Uppsala University

This is an accepted version of a paper published in *Biochemical and Biophysical Research Communications - BBRC*. This paper has been peer-reviewed but does not include the final publisher proof-corrections or journal pagination.

Citation for the published paper:

Hindlycke, H., Lu, T., Welsh, N. (2012)

"Cytokine-induced human islet cell death in vitro correlates with a persistently high phosphorylation of STAT-1, but not with NF- κ B activation"

Biochemical and Biophysical Research Communications - BBRC, 418(4): 845-850

Access to the published version may require subscription.

Permanent link to this version:

<http://urn.kb.se/resolve?urn=urn:nbn:se:uu:diva-167882>

<http://uu.diva-portal.org>

Cytokine-induced human islet cell death in vitro correlates with a persistently high phosphorylation of STAT-1, but not with NF- κ B activation

Henrietta Hindlycke¹, Tao Lu² and Nils Welsh¹

¹Dept. of Medical Cell Biology, Uppsala University, Uppsala, Sweden

² Department of Pharmacology and Toxicology, Indiana University School of Medicine, Indianapolis, IN46202, USA".

Address correspondence to: Nils Welsh, Dept. Of Medical Cell Biology, Uppsala University, Biomedicum, P.O. Box 571, S-751 23, Uppsala, Sweden. Telephone +46-18 471-42 12,

Telefax: +46-18 471 40 59

Email: nils.welsh@mcb.uu.se

ABSTRACT

Studies of insulin producing β -cells have reported conflicting responses to NF- κ B activation, encompassing both pro- and anti-apoptotic effects, possibly reflecting the use of β -cells from different species. Therefore, the aim of this study was to compare the temporal activation of NF- κ B in rat and human insulin producing cells and relate this to the dynamics of cell death, STAT-1 activation and the production of nitric oxide (NO). Rat RIN5AH and human islet cells were exposed to the cytokines IL-1 β and IFN- γ and the NOS inhibitor aminoguanidine. Cell death, NO production, I κ B α phosphorylation, p65 methylation, STAT-1 phosphorylation and cIAP-2 levels were analyzed at different time-points. Cytokine-induced RIN5AH cell death occurred on day 1, and this was paralleled by NF- κ B activation, STAT-1 phosphorylation and production of NO. On the other hand, the human islet cells instead died by an NO-independent mechanism on day 3 and 5. This later occurring cell death was associated with a gradual decrease in I κ B α phosphorylation and p65 methylation, and a lowered expression of the NF- κ B target genes I κ B α and cIAP-2. STAT-1 phosphorylation was persistently high during the entire cytokine exposure period in human islet cells. The results favor a pro-survival role of NF- κ B and a pro-apoptotic role of STAT-1 in human islet cells. Thus, rodent insulin producing cells may not be suitable as models for human β -cells in the context of cytokine-induced damage.

Keywords: Human islets, apoptosis, NF- κ B, STAT-1, cytokines, nitric oxide

1. Introduction

In both Type 1 and Type 2 diabetes mellitus the cytokines interleukin 1 β (IL-1 β) and interferon- γ (IFN- γ) play significant roles in disease onset by contributing to a state of inflammation, which disrupts the normal β -cell function [1,2]. These cytokines activate the transcription factors nuclear factor kappa-light-chain enhancer of activated B-cells (NF- κ B) and signal transducers and activators of transcription-1 (STAT-1), respectively [3]. By promoting the production of chemo/cytokines and chemo/cytokine receptors, these factors play a crucial role in the innate as well as the specific immune system [4]. This means that NF- κ B and STAT-1, by controlling inflammation and immunity, are possible key factors in the destruction of β -cells [5]. NF- κ B may also affect the development of diabetes by its ability to modulate apoptosis. The anti-apoptotic effect of NF- κ B is well documented in a majority of cell types [6]. However, the effect of this transcription factor on apoptosis rates in β -cells is far from clear. In some cases genes activated by NF- κ B in response to cytokines are mainly thought to be pro-apoptotic [7]. For example, it has been shown that IL-1 β -induced NF- κ B activation initiates excessive nitric oxide (NO) production and that this is followed by apoptosis [8]. In other cases it has been shown that NF- κ B activates anti-apoptotic genes or promotes survival and maintained function in islet cells [9-11]. The NF- κ B-dependent anti-apoptotic pathway is thought to be vital for preserving islet viability as inflammation and insulinitis build up in vivo [9].

