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ARTICLE

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# Growth arrest- and DNA-damage-inducible 45β gene inhibits c-Jun N-terminal kinase and extracellular signal-regulated kinase and decreases IL-1β-induced apoptosis in insulin-producing INS-1E cells

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**Abstract** *Aims/hypothesis:* IL-1β is a candidate mediator of apoptotic beta cell destruction, a process that leads to type 1 diabetes and progression of type 2 diabetes. IL-1β activates beta cell c-*Jun* N-terminal kinase (JNK), extracellular signal-regulated kinase (ERK) and p38, all of which are members of the mitogen-activated protein kinase (MAPK) family. Inhibition of JNK prevents IL-1β-mediated beta cell destruction. In mouse embryo fibroblasts and 3DO T cells, overexpression of the gene encoding growth arrest and DNA-damage-inducible 45β (*Gadd45b*) downregulates pro-apoptotic JNK signalling. The aim of this study was to investigate if *Gadd45b* prevents IL-1β-induced beta cell MAPK signalling and apoptosis. *Materials:* Rat insulinoma INS-1E cells and mouse beta-TC3 cells stably expressing *Gadd45b* were generated. The effects of *Gadd45b* expression on signalling by JNK, ERK and p38 were assessed by Western blotting and kinase assays. Apoptosis rate was measured by terminal deoxynucleotidyl-mediated dUTP-biotin nick end-labelling (TUNEL) and an ELISA designed to detect apoptotic nucleosomes. Expression of endogenous *Gadd45b* mRNA was measured by RT-PCR. *Results:* In INS-1E and beta-TC3 cells, expression of *Gadd45b* inhibited IL-1β-induced activation of JNK and ERK, but augmented IL-1β-mediated p38 activity. IL-1β-induced nitric oxide production and decreases in insulin content and secretion were reduced by GADD45β. IL-1β-induced apoptosis was reduced by GADD45β by up to 77%. Although IL-1β stimulated the time-dependent induction of endogenous *Gadd45b* in INS-1E cells and rat islets, expression levels were lower in these cells than in IL-1β-exposed NIH-3T3 and 3DO T cells. *Conclusions/interpretation:* Inadequate induction of *Gadd45b*, which encodes a novel beta cell JNK and ERK inhibitor, may in part explain the pro-apoptotic response of beta cells to IL-1β.

**Keywords** Apoptosis · Beta cells · *Gadd45b* · IL-1β · INS-1E · iNOS · ERK · JNK · p38

**Abbreviations** CHX: cycloheximide · ERK: extracellular signal-regulated kinase · GADD: growth arrest and DNA-damage-inducible · iNOS: inducible nitric oxide synthase · IκBα: inhibitor protein κBα · JNK: c-Jun N-terminal kinase · MAPK: mitogen-activated protein kinase · MEF: mouse embryo fibroblast · MEK: mitogen-activated protein kinase/extracellular signal-regulated kinase · MKK: mitogen-activated protein kinase kinase · MEKK: mitogen-activated protein kinase kinase kinase · NFκB: nuclear factor κB · NO: nitric oxide · PMA: phorbol myristate acetate

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## Introduction

Destruction of the pancreatic beta cells is the hallmark of type 1 diabetes [1] and is involved in the development and progression of type 2 diabetes [2]. The cytokine IL-1β has been proposed to be a mediator of the autoimmune destruction that leads to type 1 diabetes [3] and it has recently been suggested that IL-1β is involved in the glucotoxic beta cell destruction associated with type 2 diabetes [4].

In beta cells IL-1β causes activation of the c-*Jun* N-terminal kinase (JNK), extracellular signal-regulated kinase (ERK) and p38, members of the mitogen-activated protein kinase (MAPK) family of threonine or serine kinases [5, 6]. ERK and p38 are both involved in the IL-1β-mediated beta cell induction of inducible nitric oxide (NO) synthase (iNOS), leading to NO formation [6]. NO is a secondary messenger for the beta cell cytotoxic effect of IL-1β [7, 8], especially in rodent islets. However, NO-independent cytokine-mediated destruction of beta cells is possible [9–11] and of particular relevance in human beta cells [9, 11]. Inhibition of JNK activation protects beta cell lines against IL-1β-induced apoptosis [5, 12] and human islets against the destruction mediated by IL-1β, TNF-α and IFN-γ [13, 14]. Disruption of JNK activation also protects beta cells against T-cell-mediated killing [15], increases islet survival after isolation [13, 16] and improves islet graft function [16, 17]. Taken together, these observations demonstrate that JNK inhibition is crucial in maintaining the function and survival of beta cells when exposed to multiple stressors.

Prolonged JNK activation promotes apoptosis in several cell types [18, 19]. IL-1β alone or in combination with TNF-α and IFN-γ causes sustained activation of JNK in beta cells [13, 20], indicating insufficient downregulation of JNK activity in cytokine-exposed beta cells.

*Gadd45b* (which encodes growth arrest and DNA-damage-inducible 45β), *Gadd45a* and *Gadd45g* are members of the *Gadd45* gene family, which is involved in growth arrest, apoptosis and DNA repair [21]. We have recently shown that overexpression of *Gadd45b* down-regulates pro-apoptotic JNK signalling in mouse embryo fibroblasts (MEFs) and 3DO T cells, without affecting the ERK and p38 activities [22]. However, the involvement of *Gadd45b* in MAPK signalling seems to be cell-specific, because overexpression of *Gadd45b* in other cell types augments JNK and p38 activities and promotes apoptosis [23].

The aim of the present work was therefore to investigate the regulation of *Gadd45b* induction by IL-1β in beta cells and to explore the involvement of GADD45β in beta cell IL-1β-induced MAPK signalling and apoptosis. We report that *Gadd45b* is a novel beta cell primary response gene with insufficient induction in INS-1E and beta-TC3 cell IL-1β signalling, and that expression of *Gadd45b* inhibits IL-1β-induced JNK and ERK signalling and apoptosis.

## Materials and methods

### Reagents

Recombinant mouse IL-1β was from BD Pharmingen (Erembodegen, Belgium), Recombinant human TNF-α was from Endogen (Woburn, MA, USA) and recombinant rat IFN-γ from R & D Systems (Minneapolis, MN, USA). Phorbol myristate acetate (PMA) and cycloheximide (CHX) were from Sigma-Aldrich (San Diego, CA, USA).

### Cell culture

INS-1E cells (kindly provided by C. Wollheim, University Medical Centre, Geneva, Switzerland), beta-TC3 cells and 3DO T cells were grown in RPMI-1640 medium (11 mmol/l glucose) containing 10% FCS, 100 U/ml penicillin and 100 μg/ml streptomycin. In addition, the INS-1E and 3DO T-cell culture medium contained 50 μmol/l β-mercaptoethanol. Mouse NIH-3T3 fibroblasts were maintained in DMEM supplied with FCS, penicillin and streptomycin as described above.

