The immunoproteasome is induced by cytokines and regulates apoptosis in human islets

Morten Lundh^{1,2,a}, Marco Bugliani^{3,a}, Tina Dahlby¹, Danny Hung-Chieh Chou², Bridget Wagner², Seyed Mojtaba Ghiasi¹, Vincenzo De Tata¹, Zhifei Chen¹, Marianne Nissan Lund^{1,4}, Michael J Davies¹, Piero Marchetti^{3,b} and Thomas Mandrup-Poulsen^{1,b}

¹Department of Biomedical Sciences, University of Copenhagen, Copenhagen, Denmark

²Chemical Biology and Therapeutics Program, Broad Institute of Harvard and MIT, Boston, Massachusetts, USA

³Department of Clinical and Experimental Medicine, University of Pisa, Pisa, Italy

⁴Department of Food Science, University of Copenhagen, Copenhagen, Denmark

^a(M Lundh and M Bugliani contributed equally as co-first authors)

^b(P Marchetti and T Mandrup-Poulsen contributed equally as co-senior authors)

Correspondence should be addressed to T Mandrup-Poulsen
Email
tmpo@sund.ku.dk

Abstract

In addition to degrading misfolded and damaged proteins, the proteasome regulates the fate of cells in response to stress. The role of the proteasome in pro-inflammatory cytokine-induced human beta-cell apoptosis is unknown. Using INS-1, INS-1E and human islets exposed to combinations of IFN γ , IL-1 β and TNF α with or without addition of small molecules, we assessed the role of the immunoproteasome in pancreatic beta-cell demise. Here, we show that cytokines induce the expression and activity of the immunoproteasome in INS-1E cells and human islets. Cytokine-induced expression of immunoproteasome subunits, but not activity, depended upon histone deacetylase 3 activation. Inhibition of JAK1/STAT1 signaling did not affect proteasomal activity. Inhibition of the immunoproteasome subunit PSMB8 aggravated cytokine-induced human beta-cell apoptosis while reducing intracellular levels of oxidized proteins in INS-1 cells. While cytokines increased total cellular NF κ B subunit P50 and P52 levels and reduced the cytosolic NF κ B subunit P65 and I κ B levels, these effects were unaffected by PSMB8 inhibition. We conclude that beta cells upregulate immunoproteasome expression and activity in response to IFN γ , likely as a protective response to confine inflammatory signaling.

Key Words

- ▶ diabetes
- ▶ islet cells
- ▶ cytokines
- ▶ inflammatory diseases

Journal of Endocrinology
(2017) **233**, 369–379

Introduction

The proteasome is responsible for cellular protein degradation, but in addition to eliminating damaged or misfolded proteins targeted for breakdown by poly-ubiquitination, the proteasome also regulates key cellular processes such as proliferation, growth, differentiation, gene transcription, signaling and apoptosis

by modulating the half-life of cell cycle regulators and the processing of transcriptional activators and repressors (Bhattacharyya *et al.* 2014).

Proteasomal activity generates peptide fragments of exogenous or endogenous proteins that are complexed to major histocompatibility complex class I molecules

for presentation to CD8⁺ T-cells, with this process being critical for adaptive immunity, immune surveillance and immune tolerance (Ferrington & Gregerson 2012). By processing the p105 and p100 subunits of the master inflammatory transcription factor NFκB to their p50 and p52 forms, the proteasome is also a key regulator of innate immunity and inflammation (Rape & Jentsch 2002). These functions are executed by the so-called immunoproteasome (i-proteasome) consisting of the 20S core particle in which the catalytic β1, 2 and 5 constitutive subunits (also known as Psmb6/LMP19, Psmb7/LMP9 and Psmb5/LMP17, respectively) are substituted with the cytokine-inducible β1i, 2i and 5i subunits (also termed Psmb9/LMP2, Psmb10/MECL-1 and Psmb8/LMP7, respectively) (Ferrington & Gregerson 2012). The i-proteasome lid function is fulfilled by complexing with the 11S regulatory heptameric particle. Since i-proteasome processes smaller proteins and peptides, it is ubiquitin independent and does not possess ATPase activity (Ferrington & Gregerson 2012). An intermediary proteasome composed of a mixture of subunits from the s- and i-proteasomes, and a thymus-specific proteasome with a β5t for β5 substitution, also exists.

