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Role of activating transcription factor 3 in low glucose- and thapsigargin-induced apoptosis in cultured mouse islets

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ABSTRACT

In vitro, survival and function of rat pancreatic β-cells are optimally preserved in the presence of 10 mmol/l glucose (G10) and markedly altered by prolonged culture at either 2 mmol/l glucose (G2) or 30 mmol/l glucose (G30). The increase in islet cell apoptosis in G2 and G30 vs. G10 is preceded by parallel increases in the mRNA levels of the integrated stress response (ISR) gene activating transcription factor 3 (*Atf3*) and its putative target and proapoptotic gene growth arrest- and DNA damage-inducible gene 153 (*Gadd153*/*Chop*). In this study, we used islets from *Atf3* knockout ($Atf3^{-/-}$) mice to test the role of ATF3 in the stimulation of islet cell apoptosis under conditions associated with ISR activation. The glucose sensitivity of $Atf3^{-/-}$ and WT islets for the stimulation of insulin secretion and *Xbp1* mRNA splicing during 18 h culture was similar, demonstrating that glucose metabolism was unaffected by *Atf3* deletion. However, the stimulation of islet cell apoptosis by the SERCA pump inhibitor thapsigargin was slightly but significantly reduced in $Atf3^{-/-}$ vs. WT islets despite similar level of expression of *Gadd153* and *Gadd34* mRNA. Also, the stimulation of islet cell apoptosis by 7 days of culture in G2 was slightly but significantly reduced in $Atf3^{-/-}$ vs. WT islets, and this effect was accompanied by a significant reduction in *Gadd153* mRNA expression. In conclusion, the increase in *Atf3* gene expression induced by thapsigargin and low glucose concentrations slightly contributes to the stimulation of islet cell apoptosis under these culture conditions.

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1. Introduction

Glucose is the main physiological stimulus of insulin secretion by pancreatic β-cells. In addition, glucose stimulates β-cell gene expression, protein synthesis, proliferation and growth, thereby contributing to the long-term maintenance of the functional β-cell mass [3]. *In vitro*, glucose-stimulated insulin secretion (GSIS) and survival of rat β-cells are optimally preserved during culture in the presence of 10 mmol/l glucose (G10) whereas they are markedly altered at either higher (30 mmol/l or G30) or lower (2 mmol/l or G2) glucose concentrations [2]. Interestingly, the mRNA levels of integrated stress response (ISR) genes follow an asymmetric V-shaped profile similar to that of apoptosis, suggesting that they could play a role in this deleterious effect of extreme glucose concentrations.

The ISR results from the phosphorylation of eukaryotic initiation factor 2α by eIF2α kinases under various types of stress, including endoplasmic reticulum (ER) stress, amino acid deprivation, virus infection and heme deficiency [9,10,20]. This ISR is characterized by increased expression of Activating Transcription Factor 4 (*Atf4*)-target genes, such as the proapoptotic transcription factor *Atf3*, its potential target growth arrest- and DNA damage-inducible gene 153 (*Gadd153* or *Chop*) and *Gadd34* [6,13,19,21,22].

The *Atf3* gene encodes a member of the ATF/CREB family of transcription factors that share the basic region/leucine zipper DNA binding motif and bind to the ATF/CRE consensus sequence TGACGTCA [7]. Interestingly, *Atf3* expression is greatly increased in various cell types including rodent β-cells exposed to stress signals such as cytokines, nutrient deprivation, serum stimulation and calcium signaling, and this increase has been proposed to contribute to β-cell apoptosis in a number of studies [1,8,11,11,17,24]. We therefore tested whether *Atf3* plays a role in the stimulation of mouse islet cell apoptosis under conditions associated with ISR activation.

2. Materials and methods

2.1. Animals

Atf3 knockout ($Atf3^{-/-}$) [11] and C57BL6/J wild type (WT) mice were bred under standard conditions. They were matched for sex and age (8–18 months) within each experiment.