### Islet isolation and culture

Pancreatic islets of Langerhans from 3- to 5-day-old Wistar Furth rats (Charles River, Sulzfeldt, Germany) were isolated by hand-picking after collagenase digestion as described previously [6]. Islets were cultured in RPMI-1640 supplemented with 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin and 0.038% $NaHCO_3$ for 7 days prior to experimentation.

### Stable transfection

INS-1E and beta-TC3 cells were transfected with pcDNA3.1/Flag (Invitrogen, Carlsbad, CA, USA) or pcDNA3.1/Flag-*Gadd45b* [24] by the use of FuGENE 6 Transfection Reagent (Roche, Basel, Switzerland) following the manufacturer's instructions. The cells were cultured for 2 days, then trypsinised and reseeded in 100 mm dishes at two densities (1:10 and 9:10). Based on kill-curve experiments, 100 μg/ml G418 (Life technologies, Grand Island, NY, USA) was added for the selection of geniticin-resistant cells. Geniticin-resistant colonies were selected after 2–4 weeks and propagated in 50 μg/ml G418. The selected clones were assayed for *Gadd45b* (Fig. 1) expression by RT-PCR and Western blotting.

### Protein extraction

Following IL-1β stimulation $5\times10^5$ INS-1E or beta-TC3 cells were washed once in cold PBS and then lysed in 75 μl lysis buffer containing 20 mmol/l Tris acetate, pH 7.0, 0.27 mol/l sucrose, 1 mmol/l EDTA, 1 mmol/l EGTA, 1 mmol/l $Na_3VO_4$, 50 mmol/l NaF, 1% Triton X-100, 5 mmol/l sodium pyrophosphate, 10 mmol/l β-glycerophosphate, 1 mmol/l dithiothreitol, 1 mmol/l benzamidine and 4 μg/ml leupeptin. The detergent-insoluble material was pelleted by centrifugation at 15,000× *g* for 5 min at 4°C. The protein concentration in the supernatant was measured by Bradford assay (Bio-Rad, Hercules, CA, USA). The supernatants containing whole-cell lysate were either used immediately (for kinase assay or Western blotting) or stored at −80°C.

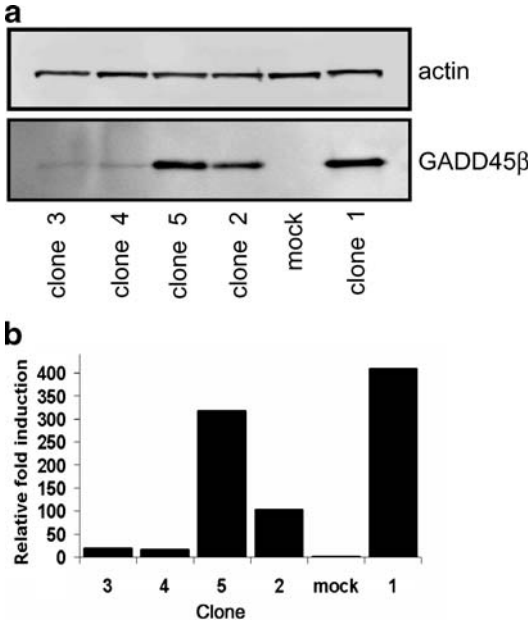



**Fig. 1** *Gadd45b*-transfected INS-1E clones. INS-1E cells were stably transfected with Flag-*Gadd45b* (clones 1–5) or empty Flag vector (*mock*). **a** INS-1E cells were lysed and Western blotting was performed with anti-GADD45β and -actin antibodies. **b** Total RNA was extracted and real-time RT-PCR was performed using *Gadd45b*-specific primers. Results are relative expression normalised to *Sp1*

## Western blotting

Protein (15–20 μg) was separated on 10% BisTris gels (Invitrogen, Carlsbad, USA) by gel electrophoresis using NuPAGE technology (Invitrogen). Following electrotransfer to nitrocellulose membranes (Invitrogen) and blocking in 5% milk dissolved in Tris-buffered saline containing 0.1% Tween-20, membranes were incubated in primary antibody at 4°C overnight. The following antibodies were used: rabbit anti-JNK (diluted 1:1000; Cell Signaling Technology, Beverly, MA, USA); rabbit anti-phospho-JNK (1:1000; Cell Signaling Technology); rabbit anti-ERK (1:1000; Cell Signaling Technology); rabbit anti-phospho-ERK (1:1000; Cell Signaling Technology); rabbit anti-p38 (1:500; Cell Signaling Technology); rabbit anti-phospho-p38 (1:500; Cell Signaling Technology); rabbit anti-phospho-MKK7 (1:250; Cell Signaling Technology); mouse anti-IκBα (1:500; Active Motif, Rixensart, Belgium); mouse anti-iNOS (1:5000; BD Biosciences, San Jose, CA, USA); mouse anti-actin (1:10 000; Abcam, Cambridge, UK) and rabbit anti-GADD45α (1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Mouse anti-GADD45β (1:500) was used as described previously [22]. Following addition of secondary peroxidase-conjugated anti-rabbit or -mouse antibodies (both from Cell Signaling Technology), binding of antibody was detected with enhanced chemiluminescence using SuperSignal (Pierce, Rockford, IL, USA). Bands were visualised with a Luminescent Image Analyzer LAS-3000 and the optical densities were quantitated using Multi Gauge Software (both from Fujifilm, Stamford, CT, USA).

## Kinase assay

The kinase assays measuring phosphotransferase activities in the cell lysates towards exogenous c-*Jun* (Calbiochem, San Diego, CA, USA), Elk-1 (a kind gift from K. Seedorf, Lilly, Hamburg) and Hsp25 (Stressgen, Victoria, BC, Canada) were performed as described previously [6] except that 2 μg GST-c-*Jun* was used instead of activating transcription factor-2. The incorporation of $\gamma$-$^{32}$P from [$\gamma$-$^{32}$P]ATP (Amersham, UK) in the substrates was measured by autoradiography and quantitated using Multi Gauge Software (Fujifilm Medical systems).

## RNA extraction and cDNA synthesis

Total RNA from $1\times10^6$ INS-1E and beta-TC3 cells, $5\times10^5$ NIH-3T3 and 3DO T cells or 300 islets was extracted by the use of Trizol reagent (Invitrogen) following the manufacturer's instructions. cDNA was synthesised from 400 ng of RNA using the TaqMan kit from Perkin Elmer (Wellesley, MA, USA).