Emerging evidence supports the hypothesis that the s-proteasome has important functions in pancreatic beta cells (Hartley *et al.* 2009). The s-proteasome balances beta-cell proinsulin levels (Kitiphongspattana *et al.* 2005) and K/ATP channel expression (Yan *et al.* 2005), and degrades Ucp2 and 3 proteins, improving glucose-stimulated insulin secretion (Brand *et al.* 2010). The s-proteasome protects beta cells from lipotoxic ER stress and maintains an anti-apoptotic Bcl2 protein family balance (Litwak *et al.* 2015). Glucotoxicity inhibits s-proteasome activity leading to caspase-3-dependent apoptosis in INS-1E cells and human islets, an effect mimicked by s-proteasome inhibition (Broca *et al.* 2014).

Although the proteasome degrades the interleukin-1 receptor-activated kinase IRAK after IL-1-induced phosphorylation (Yamin & Miller 1997), inhibition of the s-proteasome reduces IL-1-induced beta-cell iNOS expression, NO synthesis and PGE2 production, possibly by preventing s-proteasome-dependent activation of the NFκB pathway (Kwon *et al.* 1998). Accordingly, s-proteasome inhibition modestly decreases cytokine-induced islet toxicity, while suppressing islet insulin release (Storling *et al.* 2005).

The function(s) of the i- or intermediate proteasomes in beta cells are poorly understood. The 11S regulatory particle of the i-proteasome has been reported to mediate

MafA degradation leading to impaired beta-cell function (Kanai *et al.* 2011). However, the role of the i-proteasome in inflammatory beta-cell dysfunction or viability is unknown. Here, we hypothesized that the expression of the s- and i-proteasome subunits are differentially regulated by inflammatory cytokines and that inhibition of i-proteasome activity affects cytokine-induced beta-cell apoptosis.

Materials and methods

Cell culture and reagents

INS-1 and INS-1E cells (generously provided by Claes Wollheim and Pierre Maechler, University of Geneva, Switzerland) were maintained as previously described (Storling *et al.* 2005, Chou *et al.* 2012). Cells were mycoplasma negative. Human pancreatic islets were isolated by collagenase digestion, density gradient purification and handpicking from the glands of 16 organ donors (for donor characteristics, Table 1) and then cultured in M199 culture medium, supplemented with 10% bovine serum and antibiotics in a CO₂ incubator at 37°C as described previously (Bugliani *et al.* 2013). No gender-related differences were noted for any outcome variable, and data are therefore combined.

Recombinant rat (rr) IL-1β and recombinant mouse (rm) TNFα were purchased from R&D Systems. Rm IFNγ and Griess reagent were purchased from Sigma. Recombinant human (rh) IL-1β and IFNγ were purchased from Roche Diagnostics. Trypsin-, chymotrypsin- and caspase-like proteasomal activity reagents were purchased from Promega. The broad proteasome inhibitor MG132 and the selective immunoproteasome inhibitor ONX-914 (200 nM), 20- to 40-fold more selective for Psmb8 than Psmb9 (Muchamuel *et al.* 2009), were from Selleckchem (Houston, Texas, USA). The histone deacetylase (HDAC)1–3 inhibitor MS-275 was from Selleckchem, whereas the inhibitor of the deubiquitinase ubiquitin-specific peptidase 9X (USP9X) BRD0476 that in turn highly selectively inhibits Janus-activated kinase (JAK)/signal transducer and activator of transcription (STAT)1 and the HDAC1–2 selective '3' inhibitor were synthesized in-house (Chou *et al.* 2012, 2015).

Gene profiling

INS-1E cells were exposed to rrIL-1β (10 ng/mL), rmTNFα (25 ng/mL) and rmIFNγ (50 ng/mL) ± MS275 or '3' (5 μM)

Table 1 Characteristics of the 16 human donor and islet preparations.

Mean age $\pm$ s.d. (years)	62 $\pm$ 18
Gender M/F	7/9
Mean BMI $\pm$ s.d. (kg/m ²)	26.0 $\pm$ 2.7
Cause of death	14 CVD*/2 trauma
Mean ICU stay $\pm$ s.d. (days)	4 $\pm$ 3
Mean plasma glucose levels during ICU stay $\pm$ s.d. (mg/dL)	149 $\pm$ 24
Mean pancreas cold ischemia time $\pm$ s.d. (h)	15 $\pm$ 2
Mean islet preparation purity** $\pm$ s.d.	69 $\pm$ 22%
Stimulated insulin release/basal insulin release $\pm$ s.d.	2.9 $\pm$ 1.0

*CVD cardio-/cerebrovascular disease; **evaluated by dithizone staining.

