Role of the AMP kinase in cytokine-induced human EndoC-βH1 cell death

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Abstract

The aim of the present investigation was to delineate cytokine-induced signalling and death using the EndoC-βH1 cells as a model for primary human beta-cells. The cytokines IL-1β and IFN-γ induced a rapid and transient activation of NF-κB, STAT-1, ERK, JNK and eIF-2α signalling. The EndoC-βH1 cells died rapidly when exposed to IL-1β + IFN-γ, and this occurred also in the presence of the actinomycin D. Inhibition of NF-κB and STAT-1 did not protect against cell death, nor did the cytokines activate iNOS expression. Instead, cytokines promoted a rapid decrease in EndoC-βH1 cell respiration and ATP levels, and we observed protection by the AMPK activator AICAR against cytokine-induced cell death. It is concluded that EndoC-βH1 cell death can be prevented by AMPK activation, which suggests a role for ATP depletion in cytokine-induced human beta-cell death.

1. Introduction

Pro-inflammatory cytokines, such as IL-1β and IFN-γ, have been suggested to participate in the destruction and suppression of function of beta-cells in Type 1 diabetes (Pirot, Eizirik, 2008). *In vitro* studies have demonstrated that the combination of IL-1β + IFN-γ promotes dysfunction and death of beta-cells, and that this is mainly mediated via activation of the NF-κB/iNOS (Eizirik et al., 1996), STAT-1 (Moore et al, 2011) and MAP kinase (Welsh, 1996) pathways. *In vivo*, increased expression of IL-1β and IFN-γ is observed at early stages of insulitis (Eizirik et al, 1996), and diabetes in NOD mice is delayed when the mice were treated with the IL-1β receptor antagonist or when lacking the IL-1 receptor (Eizirik, Mandrup-Poulsen, 2001, Thomas et al, 2004). Although there are discordant results concerning the role of IFN-γ (Thomas et al., 1998), knockout of the IFN-γ receptor resulted in one study in protection against diabetes (Wang et al., 1997). Also in Type 2 diabetes a role for pro-inflammatory cytokines has been suggested. In this case it has been proposed that islet macrophages and dendritic cells release cytokines in response to pro-amyloid IAPP fibrils (Masters et al, 2010, Skeldon et al, 2014), causing a vicious cycle with increasing inflammation and amyloid deposits.

Our knowledge on IL-1β- and IFN-γ-induced beta-cell signalling is to a large extent based on studies with rodent beta-cells. As rodent cells behave differently from human cells (Seok et al, 2013), it may be that the cytokine-induced events delineated above are not representative for the human beta-cells. For example, it has been reported that cytokines promote the expression of anti-apoptotic genes in human islets (Sarkar et al, 2009), and that NF-κB is mainly anti-apoptotic in human beta-cells (Hindlycke et al, 2012, Hägerkvist et al, 2007, Mokhtari et al, 2009), whereas it is clearly pro-apoptotic in rodent beta-cells (Eizirik et al, 1996, Pirot, Eizirik, 2008). In

addition, human islets contain typically only 30-50% beta-cells (Scharfmann et al, 2014, Welsh et al, 2005), as opposed to mouse islets that contain on average 77% beta-cells (Cabrera et al, 2006), which makes it uncertain to what extent effects observed in human islets actually reflect the beta-cell population. To address this problem we have presently utilized the recently generated human beta-cell line EndoC-βH1 (Ravassard et al, 2011). These cells are transformed human beta-cells that contain one order in magnitude less insulin than primary human beta-cells, but respond to both glucose and GLP-1 with an increased insulin release (Ravassard et al, 2011). Moreover, a recent study has reported that the glucose-induced respiration and stimulus-secretion coupling responses of EndoC-βH1 cells resemble those of human islets (Andersson et al, 2015). Thus, the EndoC-βH1 cells share important characteristics with primary beta-cells, which allows us to study cytokine-induced signalling events in a pure human beta-cell population.

2. Methods

2.1. Materials

Lipofectamine 2000 was obtained from Invitrogen. Human recombinant IL-1β, murine- and human IFN-γ were from Peprotech. PD98059 was from Calbiochem. Indomethacin, aminoguanidine and bromophenacyl bromide were from Sigma-Aldrich. IKK inhibitor X and epigallocathecin gallate was from Santa Cruz. SP600125 was from Tocris Bioscience. Cyclosporine A, Fludarabin was from Sellec Chemicals. Compound C, 5-Aminoimidazole-4-carboxamide ribonucleotide (AICAR), PD98059 and SB302480 were from Calbiochem. Actinomycin D was from Amresco. 5,8,11-Eicosatriynoic acid was from Enzo Life Sciences and PGE_2 was from Cayman Chemical Company.

2.2. Cell culture

Murine betaTC-6 cells, which respond to cytokines with increased nitric oxide production and cell death similarly to other rodent beta-cell lines (Makeeva et al, 2008), were cultured in RPMI-1640 supplemented with 10% fetal calf serum + 2 mM L-glutamine and penicillin + streptomycin. Human EndoC-βH1 cells were cultured in ECM/fibronectin-coated plates in low-glucose DMEM with supplements as previously described (Ravassard et al, 2011).

2.3. siRNA mediated silencing of STAT-1

For knock-down of STAT-1, EndoC-βH1 cells were plated one day before transfection to achieve 50% confluency at the time of transfection. On the day of transfection cells were incubated in serum- and antibiotic free medium, and Mission

siRNA Universal Negative Control #1 (Sigma) or human STAT-1 siRNA (NM-007315, Sigma) were combined with Lipofectamine 2000 for 30 min at room temperature. Cells were incubated over night with the siRNA/liposome mixtures (30 nM), after which full culture medium was added.

2.4. Insulin release

Cells were preincubated for 30 min in Krebs Ringer bicarbonate buffer (Krebs and Henseleit, 1932) containing 10 mM HEPES pH 7.4, 2 mg/ml bovine serum albumin and 1 mM glucose, and then incubated for 1h in either 1 mM glucose, 20 mM glucose, 1 mM glucose + 0.5 mM IBMX or 20 mM glucose + 0.5 mM IBMX, at 37°C in Krebs Ringer Bicarbonate buffer with the same additions as during the pre-incubation. Insulin concentrations were measured using an Insulin Assay Kit (catalog #: 10-1113-01, Mercodia).

2.5. Evaluation of cell viability

The cell viability of EndoC-βH1 was determined by staining the cells with propidium iodide (Sigma) (20 μg/ml) for 10 min at 37 C. After washing, cells were trypsinized and analyzed for red fluorescence (FL-3) using flow cytometry (FacsCalibur, BD). In some experiments cells were inspected and photographed with a Nikon fluorescence microscope.

2.6. ATP contents

Cells were washed in ice-cold phosphate buffer saline (PBS), sonicated for 10 seconds and then centrifuged for 3 min at 12000g. The supernatants were then analyzed for ATP content using the Human Adenosine Triphosphate (ATP) ELISA Kit from

MyBiosource (catalog #: MBS9310359). ATP contents were normalized to total protein obtained by Bradford analysis.

2.7. Oxygen consumption

Oxygen consumption rates (OCR) were determined by Seahorse Extracellular Flux Analyzer XFe96 (Seahorse Bioscience, Billerica, MA, USA) as previously described (Malmgren S et al). EndoC-βH1 cells were cultured with or without the cytokines IL-1β (20 ng/ml) and IFN-γ (20 ng/ml) and 1 mM AICAR at 5.6 mM glucose for 6 hours. Prior to assay, cells were preincubated with 175 µl assay medium (Malmgren S et al, 2009) supplemented with 5.6 mM glucose in the presence or absence of cytokines for 1 h at 37 °C in the air and OCR was then measured for 20 min at this condition. The proportions of respiration driving ATP synthesis and proton leak were determined by blocking ATP synthase by the addition of 2 µM oligomycin. After 16 min, 2 µM of the uncoupler carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) was added to determine the maximal respiratory capacity. After a further 16 min, 2 µM rotenone and 2 µM antimycin A were added together to block transfer of electrons from complex I and complex III of the respiratory chain to measure the remaining non-mitochondria-dependent respiration. Non-mitochondrial respiration was subtracted from the other values when calculated different portions of respiration. Data were normalized to protein contents determined by the DC protein assay (Bio-Rad).