## Quantitative RT-PCR

Real time RT-PCR was performed on the 7900 HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA) using cDNA and SYBR Green master mix (Applied Biosystems). Expression levels of *Gadd45a*, *Gadd45b* and *Gadd45g* were calculated by the standard curve method and the quantity of each cDNA was normalised for transcription factor *Sp-1*, showing no regulation by IL-1β [25]. The primers were as follows: *Gadd45a*, 5′-TGAGCTGCTGC TACTGGAGA-3′ and 5′-TGTGAT GAATGTGGGTTCGT -3′; *Gadd45b*, 5′-ATTGACATCG TCCGGGTATC-3′ and 5′-TGACAGTTCGTGACCAGG AG-3′; *Gadd45g*, 5′-GC ATCCTCATTTCGAATCCT-3′ and 5'-CACCCAGTCGTT GAAGCTG-3′; *Sp-1*, 5′-GG CTACCCCTACCTCAAAGG -3′ and 5′-CA CAACAT ACTGCCCACCAG-3′.

## NO synthesis

INS-1E NO production was measured as nitrite accumulation in conditioned medium determined by the Griess reaction, as described previously [6]. The detection limit was 1 μmol/l.

## Insulin content and secretion

The insulin content of protein extracts from INS-1E and beta-TC3 cells and the accumulated insulin secretion, measured as insulin in the conditioned medium, were determined by radioimmunoassay [6].

### Detection of apoptosis

To determine the apoptosis rate two assays were used.

For the Cell Death Detection ELISA$^{plus}$ kit (Roche), which detects apoptotic nucleosomes in the cytoplasmic fraction of cell lysate, $5\times10^4$ INS-1E cells were seeded in 48-well dishes. Following exposure to IL-1β, the assay was performed as described by the manufacturer. The nucleosomes were measured by sandwich ELISA and data are presented as fold induction relative to untreated cells. For the detection of apoptosis with the TUNEL (terminal deoxynucleotidyl-mediated dUTP-biotin nick end-labelling) assay, $2\times10^5$ INS-1E cells were seeded in two-chamber wells. Following exposure to IL-1β, cells were fixed in 4% paraformaldehyde and the free 3′-OH strand breaks were detected by the TUNEL labelling technique according to the manufacturer's instructions (ApopTag In Situ Apoptosis Detection Kit; Chemicon International, Temecula, CA, USA). Staining with DAPI (4′,6-diamidino-2-phenylindole; 1 μg/ml) was used to assess the total number of cells. By fluorescence microscopy, a total of at least 500 cells in each condition were counted in a randomised manner by an investigator (M.G.D.) blinded to the treatment conditions. The number of TUNEL-positive cells was expressed as the percentage of the number of DAPI-stained cells.

### Statistical analysis

In histograms, data are mean±SEM. Statistical analysis was performed with the two-tailed paired Student's *t* test and $p<0.05$ was chosen as the level of significance.

## Results

### Selection of *Gadd45b*-expressing INS-1E clones

To investigate the level of GADD45β production in the stably transfected INS-1E clones, we performed Western blotting of five clones. As seen in Fig. 1a, clones 1 and 5 produced the highest levels of the GADD45β protein, with similar findings at the mRNA level (Fig. 1b). To investigate a possible dose-dependency of the effects of *Gadd45b* expression, clones 1 and 2 were chosen for further experiments.

### GADD45β attenuates IL-1β-induced JNK and ERK activation, but augments p38 activation

To determine the involvement of GADD45β in the regulation of IL-1β-induced MAPK signalling, we first performed Western blotting with phosphospecific antibodies recognising the phosphorylated (activated) kinases. As shown in Fig. 2a,b, high levels of GADD45β production (clone 1) reduced IL-1β-induced INS-1E JNK activation by 60%. GADD45β production dose-dependently inhibited IL-1β-induced ERK activity by up to 86%. On the other hand, high levels of GADD45β production augmented both basal and IL-1β-mediated p38 MAPK activation, by 400 and 70%, respectively. Similar inhibition of IL-1β-induced JNK and ERK activation, but less enhancement of p38 activation, was detected in a pool of GADD45β-producing mouse insulinoma beta-TC3 cells (Fig. 2c,d).

To examine the in vitro kinase activities of JNK, ERK and p38 MAPK in *Gadd45b*-expressing INS-1E cells, we determined the phosphotransferase activities toward exogenous c-*Jun*, Elk-1 and Hsp25 [6]. GADD45β dose-dependently reduced IL-1β-induced, ERK-mediated Elk-1 phosphorylation by up to 70% (Fig. 3). IL-1β-induced c-*Jun* phosphorylation mediated by JNK was inhibited by 54% by high levels of GADD45β (clone 1). IL-1β-mediated p38 MAPK activity, measured by the phosphorylation of p38-activated MAPK-activated protein kinase 2 substrate Hsp25, was enhanced by up to 47% by *Gadd45b* expression.

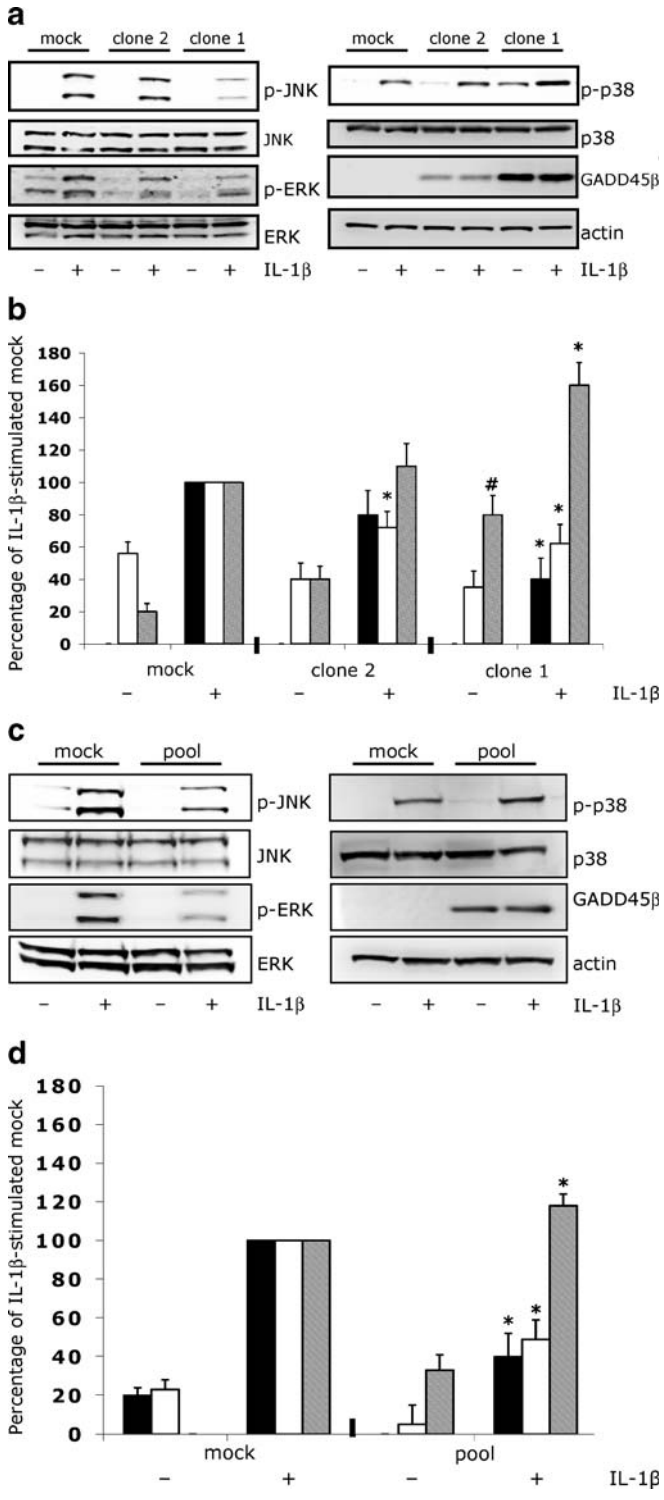
### GADD45β decreases IL-1β-induced MKK7 activation but not inhibitor protein κBα degradation