for 6 h. Gene expressions (triplicates) were analyzed using the Affymetrix rat 230.2 array (Genomic Services, Broad Institute, MA, USA) and regulated gene sets were determined using the Gene Set Enrichment Analysis (GSEA) method (Subramanian *et al.* 2005).

qPCR

INS-1E cells were exposed to recombinant rodent cytokines $\pm$ MS-275 or BRD0476 (10 μ M) for the indicated time points. Gene expressions were determined using SYBR green (Applied Biosystems) with selective primer sets. Hprt1 was used as reference gene. Human islets were exposed from 6 to 24 h to 50 U/mL (1 ng/mL) rhIL-1 β and/or 1000 U/mL (50 ng/mL) rhIFN- γ . At the end of the incubation period, total RNA was extracted using the PureLink RNA Mini Kit (Life Technologies) and quantified by absorbance at A260/A280 nm (ratio >1.9) in a NanoDrop 2000C spectrophotometer (Euroclone Spa, Pero, Italy). For quantitative real-time PCR experiments, 1 μ g total RNA was reversely transcribed using a SuperScript VILO cDNA Synthesis Kit (Life Technologies). The primers of interest were obtained from assay-on-demand gene expression products (Life Technologies). mRNA levels were quantified and normalized for β -actin using a ViiA 7 analyser (Life Technologies). Rat primer sequences are listed in Table 2.

Table 2 Rat primer sequences.

Target	Forward primer	Reverse primer
<i>Psm2</i>	CATTGAGCCATCTGGTAAA	GGTTATGGGCTCCACTTG
<i>Psm7</i>	CAGTCAGGTGGCAAAACAT	ACTTAGAATCTTGAGGGGCTGA
<i>Psm10</i>	GAATGCGTCTTGGAACAC	AGCCCCACAGCAGTAGATTT
<i>Pmse1</i>	AGCCAACTGAGCAATCTG	TCCTTCTCTGTTGCTTCTTC
<i>Hprt1</i>	GCAGACTTTGCTTTCCTTGG	CCGCTGTCTTTAGGCTTTG

Western blotting

INS-1E cells were exposed to 1 ng/mL IL-1 β and 50 ng/mL IFN γ for 30 min or 6 h as indicated in figure legends with or without 200 nM of ONX-914. Cells were lysed in RIPA buffer (for NF κ B subunits p50 and p52), and cytosolic extracts (for p65) were prepared according to the protocol from Abcam: http://www.abcam.com/ps/pdf/protocols/subcellular_fractionation.pdf. Western blots were performed using antibodies against 46 and 54 kDa phosphor-JNK isoforms, p50, p52, p65, I κ B or β -actin/ β -tubulin (reference protein). Primary antibodies: pJNK 1:1000 (Cell Signaling), I κ Ba 1:200, cat.no: sc-371 (Santa Cruz Biotechnology), 1:4000, cat.no: T6074 (Sigma-Aldrich). Secondary antibodies: 1:5000 anti-rabbit IgG, cat.no: 7074S (Cell Signaling), 1:5000 anti-mouse IgG, cat.no: 7076S (Cell Signaling).

Proteasomal activity

INS-1E cells were exposed to IL-1 β +TNF α +IFN γ cytokine combination $\pm$ MS275 (5 μ M) or BRD0476 (10 μ M), while human islets were exposed to the IL-1+IFN cytokine combination or individual cytokines for the indicated times. Trypsin-, chymotrypsin- and caspase-like proteasomal activities were determined by a luminescent assay (Promega). The proteasome inhibitor MG132 (0.5 μ M) was included as a positive control.

Electron microscopy analysis

Quantification of apoptotic beta cells was performed as previously detailed (Masini *et al.* 2009). Beta cells were identified based on the presence of typical beta granules, while apoptotic beta cells were identified based on the appearance of marked chromatin condensation and/or blebs, as previously reported (Masini *et al.* 2009). Beta cells (400–450 per experimental condition) were counted by a blinded observer, from a total 7 islets from two separate islet preparations.