The uncertainty as to whether NF- κ B is pro- or anti-apoptotic in β -cells may relate to the use of different species as sources of insulin producing cells. For example, it has been reported that human β -cells are considerably more resistant to oxidative stress than rodent β -cells [12]. Thus, rodent β -cells may be more vulnerable to cytokine-induced NO production than human β -cells, leading to completely different viability outcomes in response to NF- κ B and iNOS activation. In addition, the time course of cytokine-induced human islet cell death may be different from that of rodent cells [13], suggesting that human islet cell death, as opposed to that of rodent islet cells, may not parallel cytokine-induced NF- κ B activation. Despite this, it is often postulated that NF- κ B exerts a pro-apoptotic role in the pathogenesis of diabetes. Unfortunately, no systematic comparisons of human and rodent β -cell transcription factor activation/cell death time courses have, to our knowledge, hitherto been performed. Therefore, the aim of the present investigation was to compare the time-dependency of cytokine-induced NF- κ B and STAT-1 activation, NO production and cell death in a rodent β -cell line with that in human islet cells.

2. Materials and methods

2.1 Rat and human insulin producing cells

Rat RIN5AH cells were cultured in RPMI-1640 medium (Sigma) with 10 % fetal bovine serum, L-glutamine and penicillin. The human pancreatic islets were isolated from the pancreas of brain-dead organ donors using collagenase digestion and Biocoll gradient centrifugation. After isolation, the islets were cultured free-floating in CMRL 1066 medium containing 5.6 mM glucose, 10% fetal calf serum (FCS), and 2 mM L-glutamine for 1–5 days. To evaluate islet functional quality, batches of twenty islets were perfused with Krebs-Ringer bicarbonate HEPES buffer (KRBH) containing 2 mg/ml human albumin and 1.67 mmol/l glucose at a flow rate of 0.3 ml/min. The glucose concentration was increased to 16.7 mM after a 30-min period. Fractions for insulin measurement were collected every 4 min.

For the measurement of cell viability, nitric oxide and protein phosphorylation and levels, the RIN5AH cells were treated with human recombinant IL-1 β (50 U/ml) and murine IFN- γ (1000 U/ml) for 20 min, 1 day, 3 days and 5 days. Medium was changed day 2 and 4. Some groups were also supplemented with 0.75 mM aminoguanidine (AG). The human islets were treated with human IL-1 β (50 U/ml), human IFN- γ (1000 U/ml) and 0.75 mM AG.

2.2 Cell death and nitrite analysis

The RIN5AH cells were kept in black 96 well plates, and cell death was measured on day 1, 3 and 5. The cell death was measured by incubating for 15 min with 10 μ M of propidium iodide and 10 μ M bisbenzimidide. The cells were then washed with PBS and photographed for both blue (440 nm) as well as red (600 nm) light in the Kodak 4000 MM Image Station. Relative cell death was then obtained by calculating the ratio between dead cells (total propidium iodide) and all cells (bisbenzimidide fluorescence). The human islets were stained for 30 minutes before being washed with PBS. They were then put on a microscopic glass and the islets were photographed under an ultraviolet (UV)-light microscope (fluorescence stereo microscope) showing blue and red fluorescence. The intensities were quantified using Adobe Photoshop and the ratios were calculated. At least 10 islets per group were photographed and analyzed. Nitrite levels were analyzed using the Griess reagent [14].

2.3 Immunoblot blot analysis

The RIN5AH cells and the human islets were washed and solubilized in SDS-sample buffer containing the Halt Protease & Phosphatase Inhibitor Cocktail (Thermo Scientific).