We have previously shown that GADD45β inhibits JNK signalling in 3DO T cells and MEFs via inhibition of the JNK upstream kinase MAPK kinase (MKK) 7 [24]. Similarly, high levels of GADD45β production decreased the IL-1β-induced phosphorylation of MKK7 in INS-1E cells (Fig. 4). Having found that GADD45β modulated IL-1β-induced INS-1E MAPK signalling, we now asked whether GADD45β could interact with nuclear factor κB (NFκB) signalling, another major beta cell IL-1β signalling pathway [26]. As shown in Fig. 4, GADD45β production did not affect IL-1β-mediated degradation of the NFκB repressor inhibitor protein κBα (IκBα).

### GADD45β production decreases IL-1β-induced NO synthesis and inhibition of insulin content and release

To assess the effect of stable *Gadd45b* expression on INS-1E cell function, we first measured NO accumulation in the medium following 48 h of IL-1β exposure. As shown in Fig 5b, low and high concentrations of GADD45β decreased IL-1β-induced NO accumulation by 50% and 72%, respectively. The reduced amount of IL-1β-induced NO accumulation by *Gadd45b*-expressing cells was due to decreased production of iNOS protein (Fig. 5a).

Forty-eight hours of exposure to IL-1β caused 82% and 72% reduction of INS-1E cell insulin content (Fig. 5c) and accumulated insulin release (Fig. 5d), respectively. Stable *Gadd45b* expression neither affected the insulin content (Fig. 5c) nor the accumulated insulin release (Fig. 5d) of control cell cultures; however, high levels of GADD45β production reduced the IL-1β-mediated inhibition of insulin content and release to 47% and 38%, respectively,




◀ **Fig. 2** GADD45β inhibits IL-1β activation of JNK and ERK, but augments p38 activation in INS-1E and beta-TC3 cells. INS-1E or beta-TC3 cells stably transfected with mock and Flag-*Gadd45b* (clones 1 and 2 or pool) were exposed to 160 pg/ml IL-1β for 30 min, lysed and subjected to Western blotting. **a**, **c** Membranes were probed with antibodies recognising the phosphorylated forms of JNK, ERK or p38 (p-JNK, p-ERK and p-p38) and reprobed with antibodies directed against JNK, ERK or p38. Membranes were finally probed with anti-GADD45β and -actin antibodies. Representative blots of $n$=4–8 are shown. **b**, **d** The optical densities of the individual bands containing p-JNK (*black boxes*), p-ERK (*white boxes*) and p-p38 (*shaded boxes*) were measured and are presented in the histogram as the means±SEM of four to eight experiments. *$p$<0.05 vs IL-1β-stimulated mock; #$p$<0.05 vs untreated mock

## GADD45β production reduces IL-1β-induced INS-1E apoptosis

Forty-eight hours of incubation with IL-1β caused 4.7- and 4.9-fold increases in the apoptosis rate in mock transfected INS-1E cells investigated with the Cell Death Detection ELISA$^{plus}$ and TUNEL methods, respectively, (Fig. 6a,b). High levels of GADD45β production reduced the apoptosis rate by 77% and 60%, assayed by Cell Death Detection ELISA$^{plus}$ and TUNEL assays, respectively, (Fig. 6a,b). In the TUNEL assay, the basal apoptosis rate was not affected by GADD45β production.

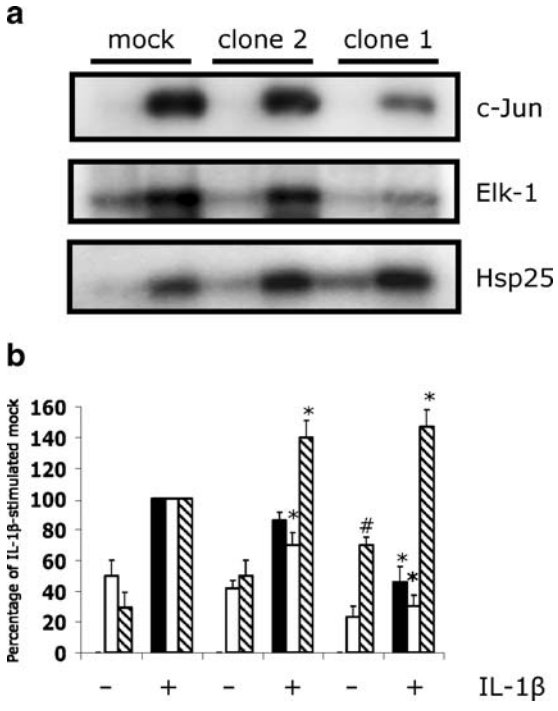



**Fig. 3** GADD45β inhibits IL-1β-induced phosphorylation of c-*Jun* and Elk-1, but enhances Hsp25 phosphorylation in INS-1E cells. INS-1E cells stably transfected with mock and Flag-*Gadd45b* (clones 1 and 2) were exposed to 160 pg/ml IL-1β for 30 min and lysed; kinase assays with [γ-$^{32}$P]ATP and c-*Jun*, Elk-1 or Hsp25 substrates was then performed. Representative autoradiograms are shown in (**a**). **b** The optical densities of c-*Jun* (*black boxes*), Elk-1 (*white boxes*) or Hsp25 (*shaded boxes*) phosphorylations are shown in the histogram as the means±SEM of four experiments. *$p$<0.05 vs IL-1β-stimulated mock. #$p$<0.05 vs untreated mock

(Fig. 5c,d). In beta-TC3 cells, 48 h of IL-1β exposure caused a 57% reduction in both insulin content (Fig. 5e) and accumulated insulin release (Fig. 5f). The insulin content and accumulated insulin release were unaffected in the control pool culture of *Gadd45b*-expressing beta-TC3 cells, but the IL-1β-induced inhibition of insulin content and release was reduced to 27 and 23%, respectively, (Fig. 5e,f).