Markers of protein oxidation

INS-1 cells were exposed to 150 pg/mL IL-1 β +0.1 ng/mL IFN γ for 6, 12, 18 and 24 h ($n=6$). The cells were then rinsed three times with cold PBS and re-suspended in cold PBS with 10 μ g/mL protease/phosphatase inhibitor (Sigma-Aldrich) plus 5 mM EDTA. The cells were lysed on ice by ultra-sonication (10 s on/off cycles for 5 min) and spun down (20,000 rcf, 4°C, 5 min). Protein concentrations

were determined by the Bradford reagent using 1 μ L of lysate in triplicate. A BSA standard curve was included for quantification. Lysate concentrations of protein carbonyls and thiols, as markers for protein oxidation, were determined using an OxiSelect protein carbonyl ELISA kit (Cell Biolabs Inc, San Diego, USA) according to the manufacturer's instructions with samples adjusted to 0.01 mg/mL protein, and by reaction of 0.20 mg/mL protein with 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) (Hawkins *et al.* 2009), respectively. In order to have all samples measured on single microplates, only the first five batches were analyzed for protein carbonyls. Sodium hydrogen phosphate, sodium dihydrogen phosphate, GSH, urea and ethanol were purchased from Sigma-Aldrich. Phosphate-buffered saline (PBS) 20 $\times$ concentrate (pH 7.5) was obtained from Amresco (Solon, OH, USA).

Statistics

Data were analyzed using GraphPad Prism or SAS 9.4 (SAS Institute Inc., Cary, NC, USA) using analysis of variance (ANOVA) followed by Bonferroni *post hoc* tests corrected for multiple comparisons where appropriate. $P < 0.05$ was considered significant. For human islet, data are presented as means $\pm$ s.d. Difference between two groups was assessed by two-tailed Student's *t*-test, or by ANOVA for multi-group comparisons. Differences between protein oxidation marker levels were compared by contrast analysis (ls means).

Results

Proinflammatory cytokines induce the expression of proteasome-coding genes

To uncover novel pathways involved in cytokine-mediated beta-cell apoptosis, we undertook an unbiased approach by performing a microarray-based gene expression analysis on INS-1E cells exposed to the cytokines IL-1 + TNF + IFN for 6 h. Enriched gene sets were identified, and among the top scoring sets was the cluster annotated as 'REACTOME_STABILIZATION_OF_P53' which consists of genes encoding the proteasome (Fig. 1A).

Heat plot analysis of this gene set revealed differential regulation of the inflammasome components by cytokines. The 20S *Psmb* 3, 4, 5 and 6 catalytic (protease) subunits (also known as β 3, β 7, β 5 and β 1, respectively), the 19S *Psmc* 1, 2, 5 and 6 regulatory ATPase subunits (also known as Rpt2, Rpt1,

Rpt6 and Rpt4, respectively), the 19S *Psm* 2, 6 and 8 non-ATPase regulatory subunits (also known as Rpn1, Rpn7 and Rpn12, respectively) as well as ubiquitin B were slightly to moderately downregulated by the cytokine combination (red $\rightarrow$ pink). In contrast, the 20S *Psm* 1, 2, 4, 5, 6 and 7 catalytic subunits (also known as α 6, α 2, α 3, α 5, α 1 and α 4, respectively) and the 11S *Psm* 1 and 2 activator subunits (also known as 11S α and 11S β , respectively) were upregulated by the cytokine combination (blue $\rightarrow$ red). Since *Psm* 8, 9 and 10 are inducible components of the immunoproteasome, and since the function of this proteasome is ubiquitin independent and thus also 19 proteasome ATPase activity independent (Ferrington & Gregerson 2012), this microarray expressional pattern is consistent with a role for inflammatory cytokines in upregulating the immunoproteasome in insulin-producing cells.

Four of the cytokine-inducible genes (*Psm* 2, *Psm* 7, *Psm* 1 and *Psm* 10) were selected on the basis of their pronounced regulation and functions in the 19S, 20S and 11S proteasome particles (Fig. 1A) for qPCR validation. We confirmed that cytokine induced the expression of these genes after 6 h (Fig. 1B, C, D and E). Induction of *Psm* 7, *Psm* 1 and *Psm* 10 was also confirmed at later time points (Fig. 1C, D and E).

In human islets, exposure to the cytokine combination IL-1 + IFN induced a significant increase in the expression of the immunoproteasomal subunits *Psm* 8, *Psm* 9 and *Psm* 10; however, the expression of the constitutive *Psm* 5, *Psm* 6 and *Psm* 7 proteasome components (Fig. 2) was not altered. A similar induction was observed in human islets exposed for 6 h to IFN- γ , but not IL-1 β (Fig. 2), consistent with the presence of an IFN- γ -inducible immunoproteasome in pancreatic islets.

