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## Interferon- $\gamma$ potentiates endoplasmic reticulum stress-induced death by reducing pancreatic beta cell defence mechanisms

Received: 26 October 2005 / Accepted: 25 January 2006 / Published online: 8 April 2006  
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**Abstract** *Aims/hypothesis:* A tight control of endoplasmic reticulum homeostasis is crucial for beta cell function and survival. We recently described that IL-1 $\beta$  plus IFN- $\gamma$  deplete endoplasmic reticulum Ca<sup>2+</sup> stores in beta cells, leading to endoplasmic reticulum stress and apoptosis. IL-1 $\beta$  alone induced endoplasmic reticulum stress but failed to induce beta cell death, while IFN- $\gamma$  alone neither caused endoplasmic reticulum stress nor induced beta cell death. This suggests that IFN- $\gamma$  aggravates endoplasmic reticulum stress induced by IL-1 $\beta$ , eventually triggering apoptosis. Here we tested this hypothesis and the mechanisms involved, by investigating the effects of IFN- $\gamma$  on endoplasmic reticulum-stress-induced beta cell apoptosis caused by a specific blocker of the sarcoendoplasmic-reticulum pump Ca<sup>2+</sup>-ATPase (SERCA). *Materials and methods:* INS-1E cells or beta cells were pretreated with IFN- $\gamma$  and then exposed to the SERCA blocker cyclopiazonic acid (CPA) for induction of endoplasmic reticulum stress. Cell death was evaluated by Hoechst 342 and propidium iodide staining. Expression of genes related to endoplasmic reticulum stress was determined by real-time RT-PCR, while activation of the endoplasmic reticulum stress response was determined by analysing X-box binding protein-1 (*Xbp1*) splicing and using a reporter construct containing five copies of the unfolded protein response element (UPRE). *Results:* CPA induces endoplasmic reticulum stress and apoptosis in insulin-producing cells. Pretreatment with IFN- $\gamma$  decreased the basal level of spliced *Xbp1* mRNA, the basal and CPA-induced activity of the UPRE reporter, and the mRNA expression of several endoplasmic reticulum chaperones (*Bip*, *Grp94* and *Orp 150*) and *Sec61a*. Furthermore, CPA-induced

*Chop* mRNA expression and beta cell apoptosis were potentiated in cells that had been pretreated with IFN- $\gamma$ . *Conclusions/interpretation:* CPA-induced endoplasmic reticulum stress and apoptosis is enhanced in IFN- $\gamma$ -treated beta cells. These effects are mediated via down-regulation of the expression of genes involved in beta cell defence against endoplasmic reticulum stress.

**Keywords** Apoptosis · Endoplasmic reticulum stress · Interferon- $\gamma$  · Pancreatic beta cells · Type 1 diabetes mellitus

**Abbreviations** ATF: activating transcription factor · BiP: immunoglobulin heavy chain binding protein · CPA: cyclopiazonic acid · CHOP: C/EBP homologous protein · ER: endoplasmic reticulum · ERAD: ER-associated degradation pathway · GRP94: glucose-regulated protein 94 · IRE1 $\alpha$ : inositol requiring ER-to-nucleus signal kinase 1  $\alpha$  · ORP150: oxygen-regulated protein 150 · SERCA: sarcoendoplasmic-reticulum pump Ca<sup>2+</sup>-ATPase · UPR: unfolded protein response · UPRE: UPR element · XBP1: X-box binding protein-1 · XBP1s: XBP1 spliced

### Introduction

A detailed understanding of the signals activating the apoptotic programme in beta cells is crucial for the design of new therapies to prevent immune-mediated beta cell death in type 1 diabetes mellitus. Accumulating evidence suggests that a tight control of endoplasmic reticulum (ER) homeostasis is important for the maintenance of beta cell function and survival [1–3]. The ER is the main site for calcium storage and signalling, protein synthesis and folding and lipid biosynthesis [4, 5]. To function properly the ER relies on numerous resident chaperone proteins, a high calcium content and an oxidative environment [4, 5]. Alterations of the ER environment, such as Ca<sup>2+</sup> depletion, altered glycosylation or perturbed redox status, may trigger accumulation of unfolded proteins and activate a specific ER stress response, known as the unfolded protein