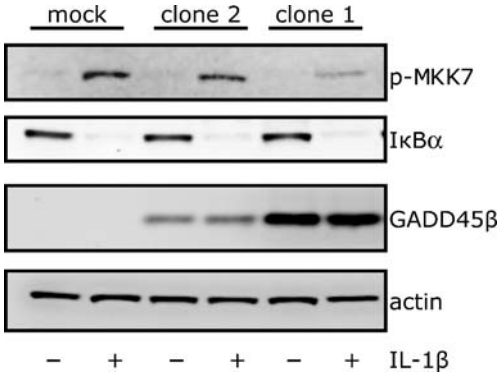



**Fig. 4** GADD45β decreases IL-1β-induced MKK7 activation but not IκBα degradation in INS-1E cells. INS-1E cells stably transfected with mock and Flag-*Gadd45b* (clones 1 and 2) were exposed to 160 pg/ml IL-1β for 30 min and lysed, and then subjected to Western blotting using anti-IκBα, anti-actin or phospho-specific anti-MMK7 (p-MKK7) antibodies. Representative blots from three experiments are shown

### *Induction of Gadd45 gene family by cytokines in islets and INS-1E cells*

Having investigated the involvement of *Gadd45b* in MAPK signalling, we now explored the regulation of endogenous *Gadd45b* by cytokines. As seen in Fig. 7a, IL-1β caused time-dependent induction of *Gadd45b* mRNA in INS-1E cells. Induction occurred within 1 h, there was a 10.9-fold peak of induction at 2 h, and the basal level was regained at 24 h. TNF-α did not induce *Gadd45b* mRNA and IFN-γ caused a weaker and more delayed induction of *Gadd45b*, with maximal 4.7-fold induction of *Gadd45b* mRNA at 4 h. Unlike *Gadd45b*, the *Gadd45a* and *Gadd45g* gene family members were not inducible by IL-1β, TNF-α or IFN-γ in INS-1E cells. The time-dependent induction of *Gadd45b* mRNA by IL-1β was reproduced in isolated rat islets. Induction was 3.7-fold at 30 min and maximal (29.8-fold) at 2 h, and sustained induction was detectable until at least 24 h of IL-1β exposure (Fig. 7b). The changes at mRNA level were mirrored at the protein level, as the GADD45α protein showed no regulation by IL-1β in INS-1E cells and islets (Fig. 7c), and the GADD45β protein was upregulated by IL-1β in both islets and INS-1E cells in a time-dependent manner (Fig. 7c). However, maximal production of endogenous GADD45β protein did not exceed the amount produced by clones 1 and 2 (Fig. 7c,d). The anti-GADD45γ antibody was not suitable for Western blotting.

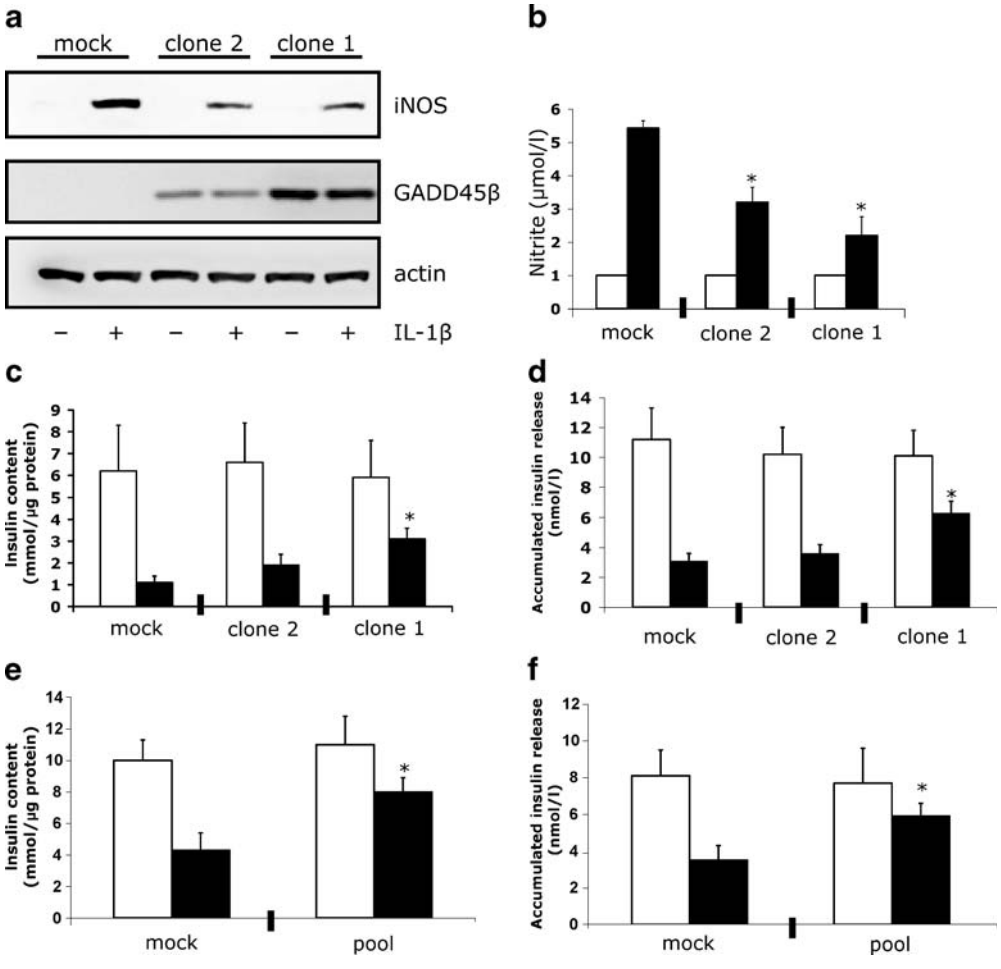



**Fig. 5** GADD45β decreases IL-1β-induced NO synthesis and inhibition of insulin content and release. INS-1E or beta-TC3 cells stably transfected with mock and Flag-*Gadd45b* (clones 1 and 2 or pool) were exposed to 160 pg/ml IL-1β. **a** After 24 h of IL-1β exposure, INS-1E cells were lysed and Western blotting was performed using anti-iNOS or -actin antibodies. A *blot* representative of three experiments is shown. **b** After 48 h of IL-1β exposure, INS-1E incubation medium was sampled; the NO released from untreated (*white boxes*) and IL-1β (*black boxes*)-treated cells is presented as the means±SEM of five experiments. **c**–**f** After 48 h of IL-1β exposure, incubation medium was sampled and the cells were lysed. The insulin content of the lysates from INS-1E (**c**) and beta-TC3 (**e**) cells and the insulin released from INS-1E (**d**) and beta-TC3 (**f**) cells (*white boxes*, untreated cells; *black boxes*, IL-1β-treated cells) are the means±SEM of four experiments. *$p<0.05$ vs IL-1β-stimulated mock

