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TNFα and IFNγ potentiate IL-1β induced mitogen activated protein kinase activity in rat pancreatic islets of Langerhans

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Abstract

Aims/hypothesis. Interleukin-1 beta (IL-1β) in synergy with tumour necrosis factor alpha (TNFα) and interferon gamma (IFNγ) is cytotoxic to pancreatic beta cells. Mitogen-activated protein kinase (MAPK) activity that is induced by interleukin-1 beta has been suggested to signal nitric oxide-dependent as well as nitric oxide-independent beta-cell destructive pathways. The aim of this study was to investigate if TNFα and IFNγ signal through mitogen-activated protein kinases in isolated rat islets of Langerhans and if they potentiate mitogen-activated protein kinase activity induced by IL-1β.
Methods. Islets of Langerhans were isolated from 5- to 7-day-old Wistar rats and precultured for 7 days before stimulation with IL-1β, TNFα and/or IFNγ for 20 min followed by lysis. Kinase activity was measured with a whole cell lysate kinase assay and after immunoprecipitation of the kinase using immunocomplex kinase assay.
Results. Exposure to IL-1β or TNFα significantly increased mitogen-activated protein kinase activity, whereas IFNγ tended to decrease extracellular-signal-regulated kinase activity. Further, TNFα and IFNγ were found to synergistically increase mitogen-activated protein kinase activity induced by IL-1β.
Conclusion/interpretation. We hypothesise that the synergistic effect of IL-1β, TNFα and IFNγ in the functional inhibition and induction of cell death in pancreatic beta cells is signalled through a synergistic activation of mitogen-activated protein kinase activity [Diabetologia (2000) 43: 1389–1396].

Keywords c-jun N-terminal kinase, cytokines, ERK, extracellular-signal-regulated kinase, insulin-dependent diabetes mellitus, JNK, MAPK, p38, SAPK, signalling.

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Abbreviations: ATF-2, Activating transcription factor 2; DTT, dithiothreitol; ERK, extracellular-signal regulated kinase; GST, glutathione S-transferase; HSP, heat shock protein; IFNγ, interferon gamma; iNOS, inducible NO synthase; JAK, Janus kinase; JNK, c-jun N-terminal kinase; MAPK, mitogen activated protein kinase; NIK, nuclear factor κB inducing factor; NO, nitric oxide; SAPK, stress activated protein kinase; STAT, signal transducer and activator of transcription; TNFR1, TNFα receptor type 1; TRADD, TNFR1-associated death domain protein; TRAF, TNFα receptor associated factor.

The cytokine interleukin-1β (IL-1β) is cytotoxic to rat [1] and human pancreatic beta cells in synergy with tumour necrosis factor-α (TNFα) and interferon-γ (IFNγ) [2, 3]. Inactivating IL-1β by treatment with soluble IL-1β receptors protects against diabetes development in non-obese diabetic (NOD) mice [4]. The three cytokines could therefore be important effector molecules in the pathogenesis of insulin-dependent diabetes mellitus by inducing beta-cell apoptosis or necrosis or both [5]. Cytokines have been proposed to induce rat beta-cell damage through expression of cytokine inducible nitric oxide synthase (iNOS) leading to nitric oxide (NO) generation [6], whereas cytokine-induced apoptosis in human islets is probably independent of NO generation [7]. Further, NO generation might not be sufficient to medi-

ate the inhibitory and toxic effects of cytokines on rat pancreatic beta cells.

Mitogen-activated and stress-activated protein kinases (MAP/SAPKs) have been suggested to signal both NO-dependent as well as NO-independent deleterious pathways in rat islets of Langerhans [8]. The MAP/SAPKs include extracellular-signal-regulated kinase (ERK) 1 and 2, c-Jun NH_2-terminal kinases (JNKs) and p38 s [9]. These kinases determine cell proliferation, differentiation or apoptosis in response to extracellular signals [9] and are activated by phosphorylation on threonine and tyrosine residues by upstream kinases [9]. The MAP/SAPKs are proline-directed threonin/serine kinases phosphorylating among other substrates Elk-1, activating transcription factor-2 (ATF-2) and c-jun [9–11], the latter together with fos or ATF subunits leading to formation of the transcription factor activating protein-1 (AP-1) [12]. The substrate specificity of MAPK could be mediated by scaffolding proteins such as JNK interacting protein 1 (JIP-1) and MEK partner 1 (MP1), targeting JNK and ERK to certain substrates, respectively [13–15]. Cellular expression of the scaffolding proteins could thus participate in directing and controlling activation of MAPK pathways.