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response (UPR) [4, 5]. This response is crucial both for cell recovery under conditions of ER stress and for the function and survival of active secretory cells, such as beta cells [1–3]. The UPR involves translational attenuation via autophosphorylation of protein kinase RNA-dependent (PKR)-like ER kinase (PERK), an increase in the folding capacity of the ER by upregulation of ER chaperones and degradation of misfolded proteins via the ER-associated degradation pathway (ERAD) [5, 6]. Transcriptional upregulation of ER chaperones is crucial for cellular recovery following ER stress because these proteins prevent additional protein aggregation by promoting proper folding and by assembling and targeting misfolded proteins for degradation [4]. Two main transcription factors regulate chaperone induction, namely activating transcription factor 6 (ATF6) and X-box binding protein-1 (XBP1). During the UPR the luminal domain of ATF6 is released from the ER chaperone immunoglobulin heavy chain binding protein (BiP; also known as heat shock 70 kDa protein 5) and translocates to the Golgi complex. ATF6 is then cleaved and its active part, encoding the basic leucine zipper transcription factor, translocates to the nucleus [4, 5]. Inositol requiring ER-to-nucleus signal kinase 1  $\alpha$  (IRE1 $\alpha$ ) is also bound to BiP in the ER lumen; BiP is removed during ER stress, leading to oligomerisation, autophosphorylation and activation of the RNase domain of IRE1 $\alpha$ . This domain will then cleave *Xbp1* mRNA, leading to the formation of an active transcription factor [4, 5]. Three *cis*-acting elements capable of binding to ATF6, XBP1, or both have been identified, namely ER stress-response element (ERSE), unfolded protein response element (UPRE) and ERSE-II [5]. Under prolonged or excessive ER stress the apoptotic machinery is induced, but the mechanisms linking ER stress and apoptosis remain to be clarified [5, 6].

Cytokines such as IL-1 $\beta$ , TNF- $\alpha$  and IFN- $\gamma$  contribute to beta cell death in type 1 diabetes [7]. We have recently described how IL-1 $\beta$  plus IFN- $\gamma$ , via nitric oxide (NO) synthesis, inhibit expression of the ER Ca<sup>2+</sup> pump SERCA2 and deplete ER Ca<sup>2+</sup> stores in beta cells, leading to ER stress and apoptosis [8]. This ER stress response includes activation of the IRE-1 $\alpha$  and PERK pathways, and increased expression of the C/EBP homologous protein (CHOP; also known as DNA-damage-inducible transcript 3) [8]. IL-1 $\beta$  alone induced ER stress but failed to induce beta cell death in the absence of IFN- $\gamma$ , while IFN- $\gamma$  neither caused ER stress nor induced beta cell death [8]. We have previously observed, by microarray analysis of primary beta cells, that exposure to IFN- $\gamma$  down-regulates the mRNAs that encode for several ER chaperones such as oxygen-related protein (ORP) 150 (also known as hypoxia up-regulated 1), calreticulin and calbindin, and a component of the ER-protein translocator, SEC61a [9]. This raised the possibility that IFN- $\gamma$  aggravates the ER stress induced by IL-1 $\beta$  by decreasing beta cell ER defences. Notably, IFN- $\gamma$  augments IL-1 $\beta$ -induced inducible NO synthase expression and NO production [8] making it difficult to discriminate between the putative effects of IFN- $\gamma$  on ER stress defences and the

effects mediated via enhanced NO production. For this reason, we used a reversible blocker of the SERCA pump, cyclopiazonic acid (CPA), to induce a ‘pure’ ER stress in beta cells and then evaluated the effects of IFN- $\gamma$  on CPA-induced beta cell UPR activation and apoptosis.

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## Materials and methods

### Cell culture and reagents

INS-1E cells were cultured in RPMI-1640 medium (Gibco, Paisley, UK) [10]. FACS-purified rat beta cells were isolated and cultured in Ham’s F-10 medium (Gibco) as described previously [8, 11]. Both INS-1E cells and primary beta cells were plated and cultured for 48 h before the addition of test agents. Recombinant rat IFN- $\gamma$  (R&D Systems, Abingdon, UK) was used at 0.036  $\mu$ g/ml (corresponding to approximately 100 U/ml of recombinant mouse IFN- $\gamma$ ) and human recombinant IL-1 $\beta$  (a gift from Dr C.W. Reynolds, National Cancer Institute, Bethesda, MD) was used at 10 U/ml. Cytokine concentrations were chosen with reference to previous dose–response experiments ([7, 8, 10]; Kutlu and Eizirik unpublished data). The reversible SERCA blocker CPA was tested at different concentrations (3.1, 6.25 and 25  $\mu$ mol/l) to induce ER stress.

### Real-time RT-PCR analysis and evaluation of Xbp-1 processing

INS-1E cells were pretreated for 24 h with IFN- $\gamma$  or left untreated. The cells were then further exposed for 6 or 12 h to 3.1, 6.25 or 25  $\mu$ mol/l CPA or to the carrier solvent of CPA (DMSO, 0.25%) as control, in the presence or absence of IFN- $\gamma$ . DMSO at this concentration affects neither INS-1E cell and beta cell viability nor gene expression (data not shown, [8]). The rationale for preculturing the cells with IFN- $\gamma$  instead of adding it in conjunction with the CPA is that we have previously observed in our microarray experiments that beta cell exposure to IFN- $\gamma$  down-regulates the expression of several ER chaperone genes and *Sec61a* after 6–24 h [9]. Thus, we intended to test whether IFN- $\gamma$ -mediated downregulation of ER ‘defence genes’ would aggravate the ER stress induced by CPA.