2.8. Immunoblotting

Cells were washed in ice-cold PBS, lysed in SDS sample buffer, boiled for 5 min and separated by SDS-PAGE. Proteins were electrophoretically transferred onto a Hybond-P membrane (GE Healthcare). Membranes were incubated with the following

primary antibodies: phospho-JNK (Thr183/Tyr185), phospho-GSK-3-alpha/beta (Ser21/9), phospho-eIF2alpha (Ser51), phospho-AMPKalpha (Thr172), phospho-ERK(thr202/tyr204), phospho-Akt(Ser473) antibodies (Cell Signaling Technology), total-ERK, total IκBalpha (C21), and total iNOS (C11) (Santa Cruz) antibodies. The immunodetection was performed as previously described (Mokhtari et al, 2009).

2.9. RNA sequencing

For whole transcriptome sequencing, input total RNA, isolated using the UltraSpec Reagent, was analyzed using the SOLiD5500XL system as previously described (Wang et al, 2013). Alignment of reads to the human reference sequence (hg19 assembly) was performed using v2.1 of the LifeScope Software. MicroRNA genes were not included in the analysis.

2.10. Gene Ontology analysis

The official gene symbols of the DE genes were submitted to the Database for Annotation, Visualization and Integrated Discovery (DAVID) Bioinformatics Resources 6.7 (http://david.abcc.ncifcrf.gov/) for the functional annotation chart analysis. The highest classification stringency setting was used for the GO analysis. We used the criteria of FDR-corrected $P < 0.001$.

3. Results

3.1. Characterization of EndoC-βH1 cells by insulin release determinations and RNA sequencing (RNA-seq)

In a typical experiment the insulin release of EndoC-βH1 cells at 1.0 mM glucose during a 60-min incubation was 0.4 ng/100000 cells. The release of insulin at 20 mM glucose, 1 mM glucose + 0.5 mM IBMX and 20 mM glucose + 0.5 mM IBMX was 0.7, 2.6 and 3.8 ng/100000 cells, respectively. This corresponds well with the results of a more detailed study on EndoC-βH1 cell insulin release and glucose-induced stimulus secretion coupling (Andersson et al, 2015). The islets from the three cadaveric organ donors responded to a 17 mM glucose stimulation with an increased insulin release in islet perifusion experiments (stimulation index 2.2, 5.9 and 8.8). The age, gender, BMI and blood group of the three donors were, 51/58/57 years, male/male/male, 28/21.4/23.4 kg/m^2 and AB/A/A, respectively.

RNA isolated from three different human islet donor preparations and three different EndoC-βH1 cell preparations was used for whole transcriptome analysis. We chose to compare intact human islets with the EndoC-βH1 cells, and not sorted human beta-cells from dissociated human islets, because dissociation and sorting of human beta-cells is a harsh procedure that may considerably affect mRNA levels. The majority of the genes were expressed at a level in the range of 1-1000 reads per kilobase per million (RPKM). The detection threshold for the RNA-seq analysis was set to a mean RPKM value of 0.5 in both of the two groups of samples. We detected 14,105 genes in human islets and 13,055 in EndoC-βH1 cells out of 23,383 annotated RefSeq genes using this criterion (Online Suppl. Table 1). EndoC-βH1 cells expressed high levels of transcripts typical for beta-cells, whereas in human islets expressed also high levels of

transcripts coding for glucagon, somatostatin, antitrypsin and cytokeratin 7 (Online Suppl. Table 1), indicating that human islets contain a mixture of alpha-, beta-, delta-, acinar and duct cells.

A comparison of the expression of genes of particular importance for beta-cell function is shown in Table 1. Expression of beta-cell transcription factors tended to be generally higher in the EndoC-βH1 cells than in human islets, suggesting that the percentage beta-cells is lower in islets than in the cell line, and that the functional state of the EndoC-βH1 cells was high. Also the higher expression of glucokinase, Glut2 and the potassium channel KCNJ11 (Kir6.2), and the lower expression of hexokinase and lactate dehydrogenase support this notion. On the other hand, EndoC-βH1 expression of the zinc transporter ZnT8 and IAPP was considerably lower than in islet cells (Table 1).

We next compared the expression of genes that mediate proximal steps in IL-1β- and IFN-γ-induced signalling (Table 2). Using $p<0.001$ as significance level, to reduce the risk of type 1 errors associated with multiple comparisons, we observed only a difference in the expression of the phospholipase C gene PLCG2, which was higher in EndoC-βH1 cells than in human islets. Besides this significant difference, expression of some other genes involved in IL-1β-induced signalling tended to be lower (IL1R1, MAP3K1 and NFKBIA) or higher (IL1RAP, MAP2K6, MAPK13 and MAPK12) in the EndoC-βH1 cells (Table 2). Among the genes that convey IFN-γ signals expression of JAK1, PTK2B and SOCS1 appeared to be lowered, whereas MAP3K4 and SRC appeared higher in EndoC-βH1 cells. As these trends may signify both increased and decreased IL-1β/IFN-γ-induced signalling, it is possible that overall cytokine signalling in EndoC-βH1 cells is similar to that of human islets. Interestingly,

Database for Annotation, Visualization and Integrated Discovery (DAVID) analysis did not reveal any significant clustering of inflammation or cytokine-signalling related genes among the differentially expressed (DE) genes (results not shown), suggesting that the cytokine-response of EndoC-βH1 cells may be similar to that of human islets.

We found 2491 differentially DE genes, out of which 1426 were lower in EndoC-βH1 cells as compared to human islets, and the remaining 1065 genes were increased in EndoC-βH1 cells (Online Suppl. Table 2). The DE-genes (increased and decreased genes separately) were submitted for functional annotation clustering analysis using DAVID, which showed that the EndoC-βH1 enriched genes, using the highest classification stringency, are involved in different proliferation-associated events, such as mitosis/cell cycle, meiosis, ATP-binding, minichromosomal maintenance and kinesin motor protein function (results not shown). Thus, the continuous proliferation of the EndoC-βH1 cells, with a doubling time of approximately 7 days (Andersson et al, 2015), as compared to the non-proliferating human islet beta-cells, is paralleled by alterations in gene expression of cell cycle/mitosis genes. Further analysis of these differences could give important clues to the reasons underlying the non-proliferative state of mature human beta-cells.

The genes which were lower in EndoC-βH1 cells than in human islets, however, were clustered in GO categories such as cadherin 1-4 domains, triple helix (collagens), laminins, IGFBP N-terminal domains, and TNFR cysteine rich domains, consistent with the notion that the EndoC-βH1 cells have fewer cell-to-cell contacts and clustering molecules as compared to cells of intact human islets (results not shown).

3.2. The combination of IL-1β and IFN-γ kills EndoC-βH1 cells rapidly by inducing apoptosis and secondary necrosis

The mode and kinetics of IL-1β and IFN-γ-induced EndoC-βH1 cell death was analysed by vital staining using propidium iodide and the Hoechst stain. We observed a rather high basal cell death rate, which was usually above 10% (Fig. 1A). The dead cells at basal conditions displayed often both chromatin condensation and fragmentation, suggesting secondary necrosis (Fig. 1B). When IL-1β and IFN-γ were added together there was a consistent increase in EndoC-βH1 cell death already after 8 h (Fig. 1A+B). At this early time point we observed both signs of increased early apoptosis (chromatin condensation and fragmentation without propidium iodide uptake) and secondary necrosis (similar to apoptosis but with propidium iodide positivity), indicating that the cells start out by executing the apoptotic programme, but that loss of plasma membrane integrity occurs before the entire apoptotic programme has been completed. To corroborate the morphological findings, we incubated the EndoC-βH1 cells with cyclosporine A and observed that cytokine-induced cell death was partially counteracted (Fig. 1C). Cyclosporine A blocks the intrinsic apoptotic pathway by preventing mitochondrial membrane permeability transition. Thus, cytokine-induced EndoC-βH1 cell death involves activation of mitochondrial events leading to early apoptosis and secondary necrosis. Similar cytokine-induced events seem to occur also in human islet cells, but at a slower rate (Delaney et al, 1997, Noguiera et al, 2013).