Proteins were separated on 10% SDS-PAGE gels and then electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes (Amersham Hybond™-P). After preblocking, membranes were incubated with the following primary antibodies: I κ B- α (C-21, Santa Cruz), Phospho-Ser32-I κ B- α (Cell Signaling), p65 (F-6, Santa Cruz), cIAP₂ (Abcam), Methyl-Lys221-p65 [15] and Phospho-Tyr701-STAT1 (Cell Signaling). As secondary antibody was used IgGHRP from GE Healthcare. The immunoreactivity was detected with the Millipore Immobilon immunoblotting detection system and the Kodak 4000 MM Image station. Total protein loading and transfer was assessed with amido black staining. The same filter were used for several consecutive immunoblottings and bound antibodies were stripped away from filters by incubating for 40 min at 55°C in 2% (wt/vol) SDS, 100 mmol/l Tris, pH 6.8 and 0.5 M β -mercaptoethanol.

3. Results

3.1 Effects of cytokines and/or aminoguanidine (AG) on RIN5AH cell death, cell number and NO production

Cytokines promoted a pronounced increase in cell death at day 1. On day 3, however, cell death rates were lower in cytokine-exposed cells, indicating that a subpopulation of cytokine-resistant RIN5AH with low cell death rates cells had been selected by the cytokine treatment. Cell death rates on day 1 were increased when AG was added together with cytokines (Figure 1A), but less dramatically than when cytokines were added alone. Also the combination of cytokines + AG resulted in a lowered cell death at day 3. AG by itself did not affect cell death rates.

From the same experiments, the relative amounts of cells, obtained by bisbenzimid staining, were calculated (Figure 1B). We observed low numbers of cells in the cytokine group at day 3 and possibly day 5, reflecting the initial loss of cells (Figure 1B). AG protected completely against the cytokine-induced lowering of total cell number.

On day 1 cytokines promoted a potent increase in nitrite levels, which was partially counteracted by AG (Figure 1C). On day 3 and 5 the cytokine-induced nitrite increase was even more pronounced, consistent with the view that the cytokine-treatment had resulted in selection of a NO-resistant cell subpopulation. Thus, most RIN5AH cells die on day 1 from cytokine-induced NO-production and the remaining cells survive in spite of increasing NO levels.

3.2 Effects of cytokines and/or AG on STAT-1 and NF- κ B activation in RIN5AH cells

STAT-1 phosphorylation was undetectable in cells not exposed to cytokines and induced by cytokines at all time points (Figure 2). AG did not affect STAT-1 phosphorylation. Interestingly, cytokine-induced STAT-1 phosphorylation was very prominent at 20 min as compared to day 1, 3 and 5. The ratio of Phospho-I κ B α to total I κ B α was markedly increased by cytokines at 20 min (Figure 2). However, as total I κ B α levels were decreased at 20 min, the increased ratio was mainly due to a rapid loss of total I κ B α and not to an increase in total P-I κ B α levels. The ratio P-I κ B α /total I κ B α was not affected by cytokines or AG on day 1, 3 or 5, indicating NF- κ B-induced IL-1 β desensitization. The methylation of p65 is considered to represent nuclear NF- κ B activation [15]. When normalizing methyl-p65 to total p65 levels, we observed that NF- κ B was activated in response to cytokines after 20 minutes, 1 day and 3 days, but not at 5 days (Figure 2). Somewhat surprisingly, NF- κ B was not methylated in the presence of AG, indicating the requirement of NO for full NF- κ B activation in RIN5AH cells. The time-dependent decrease in p65 methylation supports, similarly to the case with P-I κ B α , NF- κ B-induced desensitization.

Upon activation NF- κ B is known to bind to the cIAP-2 gene and induce expression of this anti-apoptotic protein [16]. Thus, cIAP-2 expression could be considered as an NF- κ B target gene that reflects NF- κ B *in vivo* activity. However, cytokines or AG did not affect cIAP-2 levels in RIN5AH cells at any of the time points studied (results not shown).