Cells were then collected, poly(A)<sup>+</sup> RNA was extracted and the reverse transcriptase reaction was performed as previously described [12]. Expression of *Chop*, *Xbp1*, *Bip*, *Orp150*, glucose regulated protein 94 (*Grp94*; also known as tumour rejection antigen 1), calbindin, calreticulin and *Sec61a* mRNAs was determined by real-time RT-PCR using SYBR green fluorescence on a Light Cycler (Roche Diagnosis, Mannheim, Germany) by the standard curve method [13, 14]. The housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) was used for confirmation of similar cDNA loading. Results are shown as expression of the gene of interest divided by that of *Gapdh*. The PCR amplification reactions and preparation of standards were performed as previously described [13].

Primers for real-time RT-PCR were as follows: *Xbp1*: forward (F)-GAGCAGCAAGTGGTGGATTT, reverse (R)-TCTCAATCACAAGCCCATGA (115 bp); *Orp150*: F-TCAGATGCCAAAGAGAATGG, R-TTCAGCTTCCTCCTTGAGTTC (111 bp); *Grp94*: F-AAGGTCATTGTCA CGTCGAAA, R-GTGTTCCTCTTGGGTCAGC (98 bp); calbindin: F-AAGCAAACAAGACCGTGGAT, R-CATCT CTGTCAGCTCCAGCTT (104 bp); calreticulin: F-GA TTCGAACCTTCAGCAAC, R-GGAAACAGCTTCAC GTAGCC (104 bp); *Sec61a*: F-TGTATTCATGCTGG GTTCCT, R-ATCACCATCTGCTGCTCCTT (108 bp). Primer sequences for *Bip*, *Chop*, *atf4* and *Gapdh* are described elsewhere [8]. The experiments for the determination of mRNA expression were performed in duplicate, and the means of the duplicates were considered as  $n=1$ .

IRE1 $\alpha$ -mediated *Xbp1* processing is characterised by excision of a 26-bp sequence from the coding region of *Xbp1* mRNA [15]. The cleaved fragment was evaluated by PCR followed by restriction analysis [8, 16]. Gel images were captured and optical density of the bands was determined using the KODAK 1D image analysis software. The amount of spliced *Xbp1* (*Xbp1s*) product, indicating UPR activation, was expressed as a percentage of the summed intensity of the unspliced *Xbp1* (*Xbp1u*) and *Xbp1s* bands.

#### Assessment of cell viability

INS-1E cells (6,000 cells/condition) or FACS-purified rat beta cells (10,000 cells/condition) were cultured attached to 96-well dishes and pretreated for 24 h in the presence or absence of IFN- $\gamma$  or IL-1 $\beta$ ; or for 6 h with 12.5  $\mu\text{mol/l}$  CPA or control (DMSO, 0.125%). After CPA pretreatment the cells were washed then cultured for 4 h before further treatment. INS-1E cells or beta cells were then treated with CPA in the presence or absence of IFN- $\gamma$  or IL-1 $\beta$ . The percentage of viable, necrotic and apoptotic cells was determined by inverted fluorescence microscopy with the DNA dyes Hoechst 342 (10  $\mu\text{g/ml}$ ) and propidium iodide (10  $\mu\text{g/ml}$ ) [8, 17]. Viability was evaluated by at least two independent observers, with one of them unaware of sample identity. The agreement between findings obtained by the two observers was always >90%. The apoptotic index was calculated as  $([\% \text{ apoptotic cells in experimental condition} - \% \text{ apoptotic cells in control}] / [100 - \% \text{ dead cells in control}]) \times 100$  [17, 18].

#### UPRE luciferase reporter assay

The reporter plasmid containing the luciferase gene under the control of five UPREs was kindly provided by Prof. Prywes (Columbia University, New York, NY). INS-1E cells were plated at a density of 140,000 cells/condition in 24-well plates and co-transfected with the UPRE and pRL-CMV plasmids (internal control for transfection efficiency) as described elsewhere [19]. Twenty-four hours after transfection the cells were treated with IFN- $\gamma$  and CPA

as described above. Luciferase activities in the cell lysates were determined in a TD-20/20 luminometer (Turner Designs, Sunnyvale, CA, USA) using the dual-luciferase reporter assay system according to the manufacturer's instructions (Promega, Madison, WI, USA). Test values were corrected for luciferase value of the internal control plasmid (pRL-CMV).

#### Statistical analysis

Data are shown as mean $\pm$ SEM, and comparisons between groups were made by paired *t* test or by ANOVA followed by *t* test with the Bonferroni correction, as indicated. A *p* value of  $\leq 0.05$  was considered statistically significant.