3.3. Cytokines induce phosphorylation of STAT-1, JNK, ERK, AMPK and eIF-2α, and promote degradation of IκB

Levels of P-STAT-1, IκB, P-JNK, P-ERK, P-AMPK, P-eIF-2α, P-PKB, P-GSK3 were analysed to determine which signalling pathways that are activated in response to cytokines in EndoC-βH1 cells. We observed that P-STAT-1, P-JNK and P-ERK were all increased already after 20 min (Fig. 2). Also NF-κB was activated at this time point as a decrease in IκB levels was observed. At 60 min P-JNK, P-ERK and NF-κB returned back to control levels. P-STAT-1 returned back to control levels at 300 min. P-AMPK was activated at 60 min and remained high at least until 300 min. P-eIF-2α was only increased at 60 min. We observed no effect on the phosphorylation of GSK3 and PKB. This suggests that cytokines induce a transient activation of classic cytokine-induced signalling pathways (STAT-1, NF-κB, MAPK, ER stress), but that only the AMPK was persistently activated by cytokines. This is in contrast to similar rodent experiments, in which these cytokine-induced signalling factors are often more persistently activated (Eizirik and Mandrup-Poulsen, 2001).

3.4. Cytokine-induced EndoC-βH1 cell death does not require *de novo* mRNA transcription

We next studied whether this cytokine-induced Endo-βH1 cell death requires transcriptional activation, which is the case in rodent islets (Welsh et al, 1991). For this purpose actinomycin D, an inhibitor of RNA polymerase II, was used. We observed that both cytokines and actinomycin D by themselves promoted increased EndoC-βH1 cell death (Fig. 3). However, cytokines were able to increase EndoC-βH1 cell death in the presence of all three concentrations of actinomycin D. This indicates that cytokine-induced cell death does not require *de novo* mRNA transcription.

3.5. Cytokine-induced EndoC-βH1 cell death does not require increased NF-κB or iNOS activity

To test whether NF-κB/iNOS activation promotes EndoC-βH1 cell death, we analysed cell death of EndoC-βH1 and betaTC-6 cells exposed to IL-1β + IFN-γ with or without the NF-κB inhibitor IKK inhibitor X and the iNOS inhibitor aminoguanidine. We observed that neither the NF-κB inhibitor nor the iNOS inhibitor affected cytokine-induced EndoC-βH1 cell death (Fig. 4A). In addition, we did not observe any detectable increase in nitrite production in EndoC-βH1 cells exposed to cytokines (Fig. 4B). On the other hand, both the NF-κB and the iNOS inhibitors counteracted cytokine-induced betaTC-6 cell death (Fig. 4C), which was paralleled by decreased nitrite levels (Fig. 4B). In addition, immunoblot analysis revealed that the iNOS protein was induced by cytokines in betaTC-6 cells, but not in EndoC-βH1 cells (Fig. 4D). Thus, in contrast to murine betaTC-6 cells, NF-κB activation in human EndoC-βH1 cells does not lead to induction of iNOS, the subsequent production of toxic levels of nitric oxide (NO), and cell death.

3.6. Cytokine-induced EndoC-βH1 cell death does not require increased STAT-1 activity

To investigate whether the IFN-γ-activated transcription factor STAT-1 (Pirot et al, 2008) initiates death of EndoC-βH1 cells, we knocked down STAT-1 using RNAi. This approach resulted in a 55% decrease in P-STAT-1 levels 48 h after the lipofection procedure (Fig. 5A). Yet, there was no protection against cytokine-induced

cell death (Fig. 5A). It may be that the 55% reduction in P-STAT-1 levels was insufficient to affect cytokine-induced cell death. We therefore also exposed EndoC-βH1 cells to the STAT-1 inhibitors epigallocathecin gallate (Menegazzi et al, 2001) and fludarabine (Frank et al, 1999) 15 minutes prior to and during an overnight cytokine culture period. Neither STAT-1 inhibitor protected against cytokine-induced cell death (Fig. 5B+C). This gives further support to the notion that STAT-1 does not mediate cytokine-induced EndoC-βH1 cell death.

3.7. Inhibitors of MAP kinases, the prostaglandins/leukotriene pathways and p53 did not affect cytokine-induced EndoC-βH1 cell death

Because MAP kinases have been reported to participate in cytokine-induced rodent beta-cell death (Ammendrup et al, 2000, Pavlovic et al, 2000, Saldeen et al, 2001), we incubated EndoC-βH1 cells with p38, JNK and ERK (SB203580, SP600125 and PD98059, respectively) inhibitors. None of these inhibitors counteracted cytokine-induced EndoC-βH1 cell death (Fig. 6A). We also incubated EndoC-βH1 cells with inhibitors of phospholipase A_2, lipooxygenase (Lox) and cyclooxygenase (Cox), because prostaglandins and leukotrienes have been suggested to promote beta-cell damage (Luo, Wang, 2011). Also in this case no protection was observed (Fig. 6B). The supplementation of 10 μM of prostaglandin E_2 to the culture medium did not further increase cytokine-induced cell death (Fig. 6B). Also the inhibitor of p53, pifiphirin-alpha, failed to protect against cytokine-induced cell death (Fig. 6C).

3.8. Cytokines decrease cellular ATP contents and mitochondrial respiration, and the AMPK activator AICAR protected against cytokine-induced EndoC-βH1 cell death

As the AMPK has been implicated in cytokine-induced beta-cell damage (Allagnat et al, 2013, Riboulet-Chavey et al, 2008), we next studied whether the ATP contents of EndoC-βH1 cells were affected by cytokine treatment. The combination of IL-1β and IFN-γ promoted a rapid decrease of the cell ATP content (Fig. 7A). At 6 h of cytokine treatment, ATP contents were reduced dramatically with 41% (Fig. 7A), which most probably explains the parallel activation of AMPK (Fig. 2). To explain the reduction in ATP, we next assessed EndoC-βH1 cell respiration using the Seahorse technique. A 7 h cytokine exposure resulted in a modestly reduced mitochondrial respiration at basal conditions (5.6 mM glucose) (Fig. 7B+C). The decreased respiration was mainly explained by a lowered mitochondrial ATP turnover, whereas no effect on proton leakage was observed (Fig. 7C). Cytokines also reduced the maximal respiration in the presence of FCCP (Fig. 7C). The AMPK activator AICAR promoted a lowering of basal respiration and ATP turnover rates (Fig. 7C). However, when AICAR was combined with cytokines, maximal respiration, basal respiration and ATP turnover was partially restored as compared to cytokine exposure only. These findings suggest that the cytokine-induced loss of ATP, at least in part, was caused by a lowered mitochondrial respiration, and that AICAR counteracts this effect.

Because AICAR improved mitochondrial respiration in the presence of cytokines, we next studied whether AICAR and the AMPK inhibitor Compound C affected cytokine-induced death rates. Compound C (2 μM) increased basal death rates, but did not affect cytokine-induced rates (Fig. 7D). AICAR, however, protected dose-

dependently against cytokine-induced cell death so that at a concentration of 1.0 mM of the activator cell death rates were restored back to control levels (Fig. 7D).

4. Discussion

Much due to the low availability and difficulties in isolating pure human primary beta-cells, the mechanisms by which pro-inflammatory cytokines induce death of this particular cell type are still largely unknown. However, a recent publication reported the successful generation of a new human beta-cell line, EndoC-βH1 (Rassavard et al, 2011), that displays similar insulin release and stimulus-secretion coupling as human islets (Andersson et al, 2015), which to some extent may circumvent the difficulties of obtaining pure and functional human beta-cells. We presently observed that the EndoC-βH1 cells express a majority of typical beta-cell specific genes at levels similar to those of human islet beta-cells. However, because the isolated human islet is composed of only 30-50% beta-cells, with the rest being alpha-cells, delta-cells, exocrine cells and duct cells, there were many differentially expressed genes in EndoC-βH1 cells as compared to human islets. In addition, EndoC-βH1 cells are transformed beta-cells that grow in monolayers, which is reflected by the decreased expression of cell-to-cell contact genes, and that continuously proliferate, which is reflected by the increased expression of mitosis-associated genes. Both the continuous proliferation and the monolayer growth probably explain the rather low insulin content of EndoC-βH1 cells, as a recent publication has reported that cessation of EndoC-βH2 cell proliferation results in a markedly improved insulin production of the beta-cells (Scharfmann et al, 2014), and since a three-dimensional organization of beta-cells is known to improve beta-cell function (Meda, 2013). Despite the observed

differences in gene expression between EndoC-βH1 cells and human islets, our results demonstrate that EndoC-βH1 cells retain many of the characteristics typical for primary human beta-cells, including a similar expression of genes associated with IL-1β- and IFN-γ-induced signal transduction, and it is possible that the cytokine-induced signalling events that occur in EndoC-βH1 cells, at least in part, represent those of primary human beta-cells.