3.3 Effects of cytokines and/or AG on cell death in human islets

Islet batches were first tested for glucose responsive insulin release and only glucose responsive islets (> 2-fold increased) were used for further experimentation. In addition, islets from the different donors were stained with Newport Green and studied in a fluorescence microscope for estimation of percentage cells with granular cytoplasmic staining. Only islets batches with approximate β -cell percentages above 30% were used.

We failed to observe any cytokine-induced increase in human islet cell death on day 1 (Figure 3). On day 3, however, cell death was increased and the cell death continued to increase on day 5 and 7. AG did not protect against cytokine-induced human islet cell death. Thus, human islets die later than RIN5AH cells and by an NO-independent mechanism. We also measured human islet production of NO, but due to weak signals, due to a rather small number of islets per culture dish, we could only observe cytokine-induced NO production in sporadic experiments. In

those experiments, however, cytokine-induced NO production was counteracted by AG (results not shown).

3.4 Effects of cytokines and/or AG on STAT-1 and NF- κ B activation in human islets

Cytokine exposure promoted STAT-1 tyrosine phosphorylation already after 20 min (Figure 4). This effect was somewhat weaker at 1 day, but returned to high levels on day 3 and 5. AG did not affect P-STAT-1 levels. Cytokine exposure induced also an increase in the P-I κ B α /total I κ B α ratio at 20 min and on day 1. Again, the increased P-I κ B α /total I κ B α ratio was paralleled by a lowered total I κ B α level at 20 min. On day 3 and 5, however, the P-I κ B/total I κ B α ratio was no longer increased by cytokines. AG did not affect cytokine-induced I κ B α phosphorylation. Met-p65 was augmented in cells exposed to cytokines for 20 min and for 1 day (Figure 4). Interestingly, on day 3 and 5 increased p65 methylation was no longer observed in human islets exposed to cytokines. AG did not affect cytokine-induced p65 methylation. These results indicate that STAT-1 phosphorylation remained high throughout a 5-day cytokine culture period, and that time-dependent NF- κ B desensitization occurs both in human islets cells and rat insulin producing cells.

NF- κ B activation is known to enhance transcription of the I κ B α and cIAP-2 genes. Interestingly, after the initial decrease at 20 min, I κ B α protein levels were increased by cytokines at 1 day, possibly reflecting increased NF- κ B-induced transcription (Figure 4). On day 3 and 5, however, the levels of I κ B α return back to the levels of non-stimulated islet cells. A similar pattern was observed with the other NF- κ B target gene cIAP-2 (results not shown).

4. Discussion

In the present study we performed, to our knowledge, the first systematic comparison between rat insulin producing cells and human islet cells regarding cytokine-induced NF- κ B- and STAT-1 activation, NO-production and cell death. We report that there exist major differences in cytokine responsiveness between the rodent and the human cells. It is noteworthy that human islets consist of only 30-60% β -cells and that the rat insulin producing cells are transformed insulinoma cells. However, RIN-cells have been observed to respond to cytokine exposure with similar signaling and cell death events as primary β -cells from isolated pancreatic islets [17]. Moreover, previous work has reported that FACS-purified human β -cells die when cultured in the presence of cytokines [18]. It is therefore unlikely that the presently observed cell death and signaling events of human islets would represent only non- β -cells.

In agreement with many previous studies, the RIN5AH cells responded to the cytokine treatment in line with the well established dogma stating that IL-1 β and IFN- γ activate NF- κ B and STAT-1, respectively, which in turn leads to maximal iNOS expression and pronounced cell death after only 24 hours [8]. The iNOS inhibitor AG counteracted this cell death pathway, pinpointing the pivotal role of NF- κ B-induced iNOS expression in rat β -cells. Indeed, rat β -cells are known to be very sensitive to NO, most likely due to a particularly low defense capacity against oxidative stress [12]. Therefore, in rat β -cells, NF- κ B, in synergy with STAT-1, functions as a pro-apoptotic/necrotic factor by inducing iNOS.