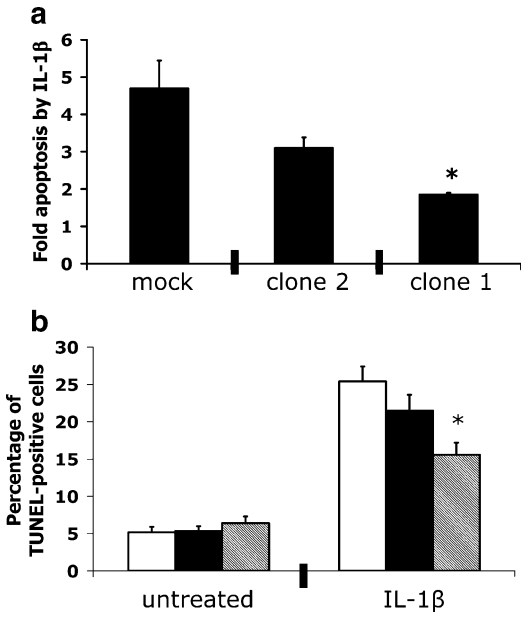



**Fig. 6** GADD45β production reduces IL-1β-induced INS-1E apoptosis. After 48 h of IL-1β exposure, apoptosis was detected by (**a**) Cell Death Detection ELISA$^{plus}$ assay and is shown as relative fold induction by IL-1β compared with untreated cells, or (**b**) TUNEL assay (mock, *white boxes*; clone 2, *black boxes*; and clone 1, *shaded boxes*), and is presented as the percentage of TUNEL-positive cells. Data in (**a**) and (**b**) are the means±SEM of four experiments. *$p$<0.05 vs IL-1β-stimulated mock

### *Gadd45b*, but not *Gadd45a* or *Gadd45g*, is a primary response gene in beta cell IL-1β signalling

To further investigate the regulation of *Gadd45b* mRNA expression, INS-1E cells were preincubated with the protein synthesis inhibitor CHX and then exposed to IL-1β or the phorbol ester PMA [27]. All three *Gadd45b* genes were induced by CHX, with 5.5-, 13.6- and 12.3-fold induction of *Gadd45a*, *Gadd45b* and *Gadd45g*, respectively, (Fig. 8). As expected, *Gadd45b*, but not *Gadd45a* or *Gadd45g*, was induced by IL-1β. PMA caused induction of *Gadd45b* mRNA but not of *Gadd45a* or *Gadd45g* mRNAs. Coincubation of CHX and IL-1β caused 4.4-fold superinduction of *Gadd45b* over the IL-1β-treated level. No superinduction of *Gadd45a* or *Gadd45g* was seen on coincubation of CHX and IL-1β. None of the *Gadd45* genes showed superinduction on coincubation with CHX and PMA. Induction of *Gadd45b* mRNA by IL-1β, but not by PMA, is thus refractory to translational blockade and *Gadd45b* is a primary response gene in IL-1β, but not PMA, signalling [28]. Neither *Gadd45a* nor *Gadd45g* is a primary response gene in beta cell IL-1β or PMA signalling.

### Reduced *Gadd45b* induction in beta-TC3 and INS-1E cells compared with NIH-3T3 and 3DO T cells

To compare the induction of *Gadd45b* mRNA in different species and cell types, mouse beta-TC3, rat INS-1E, mouse fibroblast NIH-3T3 and mouse 3DO T cells were exposed to IL-1β or PMA. As seen in Fig. 9a, IL-1β caused time-dependent induction of *Gadd45b* mRNA in all four cell lines, with maximal induction of 51.0-, 39.5-, 11.8-, 4.4-fold in 3DO, NIH-3T3, INS-1E and beta-T3 cells, respectively. PMA was a weaker inducer of *Gadd45b* mRNA and gave greater similarity of induction of *Gadd45b* mRNA in the four cell lines, with maximal induction of 13.1-, 9.2-, 7.1- and 5.4-fold in the 3DO, NIH-3T3, INS-1E and beta-T3 cells, respectively. This indicates insufficient *Gadd45b* induction specifically in response to IL-1β in beta cells.

The high levels of IL-1β-induced *Gadd45b* expression found in 3DO T cells were associated with transient IL-1β-mediated JNK activation, which was terminated within 1 h (Fig. 9b), whereas the lower level of IL-1β-induced *Gadd45b* expression in INS-1E cells was associated with sustained JNK activation lasting for at least 8 h (Fig. 9c).

## Discussion

The molecular mechanisms for the noticeable sensitivity of beta cells to apoptosis induced by IL-1β and the potentiating cytokines TNF-α and IFN-γ are incompletely understood, but include IL-1β-mediated downregulation of islet-brain 1 protein [29] and inadequate upregulation of manganese superoxide dismutase [30]. In the present paper we report decreased upregulation of beta cell *Gadd45b* by IL-1β, and that stable expression of *Gadd45b* inhibits IL-1β-induced MAPK activation, NO synthesis and apoptosis, and restores the IL-1β-induced decreases in insulin content and release.