Similar to previous finding with rodent and human islets (Pirot et al, 2008), we observe that EndoC-βH1 cells respond to cytokines by transiently activating NF-κB, STAT-1, ERK, JNK and eIF-2α. However, and in variance with what has been observed in rodent beta-cells, these particular events did not result in an increased cell death. Also inhibition of p53, p38 MAPK, phospholipase A_2, cyclooxygenase (Cox) and lipooxygenase (Lox) failed to rescue EndoC-βH1 cells from cytokine-induced death. Instead, EndoC-βH1 cell death was; a) rapid (starting at 8 hours after addition of the cytokines); b) independent from *de novo* mRNA synthesis; c) occurring via secondary necrosis; d) paralleled by a gradual AMPK activation; e) paralleled by ATP depletion and decreased mitochondrial respiration; and f) counteracted by the AMPK activator AICAR. These observations collectively suggest that the IL-1β + IFN-γ cytokine combination promotes an energy crisis of the EndoC-βH1 cells, and that a pronounced activation of AMPK rescues the cells from the lack of ATP. Indeed, the AMPK is activated by a lowered ATP/AMP ratio and protects from ATP depletion by reducing ATP consumption rates. Interestingly, cytokines have been reported to promote AMPK activation (Riboulet-Chavey et al, 2008) and a gradual loss of ATP starting at 24 h in rat islets (Collier et al, 2006), which is somewhat slower than in the EndoC-βH1 cells. However, in studies using rodent cells the AMPK was instead

assigned a pro-apoptotic role in cytokine-induced beta-cell death (Collier et al, 2006, Riboulet-Chavey et al, 2008, Allagnat et al, 2013, Santos et al, 2011). The reason for these contradictory and opposite roles of AMPK is not clear, but in rodent beta-cells AMPK activation participated in iNOS induction (Santos et al, 2011), suggesting that AMPK in these cells aggravates NO-mediated inhibition of the Krebs cycle enzyme aconitase and mitochondrial respiration (Welsh et al, 1991). This is in contrast to the human EndoC-βH1 cells, in which iNOS expression is not induced in response to cytokines, even though AMPK is activated, which means that the AMPK may play a different role in human than in rodent beta-cells.

It is unclear whether also human primary beta-cells respond to inflammatory cytokines by lowering their ATP contents. We analysed ATP in human islets, but failed to observe any effect of IL-1β + IFN-γ when present during culture for up to 3 days (results not shown). This argues against ATP depletion, but as human islets usually contain not more than 30-50% beta-cells (Welsh et al, 2005, Scharfmann et al, 2014), it can be envisaged that cytokine-induced effects on non-beta-cells might have masked any putative effect on the beta-cells. Analysis of beta-cell ATP contents and AMPK activation in cytokine-treated intact human islets, although experimentally challenging, is highly warranted.

It was early reported that human islets produce NO when cultured in the presence of cytokines (Corbett et al, 1993). However, although results are conflicting, it has been observed that the bulk of the NO produced is derived from non-beta-cells, such as duct cells (Pavlovic et al, 1999), and that cytokines damage human islets by NO-independent mechanisms (Eizirik et al, 1994, Rabinovitch et al, 1994). The findings of the present study concur to the notion that human beta-cells fail to induce

substantial expression of the iNOS gene when challenged with cytokines, which probably explains why NO inhibition, at least in some cases, does not protect human beta-cells against cytokines. Furthermore, as it has been reported that human beta-cells are more resistant than rodent beta-cells to streptozotocin, alloxan, nitric oxide donors and hydrogen peroxide (Welsh et al, 1995), it is likely that human islets not only produce less NO, but also have a better defence against oxidative/nitrosative stress.

Our finding that human EndoC-βH1 cells die from an energy crisis rather than from persistent activation of inflammatory transcription factors, for example STAT-1, was unexpected. However, it should be emphasized that the present findings do not exclude an important role of pro-inflammatory mediators in primary human beta-cells. For example, it can be envisaged that the energy crisis is particularly rapid and dramatic in proliferating EndoC-βH1 cells *in vitro*, and if the loss of ATP is less pronounced *in vivo,* other cell death mechanisms, perhaps in synergy with a partial decrease in ATP, could exert important roles in other stages of the beta-cell death process.

In summary, our present findings motivate increased focus on the AMPK as an energy saver and beta-cell anti-apoptotic factor during conditions of inflammation. Indeed, the AMPK has been reported to mediate beta-cell survival at other circumstances than those presently investigated (Meares et al, 2010). It is also noteworthy that the Type-2 diabetes drug metformin, which seems to promote some beneficial effects also in Type-1 diabetes (Lund et al, 2008), is known to activate the AMPK. Although metformin is preferentially taken up in liver cells, it is possible that it accumulates also in beta-cells when present throughout prolonged time periods (Leclerc et al,

2004), which might confer protection to beta-cells against inflammatory conditions and loss of ATP, explaining, at least in part, some of its anti-diabetic properties.

RGF, JC and CK performed the experiments and analyzed data. AA performed the bioinformatic analysis. PB, PR, RS and NW analyzed data and wrote the manuscript.

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674

GENE	Human islets (RPKM)	EndoC-βH1 (RPKM)
PDX1	15,9±3,9	81,6±8,8*
MAFA	36,4±18,2	37,3±10,9
MAFB	51,7±12,7	153±38,6
NKX2-2	9,1±2,1	15,9±0,6
NKX6-1	14,1±6,5	23,6±1,5
ISL1	33,8±6,8	55,5±9,4
FOXA2	27,8±7,0	50,9±1,8
NEUROD1	75,5±16,5	80,6±0,3
NEUROG3	0	1,4±0,25*
PAX4	0	0,5±0,18
PAX6	33,8±8,3	33,7±3,0
GCK (Glucokinase)	6,1±1,5	43,3±8,1*
HK1 (Hexokinase)	10,5±1,5	0,15±0,02*
SLC2A1 (Glut1)	17,5±4,4	14,0±3,1
SLC2A2 (Glut2)	2,9±1,9	4,2±1,1
SLC2A4 (Glut4)	0,1±0,02	0,1±0,02
LDHA (lactate dehydrogenase)	119±25,6	65,6±4,0
SLC30A8 (ZnT8)	237±58,1	21,6±3,9*
KCNJ11 (potassium inwardly-rectifying channel)	9,1±2,0	20,9±1,5
INS (insulin)	4040±1260	3120±679
IAPP (islet amyloid protein)	661±169	38,7±9,3*
PCSK1 (proprotein convertase 1)	195±32,6	119±11,3

675 *Table 1. Expression in human islets and EndoC-βH1 cells of genes with particular*
676 *importance for beta-cell function cells as determined by RNA-seq. Results are*
677 *means±SEM for three independent observations. * denotes p<0.001 using Student's t-*
678 *test.*

679

GENE	Human islets (RPKM)	EndoC-βH1 (RPKM)
IL1R1	21,6±5,0	1,0±0,1
IL1RAP	2,9±0,6	8,5±3,3
IRAK1	16,9±1,6	29,1±3,4
IRAK4	10,1±0,5	5,4±1,1
MYD88	15,2±3,2	8,9±0,9
TOLLIP	16,5±2,9	28,3±2,0
TRAF6	4,1±0,5	3,1±0,5
UBE2N	12,1±0,9	14,8±1,0
UBE2V1	15,6±1,3	28,9±1,8
ECSIT	7,0±1,0	8,0±1,2
TAB1	5,4±0,4	9,5±1,7
TAB2	31,4±5,8	25,2±5,8
MAP3K1	12,6±2,1	3,2±0,2
MAP3K7	19,8±1,9	17,1±4,1
IKBKB	6,8±1,1	4,8±0,4
IKBKG	1,1±0,2	0,9±0,2
CHUK	21,0±2,3	31,8±8,7
MAP3K14	5,3±1,1	2,0±0,3
MAP2K4	14,6±1,0	20,6±2,5
MAP2K6	2,1±0,2	14,2±4,2
MAPK8	22,6±0,8	32,3±4,4
MAPK9	12,1±1,3	9,9±0,5
MAPK10	6,4±1,0	9,8±1,7
MAPK14	14,9±0,4	23,1±1,0
MAPK13	22,8±1,1	67,2±10,2
MAPK12	1,4±0,5	6,0±1,1
NFKB1	16,4±4,7	7,7±0,2
RELA	10,4±1,4	6,5±0,4
NFKBIA	112±76,4	22,8±1,4
NFKBIB	4,2±1,1	4,0±0,3
NFKBID	1,1±0,2	1,2±0,1
IFNGR1	57,9±7,2	21,7±4,1
IFNGR2	37,1±12,4	30,4±4,0
JAK1	77,8±8,3	24,9±4,1
JAK2	4,2±0,3	2,42±0,7
MAPK1	36,9±0,4	50,9±2,5
MAPK3	16,9±1,8	20,9±2,8
PTK2B	6,35±0,8	2,2±0,1
PTPN11	15,7±1,7	15,5±4,0
MAP3K4	17,4±1,8	37,2±2,1
PLCG2	3,2±0,8	31,9±2,5*
PRKCA	6,2±1,2	13,2±1,2
SRC	6,9±1,1	14,2±0,9
PRKCD	8,6±1,3	11,0±0,7
CAMK2G	15,6±0,9	7,5±0,5
CAMK2B	4,2±0,9	2,9±0,2
CAMK2D	15,2±1,6	11,9±2,0
SOCS1	3,5±1,6	0,4±0,1