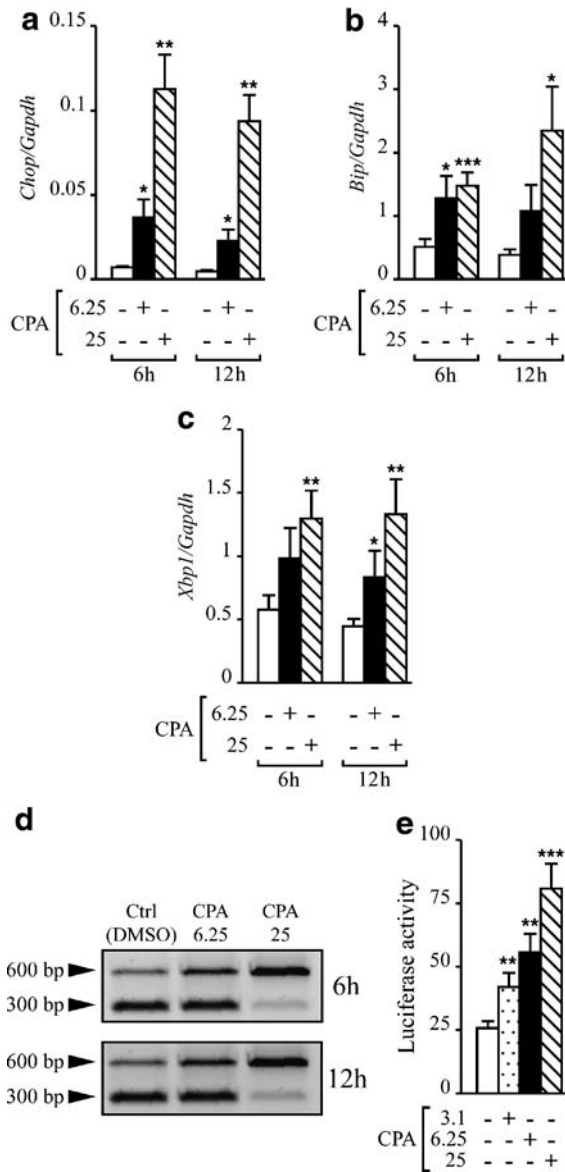
## Results

### CPA induces ER stress and UPR in INS-1E cells

To validate the use of CPA as an ER stress-inducing agent, we initially characterised different components of the UPR/ER stress response induced by CPA in INS-1E cells. For this purpose INS-1E cells were exposed for 6 or 12 h to different concentrations of CPA. A low concentration of CPA (6.25  $\mu\text{mol/l}$ ) was sufficient to trigger the UPR response, as indicated by induction of *Chop* (Fig. 1a), *Bip* (Fig. 1b) and *Xbp1* (Fig. 1c) mRNA expression. These effects were more pronounced in INS-1E cells exposed to 25  $\mu\text{mol/l}$  CPA (Fig. 1). IRE-1 $\alpha$ -mediated splicing of *Xbp1* in INS-1E cells under basal conditions (upper band 600 bp, Fig. 1d) was  $33.2\pm 2.5\%$  and  $32.8\pm 1.5\%$  of the total *Xbp1* mRNA at 6 and 12 h, respectively. Exposure to 6.25 and 25  $\mu\text{mol/l}$  CPA for 6 h increased the levels of *Xbp1s* to  $44.9\pm 2.2$  and  $77\pm 2.3\%$ , respectively ( $p < 0.001$  vs control). Twelve hours after treatment, a significant increase of the *Xbp1s* product ( $72.3\pm 8.8\%$ ,  $p < 0.01$  vs control) was still observed in INS-1E cells exposed to 25  $\mu\text{mol/l}$  CPA while it had returned to base levels in cells treated with 6.25  $\mu\text{mol/l}$  CPA. Confirming the triggering of ER stress, CPA induced a dose-dependent activation of the luciferase reporter plasmid under the control of five UPRE [20] (Fig. 1e).

### IFN- $\gamma$ potentiates ER stress induced cell death

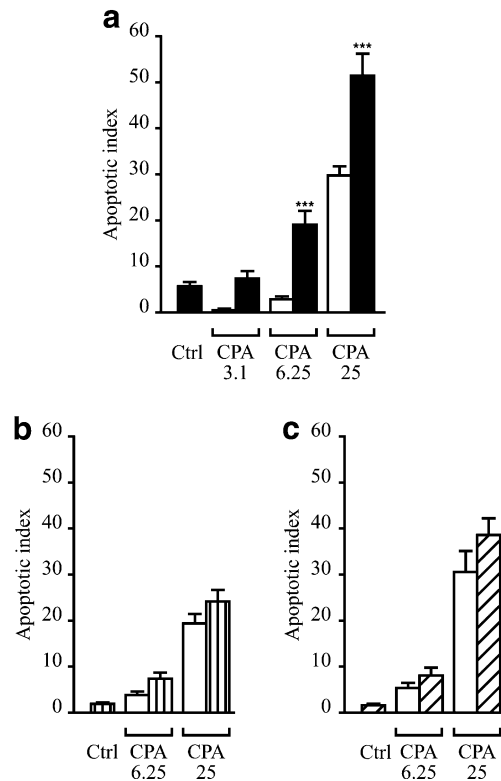
To test whether IFN- $\gamma$  indeed aggravates ER stress-induced death, we preincubated INS-1E cells for 24 h with IFN- $\gamma$  before exposing them to different concentrations of CPA (Fig. 2a). To assess the specificity of the putative effects of IFN- $\gamma$ , cells were also precultured with IL-1 $\beta$  or CPA (Fig. 2b,c). CPA induced apoptosis in INS-1E cells in a dose-dependent manner. IFN- $\gamma$  treatment alone led to a modest increase in apoptosis in INS-1E cells (apoptotic index  $5.7\pm 1$ , Fig. 2a). Pretreatment with IFN- $\gamma$  potentiated CPA-induced apoptosis in INS-1E cells, increasing by 150 and 230% the apoptosis induced by 25 and