Interestingly, the RIN5AH cell death rates in the presence of cytokines were decreased on day 3 and 5. Apparently a subpopulation of RIN5AH cells survived the high levels of NO, which resulted in the selection of cytokine-resistant cells, as observed also in previous studies [19]. It is not clear why a subpopulation of the RIN5AH cells presently survived high NO levels, but it could be speculated that a lower degree of STAT-1 activation, as observed in the surviving cells, explained the improved resistance to NO. It is also possible that the surviving cells possessed a better enzymatic defense against oxidative stress [20], a possibility not addressed in this study.

The human islet cells died not until day 3 when cultured with cytokines, and AG did not prevent this effect, suggesting that human islet cell death occurs by NO-independent pathways. This is much in line with previous studies reporting that human islet cell death occurs later than rodent islet cell death and that inhibitors of NOS do not protect against cytokine-induced cell death [21,22]. A possible explanation for this fundamental difference between human and rodent β -cells may be that human islet cells express higher levels of anti-oxidative enzymes and

other stress proteins [20]. Thus, although NO production occurs in human islet cells, it is unlikely that human islet cells die from this event. Instead, NF- κ B might exert an anti-apoptotic role in human islet cells, as observed in most other cell types. Indeed, our finding that p65 methylation and I κ B α phosphorylation rates and I κ B α protein levels subsided during prolonged cytokine exposure, both in RIN5AH and human islet cells, points to the existence of an efficient NF- κ B negative feed-back loop, resulting in NF- κ B desensitization [23,24]. As NF- κ B becomes increasingly desensitized to IL-1 β stimulation, the expression of the anti-apoptotic NF- κ B target gene cIAP-2 declines and increased susceptibility to pro-apoptotic signals ensues. This chain of events might occur also in RIN5AH cells. In these cells there is a similar early NF- κ B activation followed by a late deactivation. Thus, NF- κ B-dependent pathways are initially activated, but, due to the high susceptibility to NO, nitrosative stress prevails and the cells die despite attempts to activate anti-apoptotic genes.

Assuming that NF- κ B can no longer protect against apoptosis in human islet cells on day 3 and 5, then what promotes apoptosis? Studies have reported STAT-1 to be pro-apoptotic when activated in β -cells [5,25]. In the present investigation STAT-1 was activated by IFN- γ already after 20 minutes and remained highly phosphorylated throughout the different culture periods. It is not clear why STAT-1 phosphorylation remains high and not subsides from negative feed-back inhibition, as does NF- κ B, but it has been reported that SOCS-1 overexpression in β -cells resulted in diminished STAT-1 phosphorylation and protection against β -cell death and diabetes [26]. Therefore, it may be that the endogenous SOCS-1 activity of β -cells is inherently low resulting in a particularly high sensitivity to prolonged IFN- γ stimulation. Thus, it is tempting to speculate that the early anti-apoptotic activity of NF- κ B protects against STAT-1, but, due to the negative feed-back inhibition of NF- κ B, STAT-1 dominates over NF- κ B on day 3 to 5, and β -cells can then no longer survive. A possible extrapolation of these *in vitro* events to an *in vivo* situation could be that a prolonged islet inflammatory reaction, combined with hypersecretion of T-lymphocyte-derived IFN- γ , disturbs the β -cell balance of pro- and anti-apoptotic factors *in vivo*, causing an accelerated development of diabetes and increased β -cell death.

In summary, by systematic comparisons we have observed that human islet cells and rat insulin producing cells respond very differently to cytokine exposure. Our time course experiments indicate that rat cells die rapidly from NO-induced toxicity, whereas human islet cells die later from NO-independent mechanisms. Cytokine-induced human islet cell death parallels subsiding NF- κ B activity and persistently high STAT-1 phosphorylation rates. It may be that successful strategies to prevent β -cell death *in vivo* need to involve the inhibition of STAT-1. It may also be that great caution should be exercised when extrapolating results regarding the pro-apoptotic activity of NF- κ B in rat β -cells to the human situation.

Acknowledgements

The Nordic Network for Clinical Islet Transplantation, supported by the Swedish national strategic research initiative EXODIAB (Excellence Of Diabetes Research in Sweden), and the Juvenile Diabetes Research Foundation are acknowledged for supply of human islets (JDRF award 31-2008-413 and the ECIT Islet for Basic Research program). This work was supported in part by the Swedish Research Council (2010-11564-15-3), the Swedish Diabetes Association, the family Ernfors Fund, Barndiabetesfonden and the Novo-Nordisk Foundation.