Because TNFα and IFNγ potentiate IL-1β-induced inhibition and toxicity in beta cells [3, 7, 16, 17], the aim of this study was to investigate whether they signal through MAPK and influence IL-1β-induced MAPK activity in rat pancreatic islets.

We report that IL-1β (concentration-dependently) and to a lesser degree TNFα activate MAPKs and that combinations of IL-1β, TNFα and IFNγ synergistically increase MAPK and especially p38 activity in isolated rat islets of Langerhans.

Materials and methods

Reagents. All reagents were from Sigma (St. Louis, Mo., USA) unless otherwise specified. Recombinant human IL-1β (400 units/ng) was from Novo Nordisk (Bagsværd, Denmark). Recombinant human TNFα (2×10^7 U/mg) and recombinant rat IFNγ (5×10^6 U/mg) were from Genzyme (West Malling, Kent, England). Reagents for SDS-polyacrylamide gel electrophoresis (SDS-PAGE) were all from Bio-Rad (Richmond, Calif., USA). We obtained [γ-^{32}P]ATP (110 TBq/mmol) from Amersham Pharmacia BioTech (Amersham, UK), glutathione S-transferase (GST)-Elk-1 was a gift from K. Seedorf (Hagedorn Research Institute, Denmark), GST-ATF-2 (1–109) was a gift from R.J. Davis (Howard Hughes Medical Institute, University of Massachusetts Medical School, Worcester, Mass., USA) and recombinant murine 25000 M_r heat-shock protein (HSP25) was from Stressgen (Victoria, Canada).

Islet isolation and preculture. Islets of Langerhans from 5- to 7-day-old Wistar Furth rats (Charles River, Sulzfeldt, Germany) were isolated by handpicking after collagenase A (Boehringer-Mannheim, Mannheim, Germany) digestion of the pancreata [18]. Islets were precultured in 5-ml dishes (Nunc, Roskilde, Denmark) for 7 days at 37 °C in atmospheric humidified air in complete medium (RPMI 1640 with 11 mmol/l glucose supplemented with 100000 U/l penicillin, 100 mg/l streptomycin, 20 mmol/l HEPES buffer, 2 mmol/l L-glutamine, 0.038 % $NaHCO_3$ (all from Life Technologies, Paisley, Scotland)), and 10 % FCS (Life Technologies). Principles of animal care (NIH publication no. 85–23, revised 1985) were followed and animals were handled according to permission obtained from the Committee for Inspection of Animal Experiments under the Danish Ministry of Justice.

Islet culture. We picked 150 islets at random and cultured them in 300 µl complete medium + 0.5 % human serum in four-well plates (Nunc) before adding the cytokines. Islets were exposed to cytokines for 20 min before lysis [8].

We investigated the effect of pre-incubation on MAP kinase activity before addition of cytokines to find the effect of stress caused by handling the islets during the washing procedure. Experiments were performed with increasing pre-incubation times from 0 to 24 h followed by 20 min exposure to 0 or 60 U/ml IL-1β with no intercurrent handling.

Lysis of islets. After stimulation islets were transferred to 1.5 ml Eppendorf vials and centrifuged for 2.5 min at 5000 rpm at 4 °C. The medium was removed and the islets were lysed in 25 µl lysis buffer (20 mmol/l TRIS 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 (DTT), 1 mmol/l benzamidine and 4 µg/ml leupeptin) for 30–45 min on ice. The detergent-insoluble material was pelleted by centrifugation at 15000 rpm for 5 min at 4 °C. The supernatants containing the whole-islet lysate were stored at –80 °C until use.

Protein concentration in lysates. Using the dye reagent concentrate, Bio-Rad protein assay was done according to the manufacturers instructions.