Proinflammatory cytokines induce proteasomal activity in human islets

We next assessed the effect of cytokines on proteasomal catalytic activity. Human islets were exposed for 6–24 h to the IL-1 β + IFN- γ cytokine combination or individual cytokines. Trypsin-, chymotrypsin- or caspase-like activities, which are shared between the constitutive proteasome and the immunoproteasome, were then quantified. Twenty-four-hour exposure to the cytokine combination induced a significant increase in all three

Figure 1

Gene set enrichment analysis (GSEA) and validation of selected genes in INS-1E cells exposed to cytokines and HDAC inhibitors. (A) INS-1E cells were exposed to cytokines (IL-1 β + TNF α + IFN γ) $\pm$ the HDAC1–3 selective inhibitor MS-275 or the HDAC1–2 selective inhibitor '3' for 6 h. RNA was purified, and a microarray followed by GSEA was performed. The top scoring gene set is shown here. Selected gene expressions are highlighted in yellow, $n = 1$.

(B, C, D and E) INS-1E cells were exposed to vehicle (white bars), cytokines (black bars) $\pm$ MS-275 (light grey bars) or BRD0476 (dark grey bars) for 3, 6, 9, 12 or 24 h. Expression of (B) *Psma2*, (C) *Psme1*, (D) *Psma7*, (E) *Psmb10* was analyzed by qPCR. Results are shown as means $\pm$ s.e.m., $n = 4$. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ vs vehicle (CTRL), # $P < 0.05$, ### $P < 0.001$ vs cytokines.

activities, with the caspase-like activity being the least upregulated (Fig. 3A). In accordance with the finding that IFN γ was responsible for the transcriptional regulation of the inducible immunoproteasome components (Fig. 2), this effect was replicated by 6-h exposure to IFN- γ alone (Fig. 3B) with a preponderance of chymotrypsin-like activity. Of note, TNF alone did not induce any expression

of the selected immunoproteasomal subunits, nor did it increase proteasomal activities (data not shown). We also investigated the effect of high glucose on human islet proteasomal activity; 24-h exposure to 22.2 mM glucose caused an increase in $\sim 40\%$ trypsin-like activity ($P < 0.05$, data not shown).

Figure 2

Expression of selected proteasomal subunit genes in human islets exposed to cytokines. The expression of *PSMB5*, *PSMB6*, *PSMB7*, *PSMB8*, *PSMB9* and *PSMB10* was examined in human islets exposed to (A) a combination of IL-1 β and IFN γ (black bars) or (B) only IL-1 β (grey bars) or IFN γ (black bars). Results are shown as means $\pm$ s.e.m., $n = 4$ –8. * $P < 0.05$ and ** $P < 0.01$ vs other groups.

Effects of selective immunoproteasome inhibition on human beta-cell death

To understand whether immunoproteasomal activity mediates cytokine-induced beta-cell apoptosis, human islets were exposed for 24 h to IL-1 β +IFN- γ in the presence or absence of ONX-914, a potent and selective immunoproteasome inhibitor, with minimal cross-reactivity with the s-proteasome. As shown in Fig. 4A, these cytokines significantly increased the proportion of apoptotic human islet cells, as identified by the classical morphological criteria of chromatin condensation and/or blebs (Fig. 4B). This effect was exacerbated by concomitant exposure to ONX-914, suggesting that cytokine-inducible immunoproteasomal activity is a protective response against inflammatory stress.

Figure 3

Proteasomal activities in human islets exposed to cytokines. The trypsin-like, chymotrypsin-like and caspase-like proteasomal activities were analyzed in human islets exposed to (A) a combination of IL-1 β and IFN γ (black bars) or (B) only IL-1 β (grey bars) or IFN γ (black bars). Results are shown as means $\pm$ s.e.m., $n=4-7$. * $P<0.05$ and ** $P<0.01$ vs other groups.

Proteasomal gene expression, but not activity, is reduced by small molecule inhibitors of HDACs and the JAK1/STAT pathway

We have shown that the deleterious effects of cytokines on beta cells can be prevented with small molecule blockade of the NF κ B pathway

by HDAC inhibition (Christensen *et al.* 2014), or the JAK-STAT1 pathway (Chou *et al.* 2015). We therefore examined whether cytokine-induced expression of proteasomal genes was affected similarly. MS-275 (an HDAC1-3 inhibitor) reduced several of the cytokine-induced genes of the GSEA, an effect not mimicked by an HDAC1-2 inhibitor (Chou *et al.* 2012) (Fig. 1A), suggesting that HDAC3 is a major regulator of the beta-cell immunoproteasome. Notably, however, cytokine-induced upregulation of the immunoproteasome components Psmb8 and Psmb9 and the 11S activator subunits Psme1 and Psme2 were not counteracted by HDAC or STAT1 inhibition, suggesting that key components of the immunoproteasome were regulated by NF κ B- and JAK/STAT1-independent pathways.