**Fig. 1** CPA induces ER stress in INS-1E cells. Cells were exposed for 6 or 12 h to CPA (6.25 or 25  $\mu\text{mol/l}$ ) or control conditions (DMSO 0.25%); mRNA was then extracted and real-time RT-PCR was performed using specific primers for *Chop* (a), *Bip* (b) or *Xbp1* (c). Expression of the gene of interest was corrected for *Gapdh* expression. The results are the means $\pm$ SEM of six experiments. **d** mRNA was extracted after CPA treatment and cDNA was utilised as a template for PCR using *Xbp1*-specific primers. After amplification, PCR products were incubated with the restriction enzyme *PstI*. PCR products derived from spliced *Xbp1* are left intact (600 bp), indicating the presence of ER stress. The figure shown is representative of six experiments. **e** INS-1E cells were co-transfected with a construct containing five UPREs upstream of the luciferase reporter gene and the internal control pRL-CMV, encoding *Renilla* luciferase. After overnight transfection, the cells were exposed for 15 h to CPA (3.1, 6.25 or 25  $\mu\text{mol/l}$ ) or control and were assayed for firefly and *Renilla* luciferase activities. The results were normalised for *Renilla* luciferase activity ( $n=5$ ). \* $p\leq 0.05$ , \*\* $p\leq 0.01$ , \*\*\* $p\leq 0.001$  vs control, *t* test

6.25  $\mu\text{mol/l}$  of CPA, respectively (Fig. 2a). These joint effects were clearly higher than the sum of the isolated

effects of IFN- $\gamma$  and CPA. IFN- $\gamma$  also potentiated the pro-apoptotic effects of CPA in FACS-purified rat beta cells. Thus, 24 h incubation of primary beta cells with 25  $\mu\text{mol/l}$  CPA significantly induced apoptosis (apoptotic index of  $7.8\pm 1.3$ ,  $n=6$ ). This was increased by 175% after IFN- $\gamma$  pretreatment ( $16.1\pm 2.2$ ;  $p<0.01$  vs CPA alone, *t* test,  $n=6$ ). IFN- $\gamma$  alone did not induce apoptosis in primary cells (data not shown). The main mode of cell death observed in the experiments described above was apoptosis in both INS-1E cells and primary beta cells. The percentages of apoptosis observed for INS-1E cells and primary beta cells in the control conditions were  $3.0\pm 0.2$  and  $6.9\pm 1.3$ , respectively. CPA did not induce NO production either alone or in combination with IFN- $\gamma$  (data not shown). In contrast to the observations with IFN- $\gamma$ , neither preincubation with IL-1 $\beta$  nor CPA potentiated CPA-induced apoptosis (Fig. 2b,c) or necrosis in INS-1E cells (data not shown). In these cases, only an additive effect was observed between the different treatments.



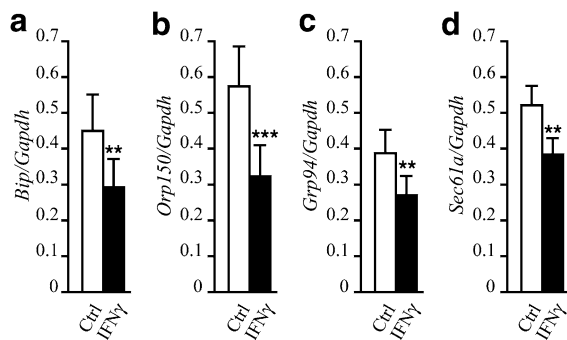
**Fig. 2** IFN- $\gamma$ , but not IL-1 $\beta$  or CPA, potentiates ER stress-induced cell death in INS-1E cells. INS-1E cells were pretreated (filled/shaded bars) or not (empty bars) with IFN- $\gamma$  (0.036  $\mu\text{g/ml}$ ) (a) or IL-1 $\beta$  (10 U/ml) (b) for 24 h or CPA (6.25  $\mu\text{mol/l}$ ) (c) for 6 h. The cells were then exposed to 3.1, 6.25 or 25  $\mu\text{mol/l}$  CPA or control (Ctrl, DMSO, 0.25%) for 15 h. Cell viability was determined with the DNA dyes Hoechst 342 and propidium iodide and apoptotic indexes were calculated as described in Materials and methods. Results are means $\pm$ SEM of six to eight experiments. \*\*\* $p\leq 0.001$  vs condition without pre-treatment, ANOVA and *t* test with Bonferroni correction

IFN- $\gamma$  decreases the expression of ER chaperones and a component of the ERAD pathway