References

- [1] W. Suarez-Pinzon, O. Sorensen, R.C. Bleackley, J.F. Elliott, R.V. Rajotte, A. Rabinovitch, Beta-cell destruction in NOD mice correlates with Fas (CD95) expression on beta-cells and proinflammatory cytokine expression in islets, *Diabetes* 48 (1999) 21-28.
- [2] S. Uno, A. Imagawa, K. Okita, K. Sayama, M. Moriwaki, H. Iwahashi, K. Yamagata, S. Tamura, Y. Matsuzawa, T. Hanafusa, J. Miyagawa, I. Shimomura, Macrophages and dendritic cells infiltrating islets with or without beta-cells produce tumour necrosis factor-alpha in patients with recent-onset type 1 diabetes, *Diabetologia* 50 (2007) 596-601.
- [3] D. Eizirik, T. Mandrup-Poulsen, A choice of death-the signal-transduction of immune-mediated beta-cell apoptosis, *Diabetologia* 44 (2001) 2115-2133.
- [4] P. Pirot, A.K. Cardozo, D.L. Eizirik, Mediators and mechanisms of pancreatic beta-cell death in type 1 diabetes, *Arq. Bras. Endocrinol. Metabol.* 52 (2008) 156-165.
- [5] C. Gysemans, H. Callewaert, L. Overbergh, C. Mathieu, Cytokine signalling in the beta cell: a dual role for IFNgamma, *Biochem. Soc. Trans.* 36 (2008) 328-333.
- [6] J. Kucharczak, M.J. Simmons, Y. Fan, C. Glinas, To be or not to be: NF-B is the answer – role of Rel/NF-B in the regulation of apoptosis, *Oncogene* 22 (2003) 8961-8982.
- [7] F. Ortis, P. Pirot, N. Naamane, A.Y. Kreins, J. Rasschaert, F. Moore, E. Thtre, C. Verhaeghe, N.E. Magnusson, A. Chariot, T.F. Orntoft, D.L. Eizirik, Induction of nuclear factor-kappaB and its downstream genes by TNF-alpha and IL-1beta has a pro-apoptotic role in pancreatic beta cells, *Diabetologia* 51 (2008) 1213-1225.
- [8] D.L. Eizirik, M. Flodstrm, A.E. Karlsen, N. Welsh, The harmony of the spheres: inducible nitric oxide synthase and related genes in pancreatic beta cells, *Diabetologia* 39 (1996) 875-890.
- [9] S. Kim, I. Millet, H.S. Kim, J.Y. Kim, M.S. Han, M.K. Lee, K.W. Kim, R.S. Sherwin, M. Karin, M.S. Lee, NF-kappa B prevents beta cell death and autoimmune diabetes in NOD mice, *Proc. Natl. Acad. Sci. USA* 104 (2007) 1913-1918.
- [10] S.A. Sarkar, B. Kutlu, K. Velmurugan, S. Kizaka-Kondoh, S.E. Lee, R. Wong, A. Valentine, H.W. Davidson, J.C. Hutton, S. Pugazhenthir, Cytokine-mediated induction of anti-apoptotic genes that are linked to nuclear factor kappa-B (NF-kappaB) signalling in human islets and in a mouse beta cell line. *Diabetologia* 52 (2009) 1092-1101.
- [11] D. Mokhtari, A. Barbu, I. Mehmeti, C. Vercamer, N. Welsh, Overexpression of the Nuclear Factor-kB subunit c-Rel protects against human islet cell death in vitro, *Am. J. Physiol. Endocrinol. Metab.* 297 (2009) E1067-E1077.
- [12] N. Welsh, B. Margulis, L.A.H. Borg, H. Jernberg-Wiklund, J. Salden, A. Andersson, D.G. Pipeleers, C. Hellerstrm, D.L. Eizirik, Differences in the expression of heat-shock proteins and antioxidant enzymes between isolated human and rodent pancreatic islets: Implications for the pathogenesis of insulin-dependent diabetes mellitus, *Mol. Med.* 1 (1995) 806-820.

