500 ng/mL; TAC 5, 15, and 30 ng/mL; n = 3; not significant.

Figure S4: Effect of the metabolic treatments and calcineurin inhibitors in cleaved caspase 3 in INS-1 cells. Histogram representing the nuclear localization of active caspase 3. Representative microphotography for each treatment.

Figure S5: Effect of different concentrations of tacrolimus (TAC) in the nuclear localization of forkhead box protein O1 (FoxO1) and v-maf musculoaponeurotic fibrosarcoma oncogene homolog A (MafA) in cells growing in 22 mM glucose and 100 μM palmitate. Data are expressed as mean plus or minus standard deviation. *$p < 0.05$ and †$p < 0.01$ versus GP5TAC. G, Glucose; P, palmitate; TAC 5, 15, 30 ng/mL.