*Table 2. Expression of genes in human islets and EndoC-βH1 cells that participate in IL-1β- and IFN-γ-induced signalling as determined by RNA-seq. Results are means±SEM for three independent observations. * denotes p<0.001 using Student's t-test.*

LEGENDS TO THE FIGURES

Fig. 1. EndoC-βH1 cells die in response to cytokine exposure by apoptosis and secondary necrosis. EndoC-βH1 cells were exposed to the cytokines IL-1β (20 ng/ml) + IFN-γ (20 ng/ml) for various time points and then labelled with propidium iodide and Hoechst for 10 min. (A) Results were quantified by fluorescence microscopy and counting of the cells showing signs of apoptosis (nuclear condensation/fragmentation) or primary/secondary necrosis (propidium iodide fluorescence). Results are sum of both apoptosis and necrosis and represent 3 independent observations ± SEM. (B) Photographs showing typical patterns of cell death. At 0 and 4 hours basal levels of apoptosis and necrosis were observed. At 8 hours an increased number of apoptotic (condensed/fragmented nuclei, arrows) and secondary necrotic (propidium iodide positive, arrows with circleheads) cells was observed. (C) EndoC-βH1 cells were cultured 21 h with cytokines (Cyt) with or without cyclosporine A (CyA, 10 μM) and then labelled with propidium iodide and analysed with flow cytometry. Results are means ± SEM for 3 independent experiments. * denotes p<0.05 vs. control.

Fig. 2. IL-1β + IFN-γ induces phosphorylation of STAT-1, JNK, ERK, AMPK and eIF-2α, but not PKB and GSK3, and induces degradation of IκB in EndoC-βH1 cells. Cells were exposed to IL-1β + IFN-γ for various time periods (x-axis) and then analyzed for activation of signaling factors using immunoblot analysis. Results are normalized to total ERK signals and are means ± SEM for 4 independent experiments. * denotes $p<0.05$ vs 0 min of cytokine exposure. The lower right panel shows bands for the different antibodies obtained from one filter stripped between the different antibody incubations.

Fig. 3. Actinomycin D does not protect against cytokine-induced EndoC-βH1 cell death. EndoC-βH1 cells were pre-exposed actinomycin D (AmD) for 15 min and then cultured for another 8 hours in the presence of IL-1β + IFN-γ before analysis of cell death using propidium iodide staining and flow cytometry. Results are means ± SEM for 4 experiments. * denotes $p<0.05$ using Students paired t-test when comparing vs. corresponding group without cytokine exposure.

Fig. 4. Inhibitors of iNOS and NF-κB do not protect against cytokine-induced EndoC-βH1 cell death. (A) EndoC-βH1 cells were pre-exposed to 750 μM of the iNOS inhibitor aminoguanidine (AG) or to 10 μM of the NF-κB inhibitor IKK inhibitor X (NF-κB inh) for 15 min and then cultured for another 18 hours in the presence of IL-1β + IFN-γ before analysis of cell death using propidium iodide staining and flow cytometry. Results are means ± SEM for 3-4 experiments. * denotes $p<0.05$ using Students paired t-test when comparing vs. corresponding group without cytokine exposure. (B) Culture medium from cells in Fig. 4A + B were analyzed for nitrite

content using the Griess reagent. * denotes $p<0.05$ using Students paired t-test when comparing vs. the group with cytokine exposure only. (C) BetaTC-6 cells were incubated and analyzed as in (A). Results are means ± SEM for 3 experiments. * denotes $p<0.05$ using Students paired t-test when comparing vs. the group with cytokine exposure only. (D) EndoC-βH1 and betaTC-6 from Figures 4A and 4C were analyzed for iNOS protein expression using immunoblotting.

Fig. 5. Inhibition of STAT-1 does not prevent cytokine-induced EndoC-βH1 cell death. (A) EnodC-βH1 cells were treated with control or STAT-1 siRNA. Two days later cells were exposed to IL-1β + IFN-γ overnight and cell death rates were analyzed by flow cytometry. For immunoblot analysis of P-STAT-1 cells were exposed to cytokines for 30 min. Upper panel shows the means ± SEM for 4 experiments and the lower panel shows a 55% knockdown of Phospho-STAT-1 induced by siRNA treatment. The intensities of the P-STAT-1 bands were normalized to total protein loading and transfer, as assessed by amidoblack staining. (B) and (C) EndoC-βH1 cells were pre-exposed to 10 μM epigallocathecin gallate (EGCG) or 50 ng/ml fludarabine (Flud) for 15 min and then cultured for another 18 hours in the presence of IL-1β + IFN-γ before analysis of cell death using propidium iodide staining and flow cytometry. Results are means ± SEM for 4 independent observations.

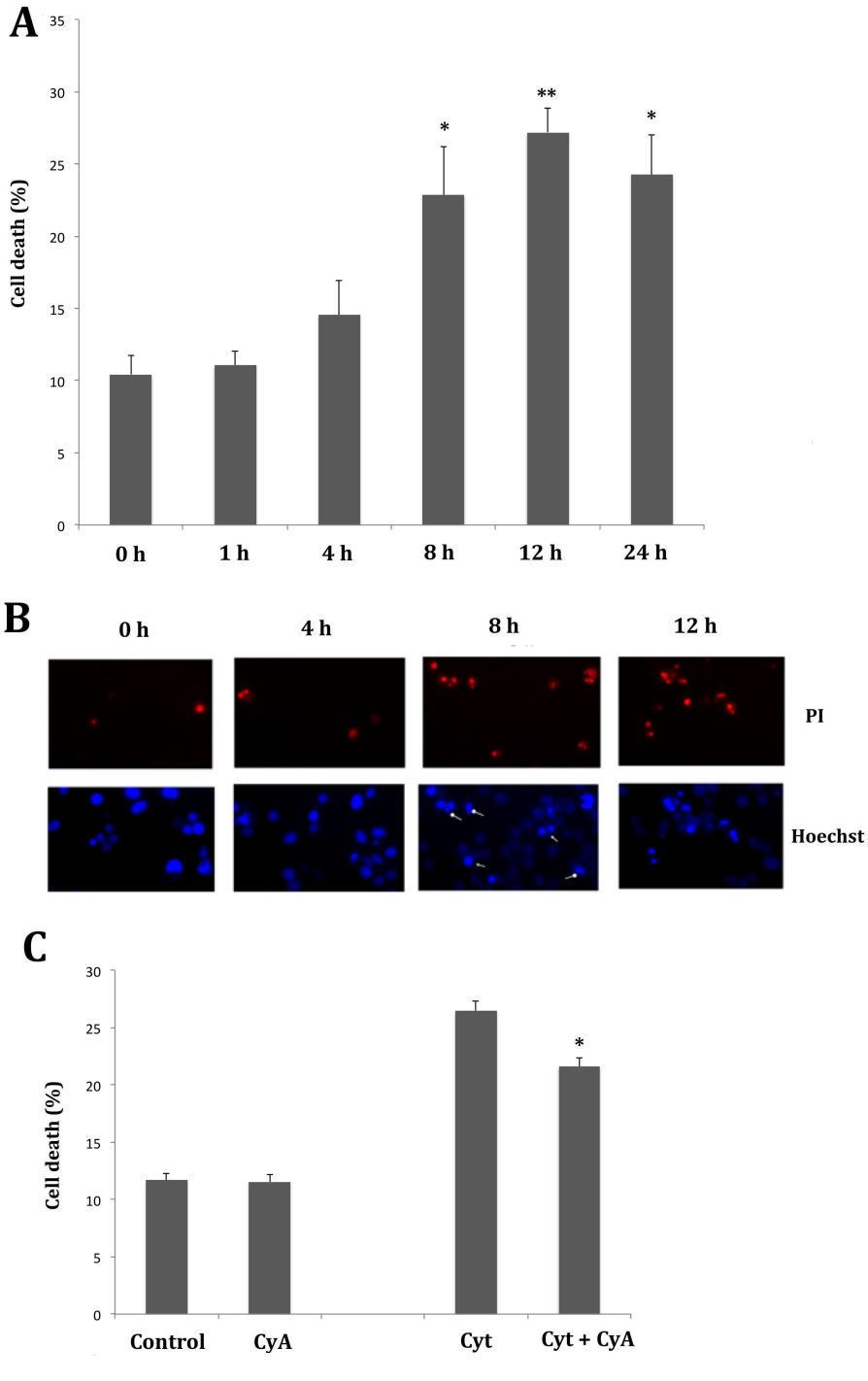
Fig. 6. Inhibitors of MAPK, Lox, Cox, phospholipase A_2 and p53 do not protect against cytokine-induced EndoC-βH1 cell death. (A) EndoC-βH1 cells were pre-exposed to 20 μM PD98059 (PD), 10 μM SB203580 (SB) or 10 μM SP600125 (SP) for 15 min and then cultured for another 18 hours in the presence of IL-1β + IFN-γ before analysis of cell death using PI-staining and flow cytometry. (B) EndoC-βH1