We validated the microarray findings by qPCR and confirmed the activity of MS-275 in reducing the expression of *Psm7* after 6 h, and *Psm10* at all time points (Fig. 1D and E). In line with the microarray data, MS-275 did not reduce *Psm2* expression (Fig. 1B) and only significantly reduced *Psm1* after 6 h (Fig. 1C). Inhibition of the JAK1-STAT1 pathway by the ubiquitin-specific peptidase 9X inhibitor BRD0476 (Chou *et al.* 2015) only reduced the expression of *Psm10* (Fig. 1E), suggesting that the JAK2-activated transcription factor ATF2 contributes to the IFN γ -mediated immunoproteasome gene expression as shown in Fig. 2.

Since these small molecule inhibitors reduced the expression of selected proteasomal genes, we investigated the ability of the compounds to reduce proteasomal activity. Cytokine exposure increased the proteasomal

Figure 4

Cytokine-induced apoptosis is exacerbated by inhibition of the proteasome in human islets. Quantification of apoptotic human islet beta cells (A), identified by chromatin condensation and/or blebs in cells containing typical β granules by transmission electron microscopy analysis of human islets (B). The quantified analysis in panel A depicts data as means $\pm$ s.e.m. for islets exposed to vehicle (white bar), ONX-914 (light grey bar), cytokines (black bar) or cytokines+ONX-914 (dark grey bar); $n=7$, * $P<0.05$ vs other groups.

Figure 5

Proteasomal activity of INS-1E cells exposed to cytokines and small molecule inhibitors of cytokine-induced apoptosis. Chymotrypsin-like proteasomal activity was measured in (A) a time-dependent manner and (B) in INS-1E cells exposed for cytokines $\pm$ MS-275/BRD0476 for 12 h. The broad proteasome inhibitor MG-132 was used as a positive control. (A) Eleven independent wells, (B) twenty-two independent wells, results are shown as means $\pm$ STD. * $P < 0.01$ and ** $P < 0.001$ vs CTRL, NS, no significance.

activity in a time-dependent manner (Fig. 5A), in line with our human islet data, suggesting a relationship of this phenomenon to the beta-cell compartment of the human islet (Fig. 4). Interestingly, none of the small molecule inhibitors affected cytokine-induced activity (Fig. 5B), indicating that the inhibition of proteasomal activity does not contribute to HDAC inhibition-mediated protection against inflammatory beta-cell stress, and that beta-cell proteasomal activation by cytokines was JAK1/STAT1- and ubiquitin independent.

Markers of protein oxidation

We next examined the mechanism underlying the i-proteasome inhibition-mediated aggravation of cytokine-induced beta-cell apoptosis. The i-proteasome plays an important role in clearing oxidatively damaged proteins generated as a consequence of inflammation, thereby preventing protein aggregation (Seifert *et al.* 2010) and the triggering of apoptosis (Chang & Chang 2015). We therefore quantified the depletion of protein thiols and formation of protein carbonyls that can be generated on a broad range of amino acids by oxidation. Cytokines did not

Figure 6

I-proteasome inhibition does not cause accumulation of oxidized proteins in cytokine-exposed INS-1 cells. (A) Thiol concentration in INS-1 cell lysates incubated with cytokines (Cyt), 200 nM Psmb8 inhibitor ONX-914 (Ct/ONX) or both (Cyt/ONX) for 6, 12, 18 or 24 h. Data are presented as mean $\pm$ S.D., $n = 6$. (B) Carbonyl concentration in INS-1 cell lysates incubated with cytokines (Cyt), Psmb8 inhibitor (Ct/ONX) or both (Cyt/ONX) for 6, 12, 18 or 24 h. Data are presented as mean $\pm$ S.D., $n = 5$. Two-way ANOVA: $P = 0.0275$; One-way ANOVAs: 6 h $P = 0.9786$; 12 h $P = 0.0421$; 18 h $P = 0.4129$; 24 h $P = 0.0835$. *Ad hoc* Student's *t*-tests for 12 h: * $P < 0.05$, ** $P < 0.01$.

alter oxidized protein levels in the presence of a functional proteasome (Fig. 6). I-proteasome inhibition did not cause an accumulation, but in fact a reduction of oxidized proteins in the presence of cytokines, as evidenced by an increase in thiol levels (Fig. 6A). Protein carbonyls were not affected by i-proteasomal inhibition (Fig. 6B). These data indicate that accumulation of damaged proteins is not the mechanism of action underlying ONX-914-mediated aggravation of inflammatory beta-cell apoptosis.