- [13] D.L. Eizirik, S. Sandler, N. Welsh, M. Cetkovic-Cvrlje, A. Nieman, D.A. Geller, D.G. Pipeleers, K. Bendtzen, C. Hellerström, Cytokines suppress human islet function irrespective of their effects on nitric oxide generation, *J. Clin. Invest.* 93 (1994) 1968-1974.
- [14] N. Welsh, D.L. Eizirik, K. Bendtzen, S. Sandler, Interleukin-1b induced nitric oxide production in isolated rat pancreatic islets requires gene transcription and may lead to inhibition of the Krebs cycle enzyme aconitase, *Endocrinology* 129 (1991) 3167-3173.
- [15] T. Lu, M.W. Jackson, B. Wang, M. Yang, M.R. Chance, M. Miyagi, A.V. Gudkov, G.R. Stark, Regulation of NF-kappaB by NSD1/FBXL11-dependent reversible lysine methylation of p65, *Proc. Natl. Acad. Sci. U S A* 107 (2010) 46-51.
- [16] H-S. Jin, T.H. Lee, Cell cycle-dependent expression of cIAP2 at G2/M phase contributes to survival during mitotic cell cycle arrest, *Biochem. J.* 399 (2006) 335-342.
- [17] J. Saldeen, N. Welsh, Interleukin-1b induced activation of NF-kB in insulin producing RINm5F cells is prevented by the protease inhibitor tosyl-lysine chloromethyl ketone, *Biochem. Biophys. Res. Commun.* 203 (1994) 149-155.
- [18] B. Movahedi, M. Van de Castele, N. Caluwé, G. Stangé, K. Breckpot, K. Thielemans, G. Vreugdenhil, C. Mathieu, D. Pipeleers, Human pancreatic duct cells can produce tumour necrosis factor-alpha that damages neighbouring beta cells and activates dendritic cells, *Diabetologia* 47 (2004) 998-1008.
- [19] G. Chen, H.E. Hohmeier, R. Gasa, V.V. Tran, C.B. Newgard, Selection of insulinoma cell lines with resistance to interleukin-1beta- and gamma-interferon-induced cytotoxicity, *Diabetes* 49 (2000) 562-570.
- [20] E. Gurgul-Convey, I. Mehmeti, S. Lortz, S. Lenzen, Cytokine toxicity in insulin-producing cells is mediated by nitro-oxidative stress-induced hydroxyl radical formation in mitochondria, *J. Mol. Med. (Berl)* 89 (2011) 785-798.
- [21] D.L. Eizirik, S. Sandler, N. Welsh, M. Cetkovic-Cvrlje, A. Nieman, D.A. Geller, D.G. Pipeleers, K. Bendtzen, C. Hellerström, Cytokines suppress human islet function irrespective of their effects on nitric oxide generation, *J. Clin. Invest.* 93 (1994) 1968-1974.
- [22] A. Rabinovitch, W.L. Suarez-Pinzon, K. Strynadka, R. Schulz, J.R. Lakey, G.L. Warnock, R.V. Rajotte, Human pancreatic islet beta-cell destruction by cytokines is independent of nitric oxide production, *J. Clin. Endocrinol. Metab.* 79 (1994) 1058-1062.
- [23] J.E. Hutti, B.E. Turk, J.M. Asara, A. Ma, L.C. Cantley, D.W. Abbott, IkappaB kinase beta phosphorylates the K63 deubiquitinase A20 to cause feedback inhibition of the NF-kappaB pathway, *Mol. Cell. Biol.* 27 (2007) 7451-7461.
- [24] A.E. Karlsen, P.E. Heding, H. Frobøse, S.G. Rønn, M. Kruhøffer, T.F. Orntoft, M. Darville, D.L. Eizirik, F. Pociot, J. Nerup, T. Mandrup-Poulsen, N. Billestrup, Suppressor of cytokine signalling (SOCS)-3 protects beta cells against IL-1beta-mediated toxicity through inhibition of multiple nuclear factor-kappaB-regulated proapoptotic pathways, *Diabetologia* 47 (2004) 1998-2011.
- [25] H.I. Callewaert, C.A. Gysemans, L. Ladrière, W. D'Hertog, J. Hagenbrock, L. Overbergh, D.L. Eizirik, C. Mathieu, Deletion of STAT-1 pancreatic islets protects against streptozotocin-induced diabetes and early graft failure but not against late rejection, *Diabetes* 56 (2007) 2169-2173.

