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Prolactin protects against cytokine-induced beta-cell death by NF κ B and JNK inhibition

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Abstract

Type 1 diabetes is caused by an autoimmune assault that induces progressive beta-cell dysfunction and death. Pro-inflammatory cytokines, such as interleukin 1 beta (IL1B), tumor necrosis factor (TNF) and interferon gamma (IFNG) contribute for beta-cell death, which involves the activation of the nuclear factor kappa B (NF κ B) and c-Jun N-terminal kinase (JNK). Prolactin (PRL), a physiological mediator for beta-cell proliferation, was shown to protect beta cells against cytokines pro-apoptotic effects. We presently investigated the mechanisms involved in the protective effects of prolactin against cytokine-induced beta-cell death. The findings obtained indicate that STAT3 activation is involved in the anti-apoptotic role of PRL in rat beta cells. PRL prevents the activation of JNK via AKT and promotes a shift from expression of pro- to anti-apoptotic proteins downstream of the JNK cascade. Furthermore, PRL partially prevents the activation of NF κ B and the transcription of its target genes *I κ B α* , *Fas*, *Mcp1*, *A20* and *Cxcl10* and also decreases NO production. On the other hand, the pro-survival effects of PRL do not involve modulation of cytokine-induced endoplasmic reticulum stress. These results suggest that the beneficial effects of PRL in beta cells involve augmentation of anti-apoptotic mechanisms and, at the same time, reduction of pro-apoptotic effectors, rendering beta cells better prepared to deal with inflammatory insults. The better understanding of the pro-survival mechanisms modulated by PRL in beta cells can provide tools to prevent cell demise during an autoimmune attack or following islet transplantation.

Key Words

- ▶ NF κ B
- ▶ pancreatic beta cells
- ▶ pro-inflammatory cytokines
- ▶ prolactin
- ▶ STAT3

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Introduction

Type 1 diabetes (T1D) is caused by an autoimmune assault that induces progressive beta-cell dysfunction and apoptosis, consequently impairing insulin secretion. During insulinitis, pro-inflammatory cytokines such as

interleukin 1 beta (IL1B), tumor necrosis factor (TNF) and interferon gamma (IFNG), contribute to beta-cell failure and death. At the time of diagnosis, it has been estimated a 70–80% loss of beta-cell functional mass

(Kukreja & Maclaren 1999, Noble 2015). Beta-cell loss in T1D is probably slow and progressive, suggesting that preventing beta-cell death is possible.

It has been previously shown that prolactin (PRL) has beneficial effects on pancreatic islets during pregnancy or following *in vitro* exposure (Boschero *et al.* 1993, Crepaldi *et al.* 1997, Cunha *et al.* 2007). Activation of the PRL receptor is important to increase beta-cell mass and maintain physiological glucose tolerance during pregnancy (Amaral *et al.* 2004), and decrease the expression of pro-apoptotic genes (Bordin *et al.* 2004). These biological effects require the participation of receptor-associated kinases, mainly through the JAK/STAT pathway (Bole-Feysot *et al.* 1998).

PRL protects human islets against inflammatory cytokine-mediated apoptosis, and this seems to be at least in part mediated by inhibiting the cell death pathways controlled by the pro-apoptotic BCL2 family members (Terra *et al.* 2011). The members of this family of proteins are grouped into the following three categories: pro-survival (BCL2, BCLx1, MCL1, BCLw and A1), pro-apoptotic (BAX, BAK and BOK) and BH3-only proteins, which are also pro-apoptotic and divided in sensitizers (DP5, BIK, NOXA and BAD) and activators (BIM, PUMA and BID) (Kim *et al.* 2006). When the expression of BH3-only sensitizers increases, they directly bind and inhibit the pro-survival members, releasing the activators. Once free, the activators bind and activate the pro-death members to form pores in the mitochondria, inducing cytochrome c release (Kim *et al.* 2006). In beta cells, JNK is also involved in the activation of the mitochondrial pathway of apoptosis through cJUN phosphorylation, DP5 expression and downregulation of MCL1 (Gurzov *et al.* 2008, 2009, Allagnat *et al.* 2011).