Effects of i-proteasome inhibition on beta-cell inflammatory signaling

Since the i-proteasome regulates the activity of the NF κ B and MAPK pathways (Rape & Jentsch 2002) we next asked if i-proteasomal inhibition affected the processing of p105/100 or degradation of p65 and I κ B, or altered JNK activity in INS-1E cells. Cytokines increased total cellular NF κ B subunit p50 and p52 levels and reduced

Figure 7

(A, B, C and D) INS-1E cells were exposed to 1 ng/mL IL-1 β plus 50 ng/mL IFN γ for 6 h without or with ONX-914 (200 nM). Western blots were performed using anti-p50, p52, I κ B α or p65 as well as anti-beta actin and anti-tubulin antibodies. For p65 analysis (C), cytoplasmic fractions were analyzed. (E) INS-1 cells were exposed to 1 ng/mL IL-1 β plus 50 ng/mL IFN γ for 30 min without or with ONX-914 (200 nM). Western blots were performed using I κ B α and anti-tubulin antibodies. Data are expressed as ratios to house-keeping protein in percentage of control, with $n=3-5$. * $P<0.05$ vs CTRL and ONX-914 (ANOVA followed by Student's t -test with Bonferroni correction). Results are shown as means $\pm$ s.e.m.

the cytosolic NF κ B subunit p65 and I κ B levels (Fig. 7A, B, C and D). However, these cytokine-regulated changes in NF κ B subunit levels were unaffected by Psmb8 inhibition (Fig. 7A, B, C and D). Since cytokine-induced I κ B degradation was partial (Fig. 7D), we repeated these experiments in INS-1 in which the degradation was almost complete (Fig. 7E). In line with the results obtained from the INS-1E cells (Fig. 7D), Psmb8 inhibition did not affect cytokine-induced I κ B degradation (Fig. 7E) or JNK activation (data not shown). In addition, Psmb8 inhibition did not affect I κ B degradation in human islets from two donors (data not shown).

Discussion

Collectively, the data obtained in this study demonstrate that inflammatory cytokines upregulate the immunoproteasome but not the standard proteasome in human islets and the insulin-producing cell line INS-1E. The expression data (Figs 1 and 2) were remarkably similar between these systems, consistent with the highly conserved i-proteasome sequences between human and rodents (Ferrington & Gregerson 2012). IFN γ was the driver of i-proteasome expression (Fig. 2) with no detectable potentiation by IL-1 β . Accordingly, the Psmb8

and Psmb9 promoters contain interferon consensus sequence 2- and gamma-activated sites that bind STAT1 and IRF-1 transcription factors (Chatterjee-Kishore *et al.* 2000) in addition to NFκB and AP-1 binding sequences and early growth response protein (Egr1) binding sites (James *et al.* 2006). The Psmb10 promoter contains similar transcription factor binding sites (Hisamatsu *et al.* 1996). The fact that IL-1β alone neither upregulated Psmb8, Psmb9 nor Psmb10 suggests that IL-1β upregulates Egr1, also known as Zif268 (zinc finger protein 225) or NGFI-A (nerve growth factor-induced protein A), which is known to suppress the response of the Psmb9 promoter to NFκB (James *et al.* 2006). Indeed, Egr1 is highly expressed in pancreatic islets and beta cells and upregulated after 4–8 h by cytokines in INS-1E cells in an NO-independent manner (Kutlu *et al.* 2003), suggesting that the cGMP/cAMP-PKA/G-CREB pathways are not involved in regulating the beta-cell immunoproteasome in contrast to endothelial cells that have a high basal expression of the immunoproteasome.

Chymotrypsin-like activity was induced more by cytokines than caspase-like activity after 6 h (Fig. 3A), compatible with the fact that the Psmb9 catalytic activity to cleave after acidic amino acid residues is significantly reduced in the immunoproteasome compared to the standard proteasome (Ferrington & Gregerson 2012) with ensuing consequences for substrate specificity. Since the assay employed does not discern between proteasomal- and non-proteasome-dependent caspase activity, it is possible that part of the cytokine-induced caspase-like activity in Fig. 3, normally almost abolished in the i-proteasome, arises from IFNγ-induced caspase 1 (Karlsen *et al.* 2000).