Whereas GADD45α and GADD45γ are strictly pro-apoptotic proteins [23, 31], the involvement of GADD45β in the apoptotic process is debated. The present study demonstrates an anti-apoptotic effect of *Gadd45b* expression in beta cells exposed to IL-1β, and we have previously shown that enhanced *Gadd45b* expression prevents apoptosis [22, 24, 32] in other cell types. However, others have shown a pro-apoptotic effect of *Gadd45b* expression [23], whereas in two recent studies *Gadd45b* deficiency [33] or overexpression [31] had no effect on cell survival or apoptosis. The molecular switch that determines the anti- or pro-apoptotic effects of GADD45β seems to be the ability of GADD45β in a particular cell type to either inhibit or activate JNK signalling. The pro-apoptotic action of GADD45β depends on the activation of JNK by MKK7-independent signalling via activation of MAPK kinase kinase (MEKK) 4, leading to MKK4 phosphorylation and concomitant JNK activation [23]. The GADD45β-mediated inhibition of JNK activity (Figs. 2 and 3) is probably linked to the observed decrease in MKK7 activation, a known target of GADD45β inhibition

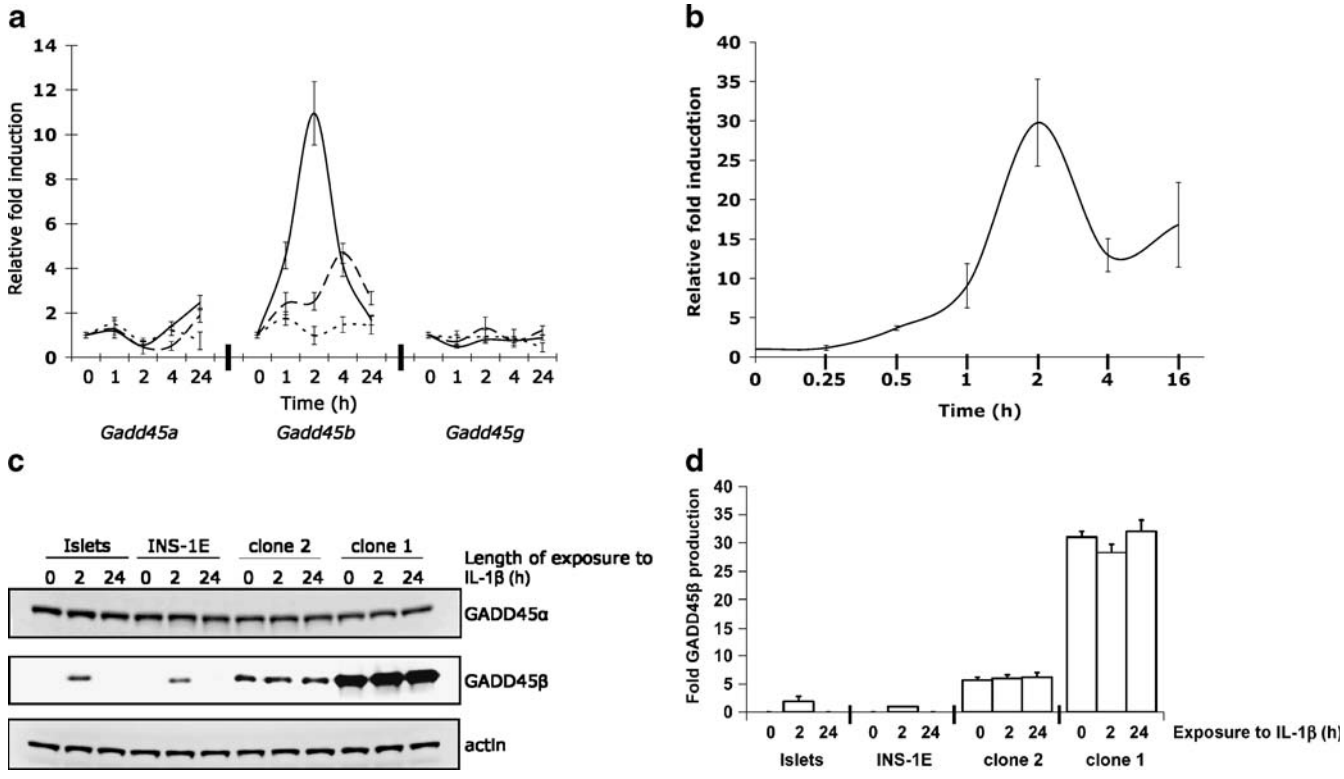



**Fig. 7** Induction of *Gadd45* gene family members by cytokines in islets and INS-1E cells. **a** INS-1E cells were exposed to 160 pg/ml IL-1β (*black line*), 20 ng/ml TNF-α (*dotted line*) or 50 ng/ml IFN-γ (*dot-and-dash line*) for 0–24 h. Total RNA was extracted and real time RT-PCR was performed using primers specific for *Gadd45a*, *Gadd45b* or *Gadd45g*. Results are presented as the means±SEM (*n*=3) of relative induction normalised to *Sp1* expression. **b** Groups of 300 islets were exposed to 160 pg/ml IL-1β for 0–24 h. Total RNA was extracted and real-time RT-PCR was performed using *Gadd45b* primers. Results are presented as the means±SEM (*n*=3) of relative induction normalised to *Sp1* expression. **c** After 0–24 h of exposure to 160 pg/ml IL-1β, islets, parenteral INS-1E cells or clones 1 and 2 were lysed and Western blotting was performed using anti-GADD45α or anti-GADD45β antibodies. Anti-actin antibody was used as loading control. **d** Optical densities of the individual bands were measured and are presented in the histogram as the means±SEM of three experiments

(Fig. 4) [24]. Inhibition of JNK has consistently been associated with decreased beta cell apoptosis [5, 12–14, 29] and is thus the probable major mechanism behind the observed beta-cell-protective properties of GADD45β. The decreased apoptosis rate observed in *Gadd45b*-expressing INS-1E cells is not likely to be a consequence of the decreased NO production, as IL-1β-induced destruction of INS-1E cells [10] is independent of NO, as in human islets [9, 11].

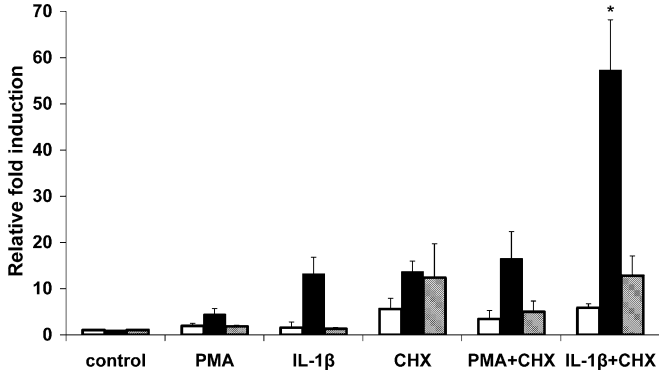



**Fig. 8** *Gadd45b*, but not *Gadd45a* or *Gadd45g*, is a primary response gene in IL-1β signalling. INS-1E cells were preincubated with or without 10 μg/ml CHX for 30 min and then exposed to 160 pg/ml IL-1β or 100 ng/ml phorbol myristate acetate (PMA) for 2 h. Total RNA was extracted and real time RT-PCR was performed with *Gadd45a* (*white boxes*), *Gadd45b* (*black boxes*) or *Gadd45g* (*shaded boxes*) primers. Results are means±SEM (*n*=4) of relative induction normalised to *Sp1* expression. **p*<0.05 vs CHX and IL-1β

The GADD45β-mediated inhibition of apoptosis in IL-1β-exposed INS-1E cells was not complete (60–77%; Fig. 6a,b). This is probably due to the incomplete JNK inhibition caused by the residual MKK7 activity found even at high levels of *Gadd45b* expression or to JNK activation mediated by MKK4 (Fig. 4). If higher levels of *Gadd45b* expression had been achieved, we would have expected a higher degree of MKK7 and JNK inhibition with concomitant inhibition of INS-1E apoptosis. However, other beta cell pro-apoptotic signalling pathways not affected by *Gadd45b* expression, such as the NFκB pathway (Fig. 4) [26, 34], may still signal apoptosis in the context of enhanced *Gadd45b* expression.