Many of the genes modified by cytokines in beta cells are regulated by the transcription factor nuclear factor kappa B (NFκB), which, directly or indirectly, controls the expression of proteins involved in the endoplasmic reticulum (ER) stress (via NO production in the case of rat beta cells) and cell death (Cardozo *et al.* 2001a, Darville *et al.* 2004). The canonical process of NFκB activation is initiated by the phosphorylation of the inhibitor of kappa B (IκB), leading to ubiquitination and proteasome degradation. This allows the nuclear translocation of NFκB and increases the transcription of target genes (Chiu *et al.* 2009). The transcriptional activity of NFκB can also be regulated by post-translational mechanisms (Vermeulen *et al.* 2002, Hayden & Ghosh 2008). Moreover, pro-inflammatory cytokines lead to a decreased expression of the PRL receptor, reducing the capacity of the beta-cell to recover after an autoimmune assault (Kutlu *et al.* 2003,

Ortis *et al.* 2010), but the nature of the crosstalk between PRL signaling and the inflammatory response regulated by NFκB (or other pathways) induced by cytokines remain to be clarified.

We have presently identified novel specific targets of PRL in beta cells in the context of inflammation. The findings obtained indicate that PRL exerts an anti-apoptotic role in rat beta cells exposed to pro-inflammatory cytokines potentially through STAT3. PRL induces AKT phosphorylation and consequentially prevents the cytokine-dependent JNK activation, promoting the differential expression of anti- and pro-apoptotic proteins downstream of the JNK cascade. Moreover, PRL partially prevents the activation of NFκB and the transcription of its target genes *IκBa*, *Fas*, *Mcp1*, *A20* and *Cxcl10*.

Materials and methods

Culture of rat islets and INS-1E cells

Wistar rats were used according to the rules of the Brazilian Regulations for Animal Care with approval of the Ethical Committee for Animal Experiments of the UNICAMP, Campinas, Brazil. Pancreatic islets were isolated from adult female Wistar rats as described (Vanzela *et al.* 2010). The islets were maintained in RPMI-1640 culture medium containing 5.6 mmol/L glucose and 10% v/v of fetal bovine serum (FBS) for 24 h prior to treatment. Insulin-producing INS-1E cells (Asfari *et al.* 1992, Janjic *et al.* 1999), a kind gift from Prof. C Wolheim (Centre Médical Universitaire, Geneva, Switzerland), were cultured in RPMI-1640 medium and supplemented with 5% v/v of FBS, HEPES 10 mmol/L, penicillin 100 U/mL, streptomycin 100 µg/mL, sodium pyruvate 1 mmol/L and 2-mercaptoethanol 50 µmol/L in the presence of 11 mmol/L glucose.

Cell treatment and NO measurement

Rat islets were cultured in RPMI medium; containing 5.6 mmol/L glucose, 1% v/v of FBS and 5% w/v of BSA, with 0.5 µg/mL of PRL or vehicle (0.01 mmol/L NaHCO₃ + 0.3% w/v BSA) for 24 h, and then challenged with 10 U/mL of human recombinant IL1B (R&D Systems) and 1000 U/mL of rat recombinant IFNG (R&D Systems) for additional 48 h. For the treatment of INS-1E cells with PRL and cytokines, cells were maintained in a medium with 1% w/v of BSA and 5.6 mmol/L glucose without FBS. The experiments were performed after 24 h pre-treatment with PRL (0.5 µg/mL) or vehicle and 24-h subsequent exposure to 10 U/mL of human recombinant IL1B (R&D Systems)

or 1600 U/mL of rat recombinant TNF (Invitrogen) combined with 100 U/mL of rat recombinant IFNG (R&D Systems). PRL or vehicle were also kept in the medium during cytokine exposure in both INS-1E and rat islets. INS-1E cells exposed to IL1B+IFNG were also used in the time course studies. Nitric oxide (NO) was measured as accumulated nitrite in the medium by the Griess reaction (Green *et al.* 1982, Schulz *et al.* 1999). The Wortmannin (Sigma-Aldrich), a specific inhibitor of PI3 kinase, used to decrease the phosphorylation of AKT, was dissolved in DMSO and used in INS-1E cells at 250 nmol/L, based on dose-response experiments (data not shown).