Interestingly, and in contrast to the effect of cytokines and high glucose reported here, prolonged glucotoxicity decreases proteasomal activities (Broca *et al.* 2014). These opposite activities may be related to the timing of the experimental conditions; we assessed the activities after just 6 and 24 h where Broca and coworkers (Broca *et al.* 2014) reported a decrease in activity after 14 days of high glucose exposure, which correlated with a substantial induction of apoptosis.

The choice of a pharmacological rather than a molecular approach to show a cause–effect relationship between i-proteasome expression and activity and islet cell damage was a deliberate strategy, since the production of a proteasome-deficiency phenotype requires triple KO of Psmb 8, 9 and 10 or double KO of Psmb9 and 10 plus inhibition with ONX 914 to inhibit Psmb8, with consequential risks of transfection artifacts. The ONX-914

inhibitor is highly selective for the i-proteasome (Muchamuel *et al.* 2009).

Psmb8 inhibition affected neither the activity of JNK nor the levels of p50, p52 and p65 or IκB degradation suggesting that i-proteasomal inhibition neither altered the turnover of these NFκB subunits nor NFκB pathway activity. Earlier studies demonstrated that the 26S proteasomal inhibitor MG-132 inhibited NFκB-dependent islet and insulin-producing cell iNOS and cyclooxygenase-2 (COX2) expression and activity (Kwon *et al.* 1998). Since NO and COX2 products are not required for cytokine-induced human islet-cell apoptosis and since we used highly selective i-proteasome inhibition, these observations are not in conflict with the data presented here. Of note, Psmb8 and Psmb9 double KO macrophages fail to activate NO production via the TRIF/TRAF3 NFκB pathway but demonstrate unaffected Myd88/TRAF6 NFκB-dependent TNF production, suggesting that the i-proteasome differentially regulates NFκB signaling (Reis *et al.* 2011).

Taken together with our observations that Psmb8 inhibition did not affect NFκB signaling, these observations do not support NO as a mediator of the increased apoptosis observed with the i-proteasome inhibitor.

In addition to affecting inflammatory signaling, proteasome inhibition would be expected to result in reduced removal of oxidized or misfolded proteins (Seifert *et al.* 2010). The i-proteasome clears *defective ribosomal products* (DRIPs), which are misfolded or oxidized proteins produced on cytokine exposure, in particular to IFNγ via mTOR signaling. DRIPs clearing is polyubiquitin and 19S ATPase independent (Shringarpure *et al.* 2003). Inhibition of the i-proteasome would therefore be expected to lead to increased levels of toxic protein aggregates (aggresomes) (Seifert *et al.* 2010), but we were unable to detect such an effect. The observed increase in protein thiols caused by the inhibitor in the presence of cytokines may be due to increased activity of γ-glutamyl cysteine ligase, the key enzyme in synthesis of the antioxidant glutathione (Dickinson & Forman 2002), although this protective response may be outcompeted by dominant pro-apoptotic signals.

I-proteasome deficiency or inhibition protects against dextran sodium sulfate (SDS)-induced enterocolitis and experimental arthritis in mice (Muchamuel *et al.* 2009, Basler *et al.* 2010, Schmidt *et al.* 2010). I-proteasome inhibition has not to our knowledge been tested in diabetic animal models, and our study clarifies the translational potential of this concept, in that our data in human islets do not support the use of inhibitors selective

for the i-proteasome in diabetes. In conclusion, beta cells upregulate immunoproteasome expression and activity in response to IFN γ , likely as a protective response to modulate inflammatory signaling.

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.

Funding

This study was supported by the University of Copenhagen career PhD fellowship and an ELITE-research grant (to M L), CSC scholarship (to Z C), Novo Nordisk Foundation – NNF13OC0004294 (to M J D), Type 1 Diabetes Pathfinder award, NIDDK (to B K W) and Novo Nordisk Foundation (to T M P O).

Author contribution statement

M L, M B, D H-C, B W, P M and T M P designed the experimental setup. M L, M B, T D, D H-C, S M G, Z H and M N L performed the experiments. M L and T M P drafted the manuscript. M J D oversaw protein oxidation experiments and edited the manuscript. All authors approved the final version.

Acknowledgements

The authors would like to thank Francesca Grano for excellent technical assistance.

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Received in final form 10 April 2017

Accepted 24 April 2017

Accepted Preprint published online 24 April 2017