The decrease in iNOS and NO synthesis by *Gadd45b* expression (Fig. 5a,b) may be mediated via ERK, as ERK inhibition decreased IL-1β-induced rat islet iNOS and NO production [6], whereas IL-1β-induced iNOS expression was independent of JNK inhibition [35]. The augmentation

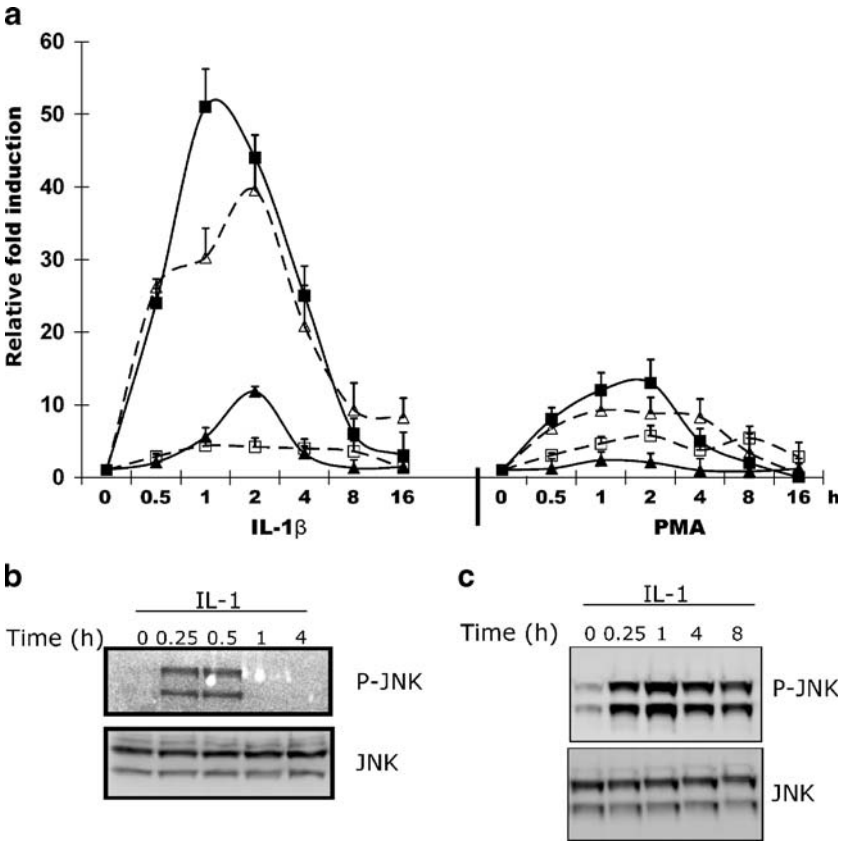



**Fig. 9** Reduced *Gadd45b* induction in beta-TC3 and INS-1E cells compared with NIH-3T3 and 3DO T cells. **a** INS-1E (*black triangles*), beta-TC3 (*white squares*), NIH-3T3(*white triangles*) and 3DO T cells (*black squares*) were exposed to 160 pg/ml IL-1β or 100 ng/ml PMA for 0–16 h, total RNA was extracted and real-time RT-PCR was performed with primers designed to target *Gadd45b*. Results are presented as means ±SEM of relative induction normalised to *Sp1* expression for four experiments. **b**, **c** 3DO T cells (**b**) and INS-1E (**c**) cells were exposed to 160 pg/ml IL-1β for 0–8 h, lysed and subjected to Western blotting with antibodies recognising the phosphorylated form of JNK (p-JNK), and reprobed with JNK antibodies. *Blots* are representative of three experiments

of p38 activation by GADD45β in INS-1E cells (Figs. 2 and 3) may explain why IL-1β-induced NO production was decreased by only 75%, as p38 activation is required for beta cell NO production [6]. The mechanism behind the observed p38 activation seems to be via GADD45β-mediated MEKK4 activation, as shown by others [23, 36, 37]. Inhibition of ERK activity by GADD45β in INS-1E cells is a novel observation, as previous studies have shown no effect on ERK activity by GADD45β [22, 23]. The mechanism of action is unclear, as IL-1β-induced beta cell ERK activation signals via MAPK/ERK kinase (MEK) 1/2 and not MKK7, on the basis of studies with the MEK inhibitor [6]. GADD45β inhibition of the ERK upstream kinases ras and raf is a possible mechanism, but this has not been investigated.

The GADD45β-mediated preservation of insulin content and release following IL-1β exposure (Fig. 5c–f) may be a consequence of the decreased apoptosis rate in these cells; however, JNK activation has been reported to suppress insulin gene expression and secretion in rat islets independently of cell death [17]. ERK does not seem to be involved in glucose-induced insulin release [38].

Expression of *Gadd45b* is regulated by the NFκB signalling pathway [22, 27, 31]. The superinduction of *Gadd45b* mRNA mediated by co-exposure of IL-1β and CHX (Fig. 8) suggests that *Gadd45b* is also regulated by NFκB in INS-1E cells. Whereas NFκB in general signals cell survival [39], NFκB signals apoptosis in beta cells [26, 34].

A limitation of this study is that it was performed in a rodent beta cell line; however, in human islets JNK and ERK also signal beta cell destruction [13, 14, 40], whereas p38 signals beta cell survival [14], making GADD45β a potentially strong tool for the protection of human beta cells against cytokine-mediated destruction. Testing this hypothesis is limited by the efficiency of transfecting human islets and the lack of small molecules able to modulate *Gadd45b* expression, but will be a future area of research.

We conclude that *Gadd45b* is a novel primary response gene in beta cell IL-1β signalling, and our finding that GADD45β is a beta cell inhibitor of JNK and ERK that is insufficiently induced may be part of the explanation for the beta cell pro-apoptotic response to IL-1β.

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