Assessment of apoptosis

The percentage of apoptotic cells was determined by the following methods: (A) after 15 min incubation with the DNA-binding dyes Hoechst 33342 (5 µg/mL; Sigma-Aldrich) and propidium iodide (5 µg/mL; Sigma-Aldrich), the viable and apoptotic nuclei were counted by two independent observers, in which one of them was unaware of sample identity (Ortis *et al.* 2006); (B) by the Guava Nexin Reagent kit (Merck Millipore), in which cells positive for annexin V were detected by flow cytometry (Guava easyCyte 8HT) according to the manufacturer's protocol and (C) by Western blot for cleaved caspase 3 (see details below). The figures were expressed by control values, equal to one, to better visualize the cell death induction.

Western blot assay

After cell culture and treatment, the cells were lysed using a Laemmli sample buffer. The total protein was fractioned in an 8–12% SDS-PAGE and transferred to a nitrocellulose membrane. Immunoblot analysis was performed using the specific antibodies for the protein of interest: phospho-SAPK/JNK (Thr183/Tyr185), phospho-c-JUN (Ser63), cleaved CASPASE 3, phospho-STAT3, STAT3, PUMA (Cell Signaling); iNOS (Abcam); phospho-AKT (ser473), phospho-AKT (thr308) and IKBA (Santa Cruz), followed by the incubation with the appropriate horseradish peroxidase-conjugated secondary antibody (Thermo Scientific). The protein intensity was detected by an ImageQuant LAS 4000 (GE Healthcare Bio-Sciences) after the chemiluminescent reaction with the SuperSignal West Femto Chemiluminescent Substrate (Thermo Scientific), according to the manufacturer instructions. The intensity values for the proteins were quantified using ImageQuant TL 7.0 Software (GE Healthcare Bio-Sciences), corrected

by the housekeeping protein alpha tubulin (αTubulin) (Sigma-Aldrich) to confirm similar protein loading and normalized by the control condition. Notice that in this case, the control condition is considered to be one. When the protein is not induced in the control, no normalization was done (to avoid division by zero).

mRNA extraction and real time RT-PCR (qRT-PCR)

Poly(A)+mRNA was isolated from the treated cells using the Dynabeads mRNA DIRECT™ kit (Invitrogen). A constant amount of the purified mRNA was reverse-transcribed using 50 U MuLV reverse transcriptase (Invitrogen) at 42°C for 60 min in a reaction containing 1× Buffer (16 mmol/L (NH₄)₂SO₄, 67 mmol/L Tris-HCl pH 8.8 (at 25°C) and 0.01% Tween 20), 5 mmol/L MgCl₂ (GeneCraft, Cologne, Germany), 2 mmol/L dNTP mix (Eurogentec, Seraing, Belgium), 2.5 µmol/L random primers and 20 U RNase inhibitor (Invitrogen). The real-time RT-PCR amplification reactions were performed using an iQ SYBR Green Supermix on Rotor-Gene Q (Qiagen), and the concentration of the gene of interest was calculated as copies per microliter using a standard curve. Gene expression values in INS-1E cells were corrected by the housekeeping gene *Gapdh*.

Promoter reporter assay

Cells were co-transfected using Lipofectamine 2000 (Invitrogen) with the pRL-CMV internal control encoding Renilla (Promega) and the pNFκB-Luciferase (BD Biosciences) (Ortis *et al.* 2008). After transfection (16 h), the cells were treated as described earlier. The luciferase activity was analyzed using the Dual-Luciferase Reporter Assay System kit (Promega), according to the manufacturer instructions. The luciferase activity values were corrected by the values of the internal control pRL-CMV of each experiment.

Small interfering RNA (siRNA) treatment

Two different siRNAs against STAT3 (Sigma-Aldrich) were used to knock down the expression of the target gene. Allstars Negative Control siRNA (Qiagen) was used as a negative control. After overnight incubation with 30 nmol/L of siRNA using 1 µL per well Lipofectamine RNAiMAX, lipid reagent (Invitrogen), the transfection medium was replaced by a regular culture medium for cell recovery. After 48 h, the cells were exposed to treatment as described earlier.

Statistical analysis

For expression comparison analysis, we used a paired *t*-test on the log-transformed data. A nominal *P*-value threshold (without correction for multiple tests) of 0.05 was considered to be statistically significant. Notice that since we applied the paired *t*-test on the log-transformed data, the effects of data normalization by the control condition is only for visual inspection. For the statistical analysis, the normalization has no effect because the data are log transformed. For illustration, consider that we would like to test whether (with PRL/control)/(without PRL/control)=1. By applying the log-transform, it is equivalent to test $\log(\text{with PRL}) = \log(\text{without PRL})$. It can be tested by a *t*-test because the logarithm is a monotonic transformation. The choice for the *t*-test rather than the usual factorial analysis of variance is because of issues of heteroskedasticity.