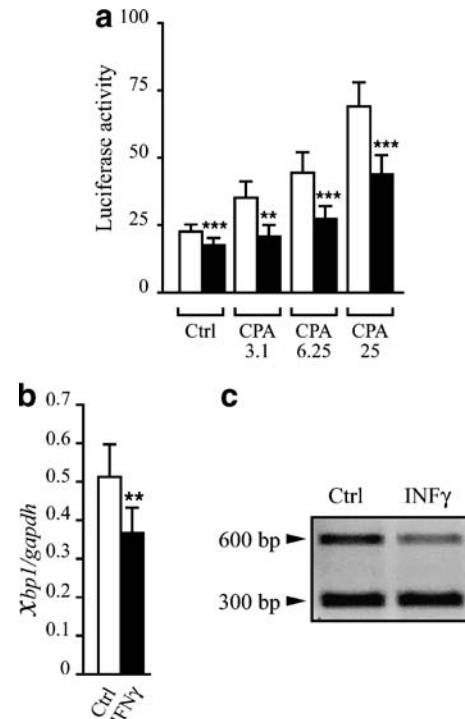
Exposure of INS-1E cells to IFN- $\gamma$  for 30–36 h caused a significant decrease of the mRNAs encoding for *Bip* (Fig. 3a), *Orp150* (Fig. 3b), *Grp94* (Fig. 3c) and *Sec61a* (Fig. 3d). This inhibitory effect of IFN- $\gamma$ , however, did not impair the induction of these genes by 6–12 h exposure to CPA (6.25–25  $\mu\text{mol/l}$ ; data not shown). IFN- $\gamma$  did not inhibit calbindin or calreticulin mRNA expression under experimental conditions similar to those described in Fig. 3 (data not shown).

IFN- $\gamma$  decreases UPR responses and increases the expression of the pro-apoptotic gene *Chop* in beta cells

The results described above suggest that IFN- $\gamma$  decreases key chaperones involved in the UPR. To evaluate the functional impact of IFN- $\gamma$  on the UPR activation we used the UPRE reporter construct. This element was initially identified as a binding site for the transcription factor ATF6 [20], but later studies indicated that it is mostly activated by XBP1 [21]. Basal activity of the UPRE reporter in INS-1E cells was decreased by IFN- $\gamma$  exposure (22%; Fig. 4a), and pretreatment with IFN- $\gamma$  hampered activation of the UPRE reporter by different concentrations of CPA (around 40% inhibition; Fig. 4a). We then examined the effects of IFN- $\gamma$  on total *Xbp1* mRNA expression and observed a 30% decrease of the *Xbp1* transcript in comparison to control (Fig. 4b). Since only spliced *Xbp1* mRNA is translated to an active XBP1 protein, we next determined the levels of spliced *Xbp1* mRNA in the presence or absence of IFN- $\gamma$ . Between 30 and 36 h exposure to IFN- $\gamma$  decreased *Xbp1s* mRNA (32.5 $\pm$ 1.6% in control conditions vs 25.4 $\pm$ 2.4% IFN- $\gamma$  exposure,  $p < 0.002$ ; Fig. 4c). On the other hand, CPA (6.25 and 25  $\mu\text{mol/l}$ ) induction of both total *Xbp1* and *Xbp1s* mRNAs was unaffected by the IFN- $\gamma$  treatment (data not shown).



**Fig. 3** IFN- $\gamma$  downregulates mRNAs for ER chaperones and *Sec61a*. INS-1E cells were exposed for 30–36 h to IFN- $\gamma$  (0.036  $\mu\text{g/ml}$ ); mRNA was then extracted and real-time RT-PCR was performed using specific primers for *Bip* (a), *Orp150* (b), *Grp94* (c) and *Sec61a* (d). Values of the gene of interest were corrected for *Gapdh* expression. The results are the means $\pm$ SEM of six experiments. \*\* $p \leq 0.01$ , \*\*\* $p \leq 0.001$  vs control,  $t$  test



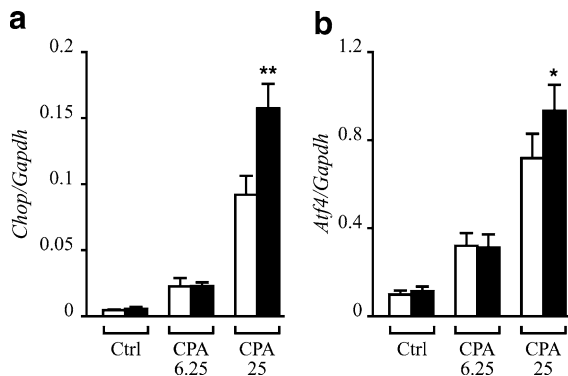
**Fig. 4** IFN- $\gamma$  decreases UPR responses in INS-1E cells. **a** INS-1E cells were co-transfected with the luciferase reporter construct containing five copies of the UPRE and the internal control pRL-CMV. Twenty-four hours after transfection, the cells were pretreated (black bars) or not (empty bars) for 24 h with IFN- $\gamma$  and then incubated with CPA (3.1, 6.25 or 25  $\mu\text{mol/l}$ ) or control (Ctrl, DMSO 0.25%) for 15 h. Results are the mean of nine experiments normalised for *Renilla* luciferase activity $\pm$ SEM. \*\* $p \leq 0.01$ , \*\*\* $p \leq 0.001$  vs no pretreatment,  $t$  test. **b** INS-1E cells were treated for 30–36 h with IFN- $\gamma$  or control as described in the [Materials and methods](#). mRNA was then extracted and real-time RT-PCR was performed using specific primers for *Xbp1* and values were corrected for *Gapdh* expression. Results are mean $\pm$ SEM of six experiments. \*\* $p \leq 0.01$  vs control,  $t$  test. **c** Cells were exposed to IFN- $\gamma$  as described above (b). *Xbp1* splicing was then assessed as described in Fig. 1. The picture is representative of six experiments