cells were incubated with 10 μM of PGE_2, 5 μM of the Lox-5 inhibitor 5,8,11-Eicosatriynoic acid (LTH), 25 μM of the Cox inhibitor indomethacin (IM) and 10 μM of the phospholipase A_2 inhibitor bromophenacyl bromide (BRB) as above. (C) EndoC-βH1 cells were incubated with 10 μM of the p53 inhibitor pifithrin-alpha (p53 inh). Results are means±SEM for 3-4 experiments.

Fig. 7. Cytokines decrease ATP contents and oxygen consumption of EndoC-βH1 cells, and the AMPK inhibitor AICAR protected against cytokine-induced cell death. *(A)* EndoC-βH1 cells were exposed to IL-1β + IFN-γ for 3, 6 or 24 h and then analyzed for ATP contents using and ELISA kit. Results are means ± SEM. N=6 and * denotes $p<0.05$ using Student's t-test when comparing vs. 0 h of cytokine exposure. (B and C) EndoC-βH1 cells were incubated for 6 + 1 h at 5.6 mM glucose with or without cytokines and 1 mM AICAR, and then analyzed for oxygen consumption rates (OCR) using the Seahorse technique. Results are means ± SEM. N=6, each performed in at least triplicates, and * denotes $p<0.05$ using one-way ANOVA and Holm-Sidak's multiple comparison test when comparing vs. control. # denotes $p<0.05$ when comparing vs. the cytokine group. *(D)* EndoC-βH1 cells were pre-exposed to 2 μM Compound C (CC) or 0.5 and 1.0 mM AICAR for 15 min and then cultured for another 18 hours in the presence of IL-1β + IFN-γ before analysis of cell death using PI-staining and flow cytometry. Results are means ± SEM for 4-7 independent observations. * denotes $p<0.05$ using Students paired t-test when comparing vs. the cytokine exposed group.

774

775 Fig. 1



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777 Fig. 2

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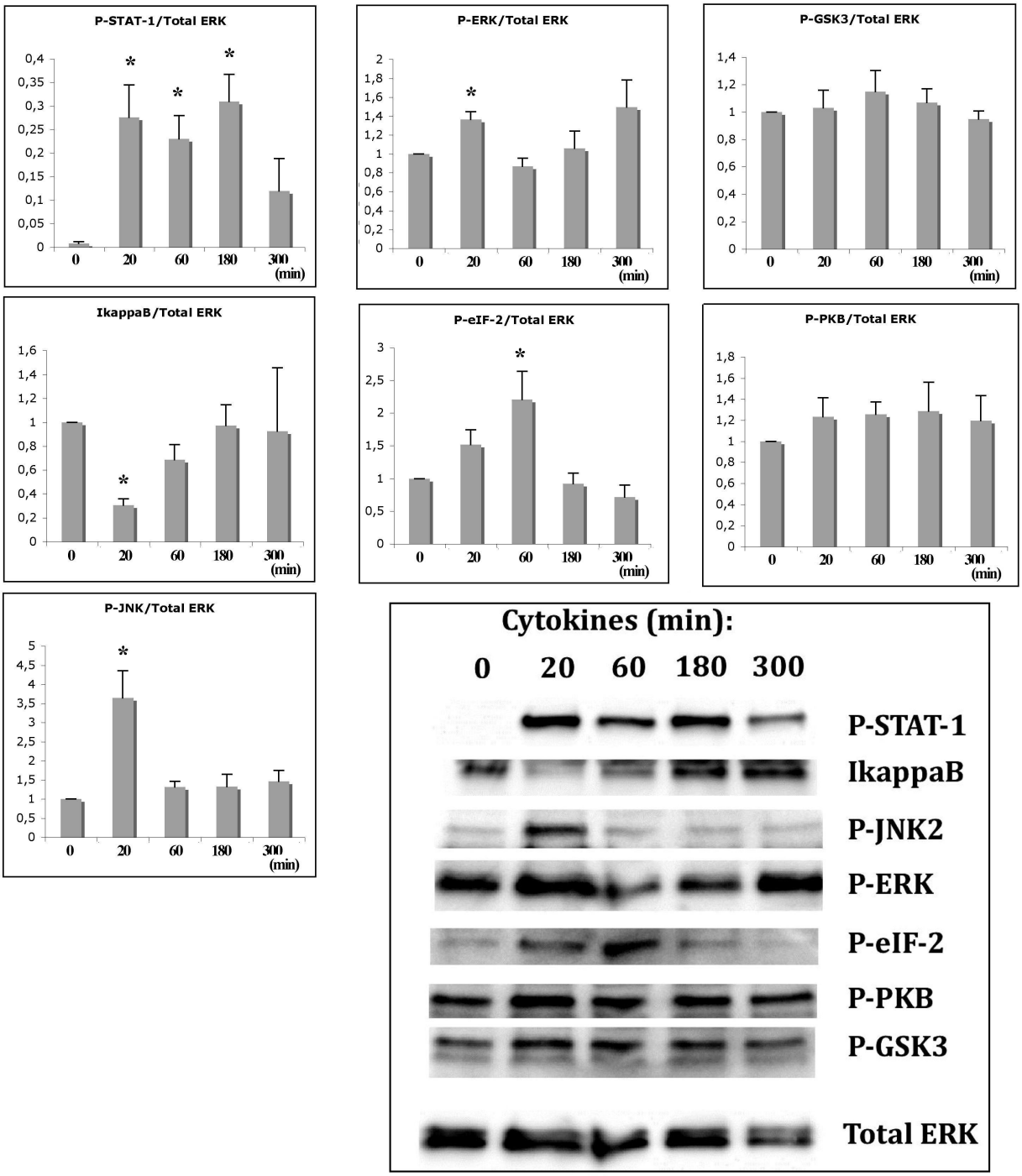