Results

PRL prevents the apoptosis induced by pro-inflammatory cytokines

Exposure of INS-1E cells for 24 h to cytokines increased the prevalence of apoptotic cells (Cardozo *et al.* 2001a,

Ortis *et al.* 2006, Allagnat *et al.* 2011), which is confirmed by three different methods (Fig. 1A, B and C). The pre-treatment of these cells with PRL prevented cell death (Fig. 1A and B) and partially the cleavage of CASPASE 3 (Fig. 1C), which was still observed after 48 h of cytokines treatment. Of note, the beneficial effect of PRL was also observed in control cells that were not exposed to cytokines (Fig. 1A and B). Because the results were similar for both cytokine combinations, the subsequent experiments were performed using only IL1B+IFNG. Importantly, the pre-treatment with PRL was also efficient in preventing (by ~40%) the activation of CASPASE 3 induced by cytokines in rat islets (Fig. 1D).

STAT3 potentially mediates the beneficial effects of PRL

We next evaluated the impact of PRL on the expression of STAT3 (Bordin *et al.* 2004, Anhe *et al.* 2007) and its phosphorylation (DaSilva *et al.* 1996). The STAT3 phosphorylation was induced by PRL treatment as observed after 15 min to 8 h of PRL exposure (Fig. 2A and B), confirming the regulatory role of PRL on STAT3 activation. To test whether STAT3 was involved in the

Figure 1

PRL prevents apoptosis induced by pro-inflammatory cytokines in rat islets and INS-1E cells. INS-1E cells (A, B and C) or rat islets (D) were pre-cultured with a vehicle or PRL and then treated with IL1B or TNF in combination with IFNG for 24 h (INS-1E), 48 h (rat islets) or for the time point indicated in the figure. (A) Apoptosis determined by percentage of Annexin V-positive cells of 5 independent experiments, normalized for the respective control (considered as one), showed as mean $\pm$ S.E.M. (B) Percentage of apoptotic cells determined by DNA-binding dyes Hoechst and propidium iodide, $n=2$ (TNF) to $n=7$, normalized for the respective control (considered as one), showed as mean $\pm$ S.E.M. (C and D) Western blot for INS-1E cells (C) and rat islets (D) cleaved CASPASE 3. One representative Western blot of 3 (islets) to 5 (INS-1E) independent experiments is shown, graphs showed the values corrected by aTubulin and normalized for the respective control values (considered as one). Results are means $\pm$ S.E.M. * $P \leq 0.05$, ** $P < 0.01$ or *** $P < 0.001$ for log-transformed paired *t*-test of vehicle vs PRL.

beneficial effects of PRL, two specific siRNAs (siSTAT3-1 and siSTAT3-2) were used to suppress STAT3 in INS-1E cells. The first siRNA was not able to significantly decrease expression of STAT3 (Fig. 2C and D), with no significant effect on cell viability (Fig. 2E). A better knockdown (KD) (60% inhibition) was observed with the second siRNA, namely siSTAT3-2 (Fig. 2C and D), which abolished the prevention of apoptosis induced by PRL following exposure to cytokines (Fig. 2E).

PRL modifies the activation of JNK and decreases cJUN-dependent *Dp5* expression

One of the mechanisms by which cytokines contribute to beta-cell apoptosis is via JNK activation (Ammendrup et al. 2000), and consequent JNK/cJUN-dependent *Dp5* expression (Gurzov et al. 2009). Pre-treatment with PRL modified the pattern of JNK activation after exposure to cytokines in the continuous presence of PRL, with an early increase in the phosphorylation of JNK after 30 min, followed by a decrease in JNK phosphorylation after 8 and 24h, compared with IL1B+IFNG alone (Fig. 3A). In

addition, INS-1E cells pre-exposed to PRL and then treated with cytokines for 8 or 24h, in the continuous presence of PRL, displayed reduced phosphorylation of cJUN (8h) (Fig. 3B), a downstream indicator of JNK activity, and *Dp5* expression (24h) (Fig. 3C), suggesting that PRL-induced inhibition of JNK phosphorylation plays a key role in preventing beta-cell death.