One of the mechanisms by which ER stress induces apoptosis is via *Chop* activation [22–24]. Thus, and because pretreatment with IFN- $\gamma$  would decrease the ability of beta cells to face an ER stress, sequential treatment with IFN- $\gamma$  and CPA would be expected to trigger a higher expression of *Chop* mRNA as compared to CPA alone. This was indeed the case, at least for the higher concentration of CPA tested. Thus, the induction of IFN- $\gamma$ +CPA (25  $\mu\text{mol/l}$ ) on *Chop* mRNA expression was almost double the sum of the individual effects of IFN- $\gamma$  and CPA on this mRNA (Fig. 5a). Induction of *Chop* by ER stress is abolished in cells that are knockout for the transcription factor ATF4 [25] and ATF4 has been shown to bind to the *Chop* promoter [26]; we therefore examined whether CPA induced *Atf4* mRNA and whether IFN- $\gamma$  preculture modified the expression of this transcription factor. As observed in Fig. 5b, CPA induces *Atf4* mRNA, and pretreatment with IFN- $\gamma$  augmented *Atf4* induction by 25  $\mu\text{mol/l}$  CPA.

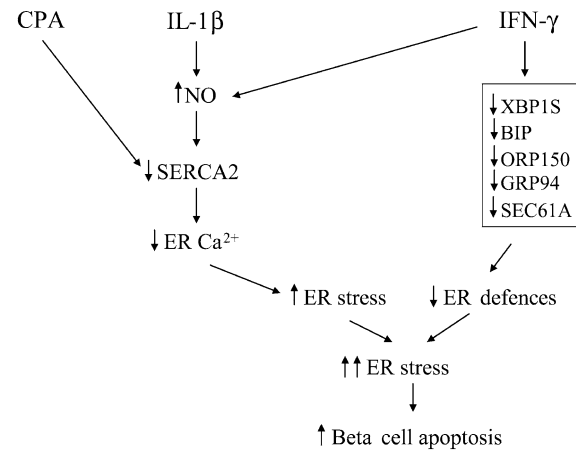
## Discussion

We have observed that low concentrations of CPA induce ER stress and UPR activation in INS-1E cells, as indicated by IRE-1-mediated *Xbp1* alternative splicing, induction of a reporter construct containing five copies of the UPRE, and upregulation of mRNAs encoding classical markers of UPR activation such as *Bip*, *Xbp1* and *Chop*. CPA induces a ‘pure’ ER stress by direct inhibition of the SERCA pump [27], depleting ER  $\text{Ca}^{2+}$  stores without inducing NO production (data not shown). Against this background, we used CPA here to study the putative potentiating effects of IFN- $\gamma$  on ER stress. The results obtained indicate that IFN- $\gamma$  potentiates CPA-induced apoptosis in INS-1E cells and in primary rat beta cells. In contrast, IL-1 $\beta$  or CPA has only a mild additive effect on CPA-induced cell death, indicating specificity for IFN- $\gamma$  in this effect. In some cell types, induction of a mild ER stress protects against cell death by a subsequent and more severe ER stress-inducing stimulus [28–30]. This protection is mediated via induction of ER chaperones such as BiP, GRP 94 and calreticulin [28–30]. The lack of such a phenomenon in beta cells exposed to sublethal concentrations of CPA or IL-1 $\beta$  (present data) probably reflects the exquisite vulnerability of beta cells to disruption of ER homeostasis.