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2.2. Islet isolation and culture

Pancreatic islets from *Atf3*$^{-/-}$ and WT mice were isolated by collagenase digestion of the pancreas followed by density gradient centrifugation as described [15], except for the volume (2 ml) and concentration (1 mg/ml) of the collagenase solution used to inflate the pancreas. They were precultured for one week at 37 °C in the presence of 5% CO_2 in serum-free RPMI 1640 medium (Invitrogen, Carlsbad, CA) containing G10, 5 g/l bovine serum albumin (fraction V; Roche Diagnostics, Mannheim, Germany), 100 IU/ml penicillin and 100 μg/ml streptomycin. Islets were then cultured for 18 h to 7 days in the same medium containing G2, G10 or G30 or G10 + 1 μmol/l thapsigargin (TG) (Sigma, St. Louis, MO, USA) and processed for further analysis. All experiments were approved by the local ethics committee for animal experimentation (Projects 2004/UCL/MD/003 and UCL/MD/2009/009) and performed according to the Principles of Laboratory Animal Care.

2.3. Measurement of insulin secretion and islet insulin content

Insulin concentration was measured in the culture media and in total islet extracts by RIA using rat insulin as standard [12].

2.4. Morphological analysis

Islets were fixed for 4 h in Bouin's fluid and embedded in paraffin. Four μm thick sections were then processed for hematoxylin and eosin staining. Images from these sections were acquired with an Axioplan microscope coupled to an Axiocam HRc digital camera (using fixed camera settings) and analyzed with Axiovision 3.1 software (Carl Zeiss, Oberkochen, Germany).

2.5. Measurement of islet gene mRNA levels

Islet total RNA was extracted and reversed transcribed into cDNA as previously described [6]. Real-time PCR was performed with an iCycler iQ Real-Time PCR Detection System (Bio-Rad, Hercules, CA). Primers sequences and reactions conditions are shown in Suppl. Table 1.

2.6. Measurement of Xbp1 mRNA splicing

cDNAs corresponding to unspliced and spliced *Xbp1* mRNA (NM_001004210) were amplified simultaneously with TATA-box binding protein (*Tbp*) cDNA by duplex PCR using the Amplitaq Gold kit (Applied Biosystems, Foster City, CA, USA) (primers sequences in Suppl. Table 2). The amplicons were then separated by electrophoresis on an Agilent 2100 bioanalyzer using the Agilent DNA 1000 Assay kit (Agilent Technologies, Waldbronn, Germany). The spliced/total *Xbp1* mRNA ratio was calculated as an indicator of *Xbp1* mRNA splicing.

2.7. Measurement of islet cell apoptosis

Cytosolic histone-associated DNA fragments in islet cells were measured with the Cell Death Detection ELISA^{PLUS} kit (Roche Diagnostics, Mannheim, Germany) following manufacturer's instructions. Briefly, cytoplasmic DNA fragments (mono- and oligonucleosomes) were separated from nuclear DNA and measured using a mixture of anti-histone-biotin and anti-DNA-Peroxidase (POD) antibodies in streptavidin-coated wells. After addition of POD-substrate, absorbance of each sample was measured at 405 nm (reference wavelength: 490 nm). The data were normalized to the absorbance of the positive control provided in the kit and corrected for differences in islet DNA content measured in the nuclear fraction by fluorimetry using SYBR Green I [18].