PRL regulates the expression of pro- and anti-apoptotic BCL2 family members

We next evaluated the involvement of PRL in modulating the pro-death BH3-only proteins BIM and PUMA, known to be activated downstream of *Dp5* (Gurzov et al. 2009, Gurzov & Eizirik 2011). After 24h of treatment, IL1B+IFNG increased *Bim* (Fig. 4A) and *Puma* mRNA (Fig. 4C) and PUMA protein expression (Fig. 4D and E). Although PRL did not prevent cytokine-induced total *Bim* mRNA expression (Fig. 4A), it decreased the expression of its most pro-apoptotic isoform, namely *Bim* small (*BimS*), in untreated condition and marginally following cytokine condition (Fig. 4B). PRL also decreased cytokine-mediated PUMA expression at the mRNA

Figure 2

The STAT3 pathway is involved in PRL protective effects against cytokine-induced apoptosis. (A and B) INS-1E cells were treated with PRL for the time points indicated in the figure. Protein cell lysates were used in Western blot for phosphorylated STAT3 (pSTAT3) and aTubulin. (A) One representative Western blot of 3 independent experiments. (B) Optical density measurements of the blots corrected by aTubulin are expressed as mean $\pm$ s.e.m. and normalized by the control value in each experiment (considered as one). * $P < 0.05$ or ** $P < 0.01$ for log-transformed paired *t*-test of each time point vs control (time point zero). (C, D and E) INS-1E cells were transfected overnight with two specific siRNAs for STAT3 (siSTAT3-1 and siSTAT3-2) or a control siRNA (siCTL). 48h after transfection, cells were treated for 24h with vehicle or PRL, then with IL1B+IFNG for additional 24h. (C and D) Protein cell lysates were used in Western blot for STAT3 and aTubulin. (C) One representative Western blot of 3 independent experiments. (D) Optical density measurements of bands corresponding to STAT3 corrected by aTubulin and normalized by the control value in each experiment (considered as one) are expressed as mean $\pm$ s.e.m. * $P < 0.05$ for log-transformed paired *t*-test of each siRNA vs siCTL. (E) Apoptosis determined by percentage of annexin V positive cells of 4 independent experiments, results were normalized by the control value in each experiment (considered as one). Data are means $\pm$ s.e.m. * $P \leq 0.05$ or ** $P < 0.01$ for log-transformed paired *t*-test of vehicle vs PRL.

Figure 3

PRL modulates the activation of JNK/cJUN-dependent *Dp5* expression in INS-1E cells. INS-1E cells were treated for 24h with PRL or vehicle and then with IL1B+IFNG for 8h (B) or 24h (C) or for the time points indicated in the figure (A) in the continuous presence of PRL. Protein cell lysates were used in Western blot for phosphorylated JNK (pJNK), phosphorylated cJUN (pcJUN) and aTubulin. One representative Western blot of 7–8 independent experiments and optical density measurements of the blots for pJNK (A) and pcJUN (B), corrected by aTubulin and normalized by the control in each experiment (considered as one) are expressed as mean $\pm$ S.E.M. (C) The expression of *Dp5* was assessed in 4 independent experiments by qRT-PCR, corrected by the housekeeping gene *Gapdh* and expressed as mean $\pm$ S.E.M. * $P \leq 0.05$, ** $P < 0.01$ or *** $P < 0.001$ for log-transformed paired *t*-test of vehicle vs PRL at the same time point.

(Fig. 4C) and at protein levels (Fig. 4D and E). Importantly, PRL alone increased the expression of the pro-survival BCL-2 family protein BCLx1 in a time-dependent manner and also increased (30%) the expression of this protein after 8h of exposure to cytokines compared with cells treated with IL1B+IFNG alone (data not shown).