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781 Fig. 3
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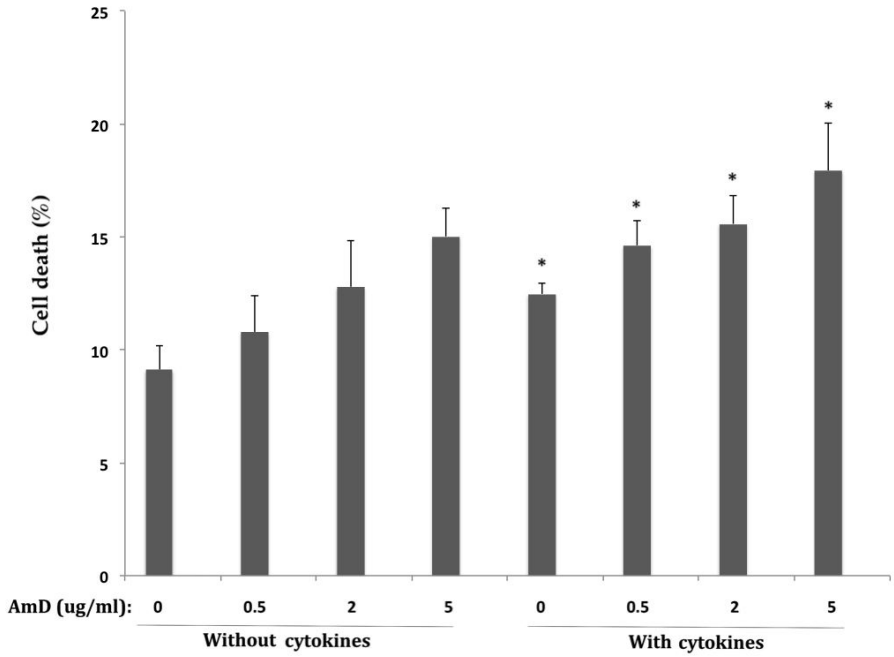

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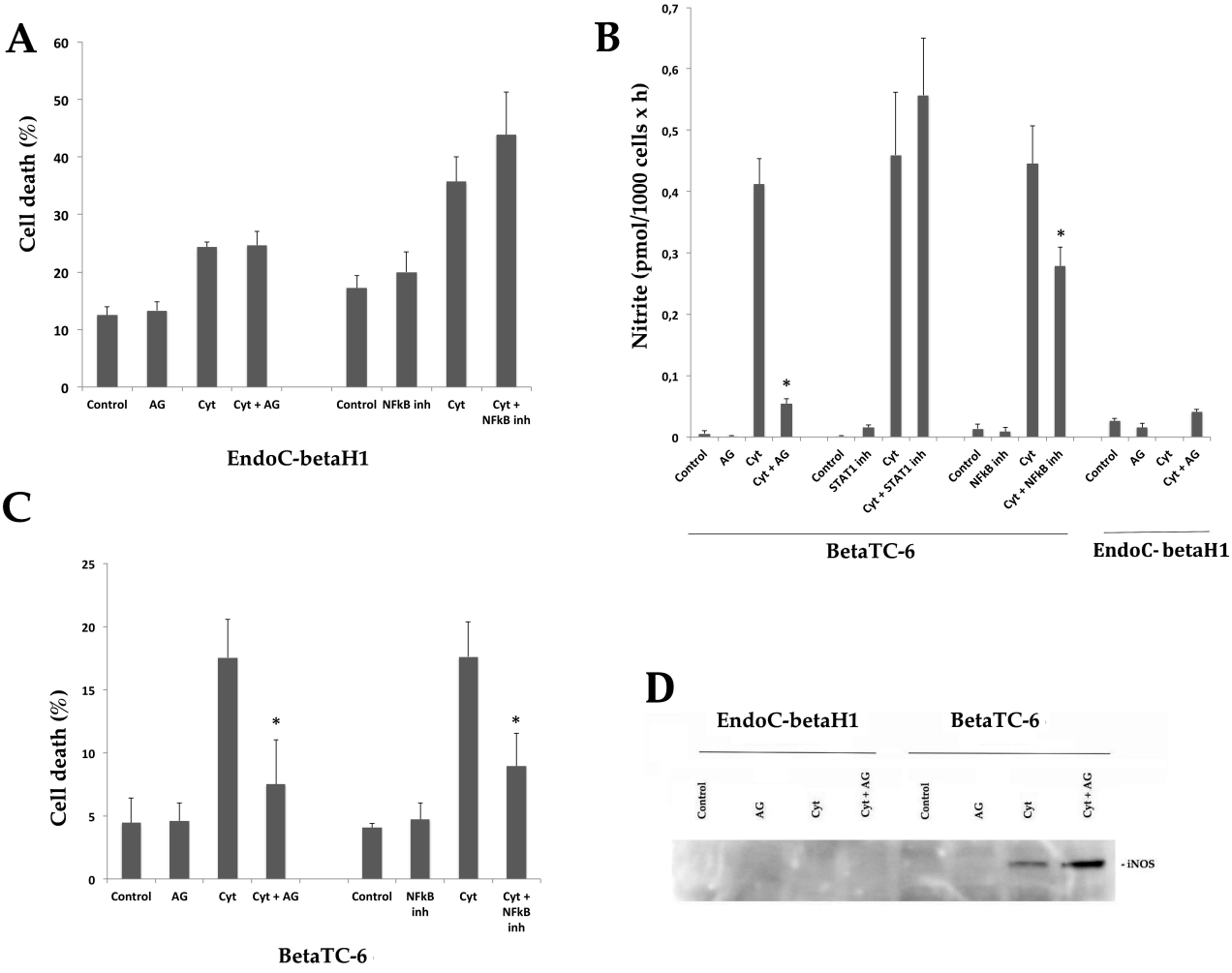
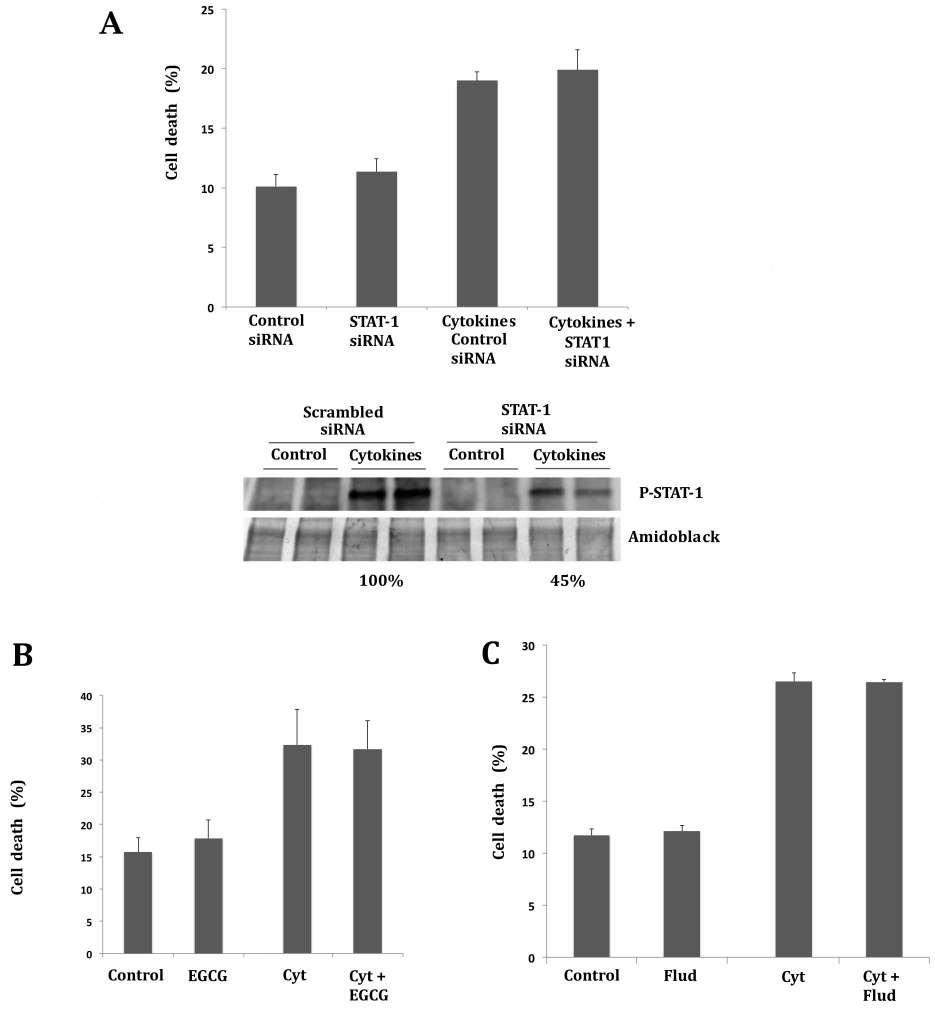
785 Fig. 4