Prolonged exposure to IFN- $\gamma$  significantly decreased UPRs, as suggested by the decreased basal *xbp1s* mRNA and the decrease in both basal and CPA-induced activity of the UPRE reporter construct. Moreover, mRNA expression of important ER chaperones, such as *Orp150*, *Grp94* and *Bip*, and of the  $\alpha$  subunit of the *Sec61* translocon, were also decreased by IFN- $\gamma$ . XBP1 is a key transcription factor for the induction of ER chaperones, including genes from the ERAD pathway during UPR [5, 31]. The spliced (active) form of *Xbp1* mRNA is detectable at basal levels in primary beta cells [8] and in INS-1E cells (present study). Taking into account the high synthesis of insulin by beta cells, this basal XBP1 activation is probably important for



**Fig. 5** IFN- $\gamma$  potentiates CPA induction of *Chop* and *Atf4* mRNAs. INS-1E cells were pretreated (black bars) or not (empty bars) for 24 h with IFN- $\gamma$  and then incubated with CPA (6.25 or 25  $\mu\text{mol/l}$ ) or control (Ctrl, DMSO 0.25%) for 15 h. mRNA was then extracted and real-time RT-PCR performed using specific primers for *Chop* (a) and *Atf4* (b). Values were corrected for *GAPDH* expression. The results are the means  $\pm$  SEM of six experiments. \* $p < 0.05$ , \*\* $p < 0.01$  vs no pre-treatment,  $t$  test



**Fig. 6** Proposed model for the synergistic effects of IL-1 $\beta$  or CPA and IFN- $\gamma$  in inducing beta cell ER stress and apoptosis. Details, see Discussion

the maintenance of beta cell ER homeostasis. BiP is a master regulator of ER functions, directing protein folding and assembly, sensing stress in the organelle and contributing to the preservation of the ER  $\text{Ca}^{2+}$  stores [32]. GRP94 also has  $\text{Ca}^{2+}$ -binding properties [33]. The high basal levels of BiP and ORP150 expression in beta cells suggest an important role for these chaperones in beta cell function and survival [24, 34]. All these chaperones, presently shown to be downregulated by IFN- $\gamma$ , have been previously shown to protect different cell types against death [29, 35–38]. The SEC61 channel is involved both in translation of ER proteins and translocation of proteins to be degraded by the ERAD pathway [39, 40]. Efficient removal of misfolded proteins via the ERAD pathway is essential for cellular recovery during the ER stress response [40]. The lower basal expression of *Xbp1*, *Bip*, *Grp94*, *Orp150* and *Sec61a* mRNAs induced by IFN- $\gamma$  probably decreases the beta cells defences against ER stress, rendering them particularly vulnerable to agents such as IL-1 $\beta$  or CPA. This is in agreement with the potentiating effects of IFN- $\gamma$  on the subsequent CPA-induced ER stress response, as measured by increased *Chop* expression and apoptosis (present findings), and with recent observations that IFN- $\gamma$  contributes to ER stress and apoptosis in oligodendrocytes [41]. *Atf4* mRNA expression was upregulated by CPA and pre-culture with IFN- $\gamma$  led to a small but significant increase in CPA-mediated *Atf4* induction. This may contribute to the potentiating effects of IFN- $\gamma$  on *Chop* mRNA expression, because ATF4 is involved in *Chop* induction during ER stress [26]. Further studies are now necessary to understand the molecular mechanisms by which IFN- $\gamma$  modulates the expression of this group of genes.

Of note, IFN- $\gamma$  stimulates MHC class I and II expression and induction of transporter associated with antigen processing (TAP) genes in beta cells [12, 42]. The MHC class I complex is assembled in the ER, while antigenic peptides generated in the cytosol are transported to the ER via the TAP transporters. Part of these peptides return to the cytosol, via the SEC61 channel [43]. The accumulation of

MHC molecules and peptides in the ER lumen leads to an ER overload that, under certain conditions, may cause ER stress [43]. In line with this, transgenic overexpression of MHC molecules in the beta cells leads to severe beta cell dysfunction and death [44–46]. It is thus possible that IFN- $\gamma$ -mediated ER overload also contributes to the potentiating effects of IFN- $\gamma$  on ER stress-induced beta cell apoptosis.

We previously observed that IL-1 $\beta$  induces an ER stress response in beta cells without leading to apoptosis [8]. IFN- $\gamma$  alone did not induce ER stress [8] (present data) but, in combination with IL-1 $\beta$ , it increased IL-1 $\beta$ -induced Ca<sup>2+</sup> depletion and triggered beta cell apoptosis [8]. Based on the present observations, we propose a model to explain the synergistic effects of the two cytokines on beta cell ER stress and apoptosis (Fig. 6). Thus, IL-1 $\beta$  induces an NO-mediated inhibition of the SERCA2 pump and a partial depletion of ER Ca<sup>2+</sup> stores, leading to ER stress in beta cells. CPA also inhibits SERCA activity and causes ER stress, but this effect is independent of NO formation. IFN- $\gamma$  enhances IL-1 $\beta$ -induced ER stress by downregulating beta cell defences against ER stress and by increasing NO production. These combined effects, if sufficiently prolonged, will eventually ‘convince’ the beta cells to start the apoptotic programme.

**Acknowledgements** This work was supported by grants from the EFSD/JDRF/Novo Nordisk European Programme in Type 1 Diabetes Research, the Fonds National de la Recherche Scientifique (FNRS; Belgium) and the Communauté Française de Belgique—Actions de Recherche Concertées (ARC). We thank the staff from the Laboratory of Experimental Medicine, ULB, and especially M. A. Neef, G. Vandebroek and R. Leeman for excellent technical support. A. K. Cardozo is the recipient of a postdoctoral fellowship from the JDRF.

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