PRL-decreased JNK activation is dependent of AKT

PRL induces activation of AKT in pancreatic beta cells (Amaral *et al.* 2004), which was confirmed in our model

by the observed increased phosphorylation of both serine and threonine residues of AKT in PRL-treated INS-1E cells (Fig. 5A and B). Cytokines have been described to impair the signal transduction of this pro-survival pathway (Storling *et al.* 2005). In line with this, after 30min of IL1B+IFNG exposure there was a clear decrease in the phosphorylation of AKT at serine 473 in both vehicle- and PRL-treated cells (Fig. 5A). At later time points, however, PRL was able to maintain and even increase AKT phosphorylation in both residues in spite of the continuous presence of cytokines (Fig. 5A and B), which

Figure 4

PRL downregulates the expression of pro-apoptotic BCL2 family members. INS-1E cells were pretreated for 24h with vehicle or PRL and then with IL1B+IFNG for additional 24h in the continuous presence of PRL. The expression of (A) *Bim*, (B) *Bim small (BimS)* and (C) *Puma* was assessed by qRT-PCR and corrected by the housekeeping gene *Gapdh*. The data represent the means $\pm$ S.E.M. of 4 independent experiments. (D and E) PUMA expression was also analyzed by Western blot. (D) One representative Western blot of 4 independent experiments, (E) optical density measurements of bands corresponding to PUMA corrected by aTubulin and normalized by the control in each experiment (considered as one) are expressed as mean $\pm$ S.E.M. * $P < 0.05$ or ** $P < 0.01$ for log-transformed paired *t*-test of vehicle vs PRL.

Figure 5

PRL upregulates the phosphorylation of AKT, which contributes for the downregulation of JNK. (A and B) INS-1E cells were treated for 24 h with PRL or vehicle and then with IL1B+IFNG in the continuous presence of PRL, for the time points indicated in the figure. Protein cell lysates were used in Western blot analysis of (A) phospho-AKT serine (pAKT ser) and (B) threonine (pAKT thr). One representative Western blot of 4 (A) to 5 (B) independent experiments is shown and the optical density measurements of the specific bands corrected by aTubulin and normalized by the control in each experiment (considered as one) are expressed as mean $\pm$ S.E.M. * P < 0.05 or ** P < 0.01 for log-transformed paired t -test of vehicle vs PRL at each time point. The inset depicts the area under the curve (AUC) for its respective graphs. (C, D and E) INS-1E cells were pretreated for 2 h with 250 nmol/L of Wortmannin or DMSO, then treated for 24 h with PRL or vehicle followed by 8 h of exposure to IL1B+IFNG in the continuous presence of Wortmannin and PRL. The expression of (C) pAKT ser, (D) pAKT thr and (E) pJNK was assessed by Western blot. One representative Western blot of 5 (C and D) and 5–9 (E) and the optical density measurements of the specific bands corrected by aTubulin and normalized by the control in each experiment (considered as one) are expressed as means $\pm$ S.E.M. $^{\$}P$ < 0.05 or $^{\$\$}P$ < 0.01 for log-transformed paired t -test of DMSO vs Wortmannin in each condition (C and D); and ** P < 0.01 for log-transformed paired t -test of vehicle vs PRL (E). (F, G and H) Isolated rat islets were treated for 24 h with PRL or vehicle and then with IL1B+IFNG for the time points indicated in the figure and (G) pAKT ser and (H) pJNK were assessed by Western blot. One representative Western blot of 5 independent experiments are shown with the optical density measurements of the specific bands corrected by aTubulin and normalized by the control in each experiment (considered as one) expressed as means $\pm$ S.E.M. * P $\leq$ 0.05 for log-transformed paired t -test of vehicle vs PRL in each time point.

is confirmed by the measurement of the area under the curve in each graph (Fig. 5A and B, smaller graph).

AKT is a modulator of JNK activation (Humphrey *et al.* 2014). We next evaluated whether the observed decrease in cytokine-induced JNK phosphorylation, induced by PRL (Fig. 3A), was caused by AKT activation. INS-1E cells treated with Wortmannin, a PI3K/AKT inhibitor, displayed ~40% less phosphorylation of both serine and threonine AKT than DMSO treated cells (Fig. 5C and D), and under this condition pre-treatment with PRL failed to decrease cytokine-induced JNK phosphorylation (Fig. 5E). Although PRL was able to promote a small increase in AKT phosphorylation in isolated rat islets exposed to cytokines at 30 min (Fig. 5F and G), this was not accompanied by modulation of cytokine-induced JNK activation (Fig. 5F and H), at least in the tested conditions.