A
Cell death (%)
60
50
40
30
20
10
0
Control
AG
Cyt
Cyt + AG
Control
NFkB inh
Cyt
Cyt + NFkB inh
EndoC-betaH1
B
Nitrite (pmol/1000 cells x h)
0,7
0,6
0,5
0,4
0,3
0,2
0,1
0
Control
AG
Cyt
Cyt + AG
Control
STAT1 inh
Cyt
Cyt + STAT1 inh
Control
NFkB inh
Cyt
Cyt + NFkB inh
Control
AG
Cyt
Cyt + AG
BetaTC-6
EndoC- betaH1
C
Cell death (%)
25
20
15
10
5
0
Control
AG
Cyt
Cyt + AG
Control
NFkB inh
Cyt
Cyt + NFkB inh
BetaTC-6
D
EndoC-betaH1
BetaTC-6
Control
AG
Cyt
Cyt + AG
Control
AG
Cyt
Cyt + AG
- iNOS

787

788 Fig. 5



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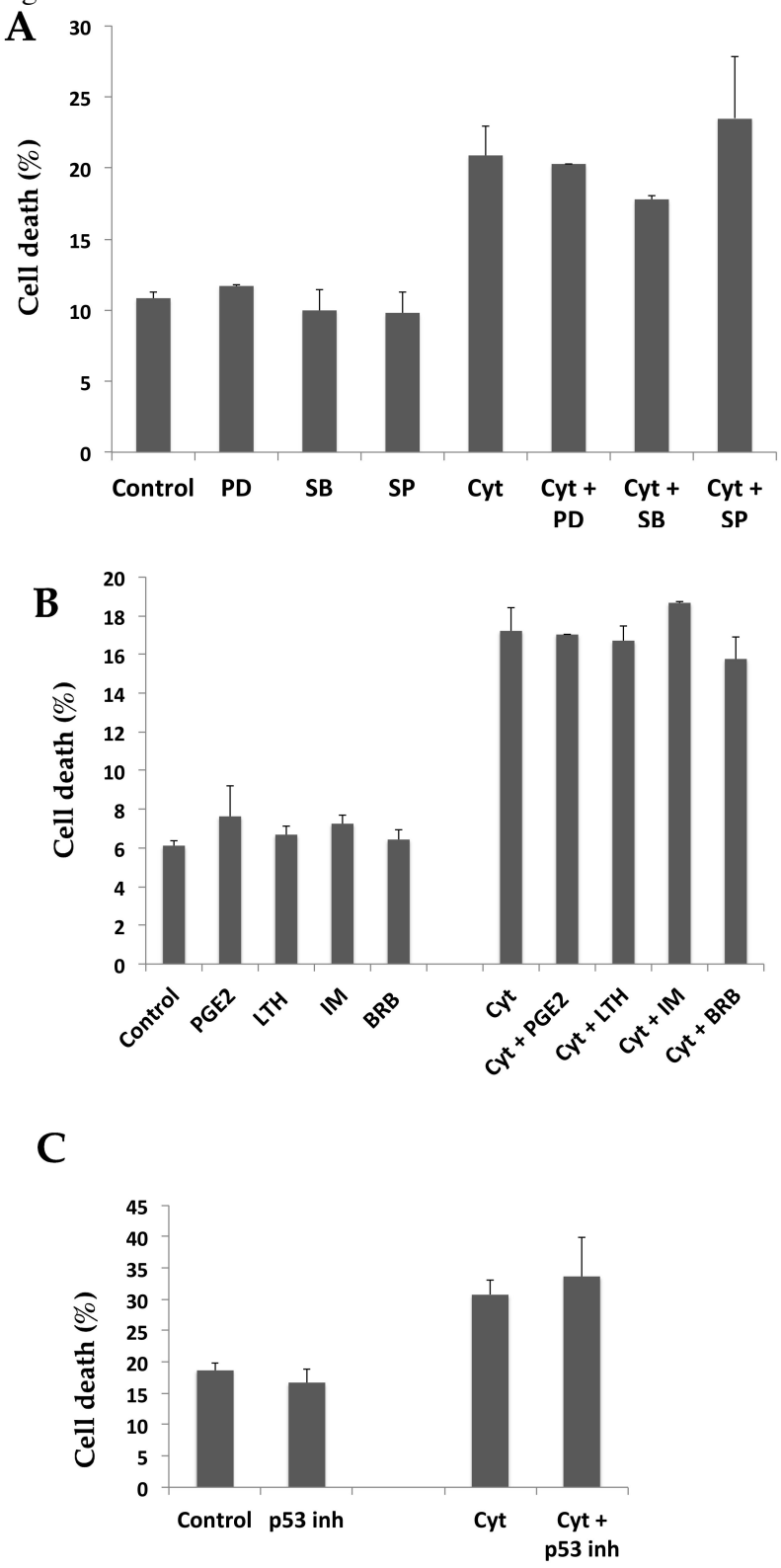
791 Fig. 6



792
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794 Fig. 7

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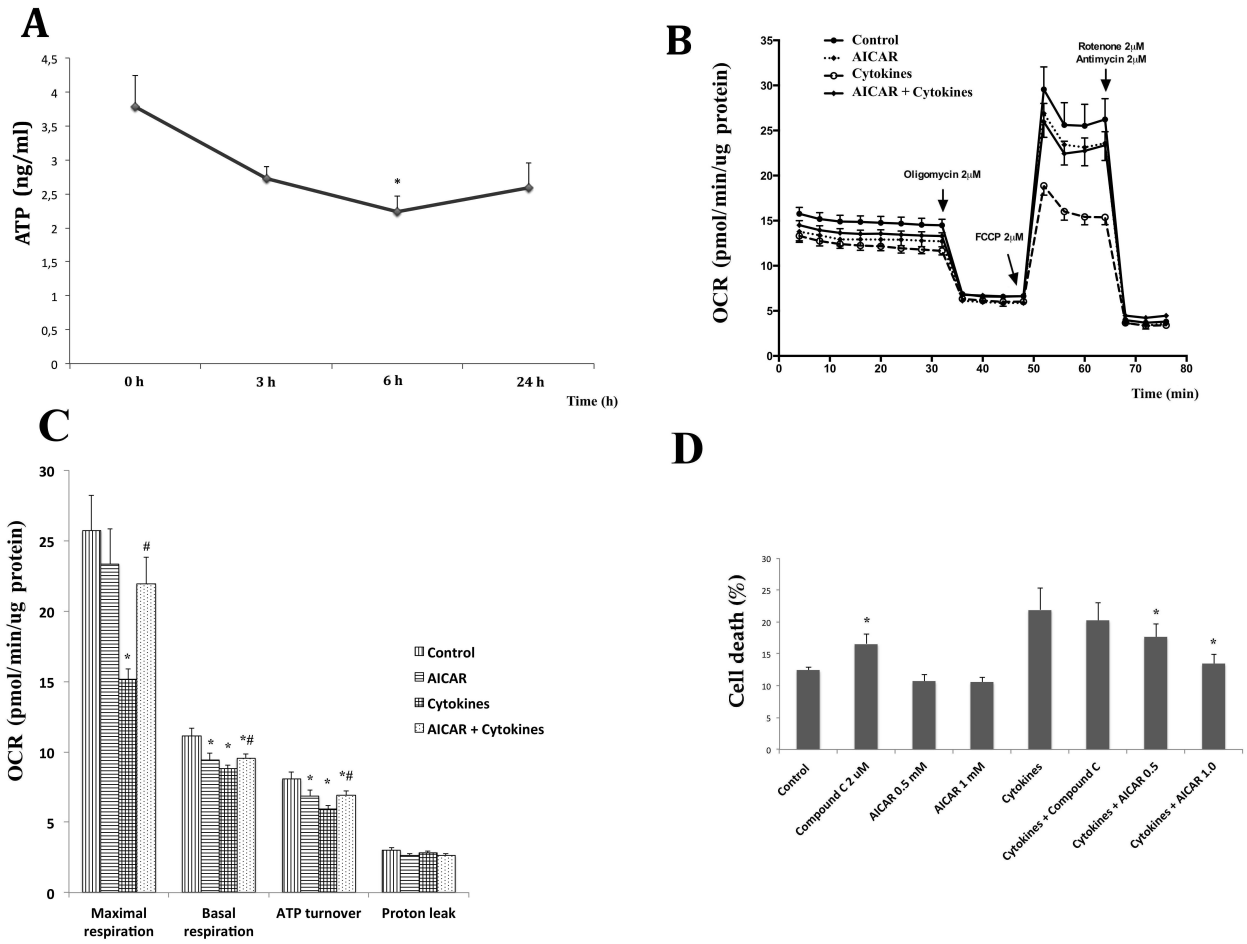

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