- [26] M. Flodström-Tullberg, D. Yadav, R. Hägerkvist, D. Tsai, P. Secrest, A. Stotland, N. Sarvetnick, Target cell expression of suppressor of cytokine signaling-1 prevents diabetes in the NOD mouse, *Diabetes* 52 (2003) 2696-2700.

Legends to the Figures

Fig. 1. Effects of cytokines and/or AG on the relative cell death (A) , cell number (B) and NO production (C) of RIN5AH cells. (A) RIN5AH cells were cultured for up to 5 days with or without IL-1 β (50 U/ml) + IFN- γ (1000 U/ml) (cytokines) and 0.75 mM aminoguanidine (AG). Relative cell death was assessed by vital staining with propidium iodide and bisbenzimidazole and calculation of the red/blue ratio. Results are expressed as ratios to the control and are means $\pm$ SEM. * denotes $P < 0.05$ and *** $P < 0.001$ compared to the control group using Student's t-test ($n=4$). + denotes $p < 0.05$ when comparing to the cytokine group. (B) The total number of cells was assessed by vital staining with bisbenzimidazole. * denotes $P < 0.05$ compared to the control group using Student's t-test ($n=4$). (C) Nitrite levels, as a measure of NO production, were analyzed using the Griess reagent. Results are means $\pm$ SEM for 4 observations.

Fig. 2. Effects of cytokines and/or AG on RIN5AH cell STAT-1- and I κ B α phosphorylation and p65 methylation. RIN5AH cells were cultured for up to 5 days with or without IL-1 β (50 U/ml) + IFN- γ (1000 U/ml) and 0.75 mM AG. Cells were then harvested and analyzed by immunoblot analysis. P-STAT-1 and Met-p65 signals were normalized to total p65 signals and P-I κ B α to total I κ B α . Total I κ B α was normalized to amido black staining from the same filters. Observe the decrease in total p65 of cytokine-treated cells on day 1, 3 and 5, which occurs as a consequence of cytokine-induced cell death. * denotes $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ compared to the control group using Student's t-test ($n=4$).

Fig. 3. Cell death in human islets after exposure to cytokines and/or AG for different time periods. Human islets were cultured for up to 7 days with or without IL-1 β (50 U/ml) + IFN- γ (1000 U/ml) (cytokines) and 0.75 mM AG. Relative cell death was assessed by vital staining with propidium iodide and bisbenzimidazole and calculation of the red/blue ratio. Results are expressed as ratios to the control and are means $\pm$ SEM. * denotes $P < 0.05$ compared to the control group using Student's t-test ($n=4-6$).

Fig. 4. Effects of cytokines and/or AG on human islet STAT-1- and I κ B α phosphorylation and p65 methylation. Human islets were cultured as in Figure 3 and then used for

immunoblot analysis of P-STAT-1, P-I κ B α , total I κ B α , Met-p65 and total-p65. P-STAT-1 and Met-p65 were normalized to total p65, P-I κ B α was normalized to total I κ B α and total I κ B α was normalized to amido black. All experimental groups were expressed as ratios to the control. Results are means $\pm$ SEM. * denotes $P < 0.05$ and ** denotes $P < 0.01$ compared to the control group using Student's t-test (n=4).

Figure 1
[Click here to download high resolution image](#)

Figure 2
[Click here to download high resolution image](#)

Figure 3
[Click here to download high resolution image](#)

Figure 4

[Click here to download high resolution image](#)