PRL attenuates the activation of NFκB by cytokines in beta cells

In parallel to JNK activation, IL1B also induces NFκB activation, a key signal for induction of beta-cell apoptosis (Heimberg *et al.* 2001, Ortis *et al.* 2006). We thus assessed the transcriptional activation of NFκB using a luciferase reporter assay regulated by 6 NFκB binding sites. INS-1E cells treated with IL1B+IFNG presented increased activation of NFκB in a time-dependent manner (Fig. 6A), and pre-treatment with PRL impaired this activation, with a ~40% decrease after 16 h of exposure to cytokines (Fig. 6A). These findings were confirmed by analyzing NFκB target genes in beta cells (Fig. 6B, C, D, E and F). Thus, PRL partially prevented cytokine-induction of *IκBα*, *Fas*, *Mcp1* and *A20* by 35–50% (Fig. 6B, C, D and E) and *Cxcl10* by ~70% (Fig. 6F). Although PRL did not change cytokine-induced iNOS expression in isolated rat islets (Fig. 6G), it decreased this protein in INS-1E cells (Fig. 6H), which was correlated with a decrease in NO production as measure by medium nitrite accumulation (Fig. 6I), and induced a delay in IκBα protein replenishment after its complete degradation by cytokine exposure (Fig. 6J). In rat beta cells, the activation of ER stress is NO dependent (Brozzi *et al.* 2015); but despite the partial inhibition of NO production (Fig. 6I) PRL did not prevent cytokine-induced ER stress (data not shown).

Discussion

PRL exerts its anabolic effects on different cell types mainly by stimulating the JAK/STAT pathway. Three members of the STAT family have been identified to

promote the activation of the PRL-responsive genes, namely STAT1, STAT3 and STAT5 (DaSilva *et al.* 1996, Bole-Feysot *et al.* 1998). STAT5 has been described to be the main mediator of the protective effects of PRL and growth hormone in human and rat beta cells exposed to stressful conditions (Jensen *et al.* 2005, Terra *et al.* 2011); while STAT3 has also been suggested to mediate the beneficial effects of PRL on insulin secretion in pancreatic beta cells (Anhe *et al.* 2007). We presently show that PRL induces STAT3 activation in INS-1E cells, which is related to the observed protective effects of PRL against cytokine-induced apoptosis.

Cytokines induce activation of JNK in rat and human beta cells (Ammendrup *et al.* 2000, Brozzi *et al.* 2015). The involvement of JNK in beta-cell death can be dependent (Ortis *et al.* 2012) or independent of NFκB activation (Gurzov *et al.* 2008). JNK contributes for the activation of the mitochondrial pathway of apoptosis (Gurzov *et al.* 2008) through cJUN phosphorylation and *Dp5* expression (Gurzov *et al.* 2009). DP5 inactivates the anti-apoptotic protein BCLxl, leading to the release of PUMA and BIM. These two BH3-only activators induce BAX translocation to the mitochondria, which releases cytochrome c and triggers the activation of caspases 9 and 3 that execute the intrinsic apoptotic pathway (Gross *et al.* 1998, 1999). We presently demonstrate that PRL decreases the expression of both cJUN and *Dp5* and the downstream pro-apoptotic proteins PUMA and *BimS*, while it increases the anti-apoptotic protein BCLxl expression. The net result is a shift from a pro- to an anti-apoptotic balance, which explains the observed protection induced by PRL against cytokine-induced beta-cell death.

In order to clarify the mechanism by which PRL modulates JNK, we assessed the role of AKT kinase in this context. The AKT pathway is an important anti-apoptotic signal that inhibits the MAPK cascade involved in cytokine-induced JNK activation (Franke *et al.* 1997, Humphrey *et al.* 2014), and we have previously shown that PRL induces PI3K/AKT during pregnancy (Amaral *et al.* 2004). We presently observed that PRL increases the phosphorylation of AKT and demonstrated that the inhibition of this AKT activation prevent PRL inhibitory effects and even increases cytokine-induced JNK activation. These observations confirm that AKT is one of the signals that mediate the beneficial effects of PRL on beta-cell survival.