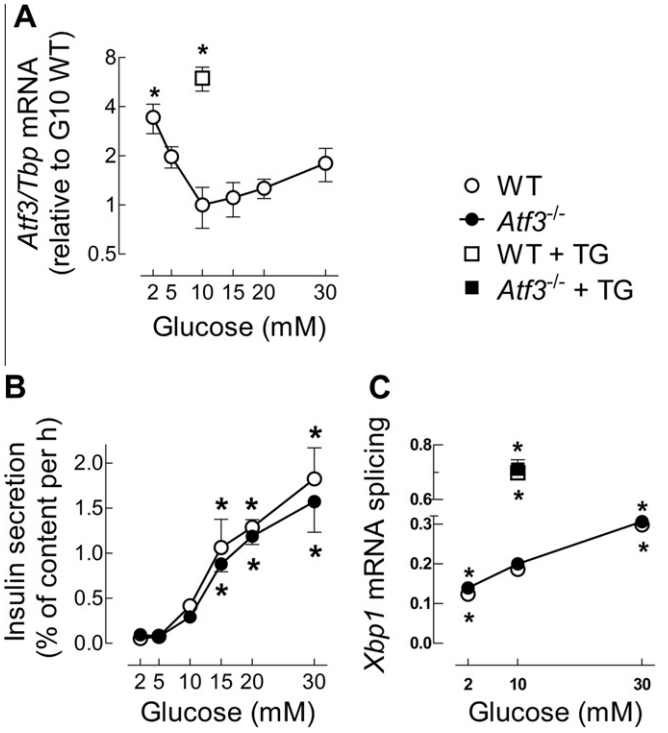

Fig. 1. Effects of glucose and thapsigargin on *Atf3* mRNA expression (A), glucose-stimulated insulin secretion (B) and *Xbp1* mRNA splicing (spliced/total *Xbp1* mRNA levels) (C) in islets from WT and *Atf3*$^{-/-}$ mice – islets isolated from Wild-Type (WT) mice (white symbols) and *Atf3*$^{-/-}$ mice (black symbols) were precultured for 1 week in serum-free RPMI medium containing G10 and 5 g/l BSA and then cultured for 18 h in the presence of G2, G5, G10, G15, G20 and G30 (A and B) or in the presence of G2, G10 and G30 or G10 + 1 μmol/l thapsigargin (TG) (C). (A) *Atf3* to *Tbp* mRNA ratio was measured by real-time PCR and expressed relative to the mRNA ratio in WT islets cultured in G10. *Atf3* mRNA was not detectable in islets from *Atf3*$^{-/-}$ mice (*Atf3*: threshold cycle (C_t) = 30.75 in WT islets cultured in G10, C_t > 40 in *Atf3*$^{-/-}$ islets; *Tbp* : C_t = 28.6 and 28.1 in WT and *Atf3*$^{-/-}$ islets cultured in G10). (B) Insulin secretion was measured and expressed as a percentage of the islet insulin content at the end of culture (mean ± SEM of insulin content in WT islets: 57.3 ± 3.1 ng/islet; mean ± SEM of insulin content in *Atf3*$^{-/-}$ islets: 62.4 ± 4.5 ng/islet). (C) Spliced/total *Xbp1* mRNA ratio were computed as an indicator of *Xbp1* mRNA splicing and expressed relative to the ratio in WT islets cultured in G10. Results are means ± SEM for three experiments. *P < 0.05 vs. G10 by two-way ANOVA and a post-test of Bonferroni.

The percentage of apoptotic islet cells was also determined using the *In Situ* Cell Death Detection Kit (Roche Diagnostics, Mannheim, Germany). DNA strand breaks (of mono- and oligonucleosomes as well as single strand breaks) were identified by labeling free 3'-OH termini with fluorescein-dUTP using Terminal deoxynucleotidyl transferase (TUNEL reaction). Reactions were performed on 4 μm-thick sections of paraffin-embedded islets. Islet cell nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI). The percentage of apoptotic cells was determined by counting fluorescein- and DAPI-positive nuclei on digital images obtained by fluorescence microscopy (FluoArc mounted on an Axioskop 40 coupled to a HBO 100 camera; Carl Zeiss, New York, NY) under standardized conditions (fluorescein: excitation/emission 475/540 nm; DAPI: excitation/emission 350/460 nm).

2.8. Statistical analysis

Results are means ± SEM for the indicated number of experiments. Statistical significance of differences between groups was assessed by two-way ANOVA followed by a test of Bonferroni. Differences were considered significant if P < 0.05.

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