NFκB is an important transcription factor involved in the induction of beta-cell death by cytokines (Giannoukakis *et al.* 2000, Baker *et al.* 2001, Heimberg *et al.* 2001, Ortis *et al.* 2006). During the activation of NFκB signaling, IκBα

is rapidly degraded allowing the nuclear translocation of NF κ B (Hayden & Ghosh 2008, Chiu et al. 2009). In the nucleus this transcription factor triggers the transcription of several target genes, including *Fas*, *Mcp1* (Cardozo et al. 2001b, Ortis et al. 2008), *Cxcl10* (Cardozo et al. 2003, Eldor et al. 2013), *A20* and *I κ B α* (Shembade & Harhaj 2012). The first three genes are involved in the amplification of the inflammatory process, while *A20* and *I κ B α* act as negative

regulatory elements retaining NF κ B in the cytoplasm and preventing its translocation to the nucleus (Shembade & Harhaj 2012). We presently observed that PRL directly reduces the promoter activity of NF κ B after a cytokine stimulus. This is accompanied by attenuation of the pro-inflammatory responses downstream of NF κ B, as evidenced by the observed down regulation of *I κ B α* , *Fas*, *Mcp1*, *A20* and *Cxcl10* expression.

Figure 6

PRL decreases the NF κ B activation induced by cytokines. (A) INS-1E cells were transfected for five hours with the NF κ B reporter and a pRL-CMV plasmid used as internal control. Sixteen hours after transfection, the cells were pretreated with PRL or vehicle and then with IL1B combined with IFNG for the time points indicated in the figure. Luciferase was assayed, and the obtained values were corrected by the internal control value of each experiment. The results are means $\pm$ S.E.M. of 3 independent experiments. (B, C, D, E and F) The expression of the NF κ B target genes (B) *I κ B α* , (C) *Fas*, (D) *Mcp1*, (E) *A20* and (F) *Cxcl10* was assessed by qRT-PCR after 24 h of cytokine treatment and corrected by the housekeeping gene *Gapdh*. The data represent the means $\pm$ S.E.M. of 4 independent experiments. Expression of (G) iNOS in rat islets ($n=3$), (H) iNOS ($n=6$) and (J) *I κ B α* ($n=3-4$) in INS-1E cells were assessed by Western blot analysis at the time points indicated in the figure (G and J) and 24 h (H). One representative Western blot and the optical density measurements of the specific bands corrected by aTubulin are shown. No normalization (G and H) or normalization by control was done in each experiment (considered as one) (J). Data are shown as means $\pm$ S.E.M. (I) The supernatant of INS-1E cells treated for 24 h with vehicle or PRL and then with IL1B+IFNG for additional 24 h was collected for determination of nitrite accumulation. * $P<0.05$ or ** $P<0.01$ for log-transformed paired t -test of vehicle vs PRL at each time point.

It was previously shown that PRL ameliorates the severity of joint inflammation and reduces chondrocyte apoptosis in models of inflammatory arthritis, possibly via the JAK2/STAT3-dependent pathway (Adan *et al.* 2013). These results are consistent with our observation that PRL reduces NF κ B activity and expression of inflammatory mediators. Of note, a constitutively activated form of STAT3 is able to suppress NF κ B transcriptional activity (Nishinakamura *et al.* 2007), while STAT3 knockdown results in significant elevation of NF κ B promoter activity (Gong *et al.* 2014) in other cell types. Therefore, the observed decrease in NF κ B activation induced by PRL could be mediated via STAT3 activation, a hypothesis that requires further investigation.

Induction of ER stress by cytokines, via activation of NF κ B and consequently NO production, is involved in the destruction of rat beta cells (Cardozo *et al.* 2005, Brozzi *et al.* 2015). Although we observed a decrease in NO production induced by PRL and previous findings demonstrated that pregnancy can improve calcium handling in islets (Vanzela *et al.* 2010); a condition that can be related to the maintenance of ER homeostasis; here we observed that the protective effects of PRL do not involve the attenuation of the main ER stress signaling pathways that contribute to cytokine-induced beta-cell death (Cardozo *et al.* 2005).

In conclusion, the present findings suggest that PRL increases the 'apoptosis threshold' in beta cells by both reducing expression/activity of pro-apoptotic effectors and augmenting anti-apoptotic mechanisms. These effects enable beta cells to better survive in face of inflammation-induced stress. Understanding the specific pro-survival proteins and pathways modulated by PRL might provide novel tools to protect beta cells during an autoimmune attack or following islet transplantation.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

F O, A C B and E C V conceived and supervised the study; F O, T R N and E C V designed the experiments; T R N, E C V, K C B and F B performed the experiments; D L E provided new tools and reagents; T R N, E C V, A F, A K C, D L E, A C B and F O analyzed the data; T R N, E C V, A C B and F O wrote the manuscript; all the authors revised the manuscript.

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