Whole-cell lysate kinase assay. Whole-cell lysate kinase assay was done as described previously [8]. In brief, 3–5 µl whole-islet lysate (volume of whole islet lysate adjusted to contain same amount of protein) was added to 17 µl reaction buffer (2 µg GST-Elk-1, 2 µg GST-ATF-2, 1 µg HSP25 (the rodent homologue of human HSP27), 25 mmol/l TRIS pH 7.5, 0.1 mmol/l EGTA, 0.1 mmol/l Na_3VO_4, 1 µmol/l cAMP-dependent protein kinase inhibitor peptide and 10 mmol/l Mg-acetate) and 3 µl ATP mixture (1 mmol/l ATP and 0.1 MBq [γ-^{32}P]ATP). The phosphotransferase reactions were carried out at 30 °C for 30 min. Reactions were terminated by addition of 25 µl cold SDS sample buffer (125 mmol/l TRIS pH 6.8, 4 % SDS, 0.1 mol/l DTT, 10 % glycerol and 0.02 % bromphenol blue) and boiling for 5 min. We did SDS-PAGE using 4 % stacking gels and 12 % separation gels. After electrophoresis, the upper part of the separation gels were washed in a mixture of 10 % acetic acid and 40 % methanol. The gels were dried, and phosphorylated proteins were visualized by autoradiography using PhosphoImager (Molecular Dynamics, Sunnyvale, Calif., USA) and quantified using ImageQuant software version 3.3 (Molecular Dynamics).

Immunoprecipitation and immunocomplex kinase assay. The immunoprecipitation and immunocomplex kinase assays were done as described previously [8] with minor modifications and using GST-ATF-2 and HSP25 as substrates for ERK1/2 and MAPKAP-K2, respectively. In brief, lysate containing 400 µg protein diluted to a final volume of 1.0 ml in washing buffer (lysis buffer containing 0.1 % Triton X-100) was immunopre-

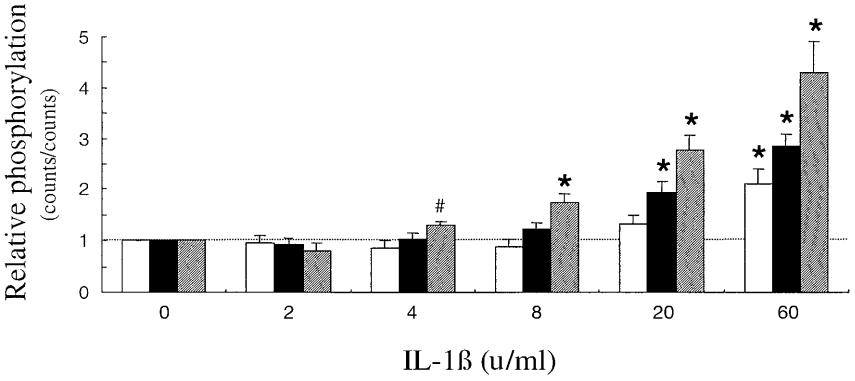


Fig. 1. Relative phosphorylation of the substrates Elk-1 (□), ATF-2 (■) and HSP25 (▨) after exposure to IL-1β for 20 min. Data are means ± SEM ($n = 6$). # $p < 0.05$ and * $p < 0.025$ vs control

cipitated by incubation for 2 h at 4 °C with 2 µl anti-ERK1/2 (New England Biolabs, Hitchin Hertfordshire, UK) or 2 µg anti-MAPKAP-K2 antibodies (Upstate Biotechnology, Lake Placid, N. Y., USA) followed by 2 h of incubation at 4 °C with 30 µl Sepharose-protein A beads (Pharmacia Biotech, Uppsala, Sweden). The beads were washed four times in washing buffer and three times in kinase buffer (20 mmol/l HEPES at pH 7.5, 20 mmol/l β-glycerophosphate, 10 mmol/l $MgCl_2$, 1 mmol/l DTT and 50 µmol/l Na_3VO_4). Kinase reactions were carried out for 30 min at 30 °C in 35 µl kinase buffer containing 0.4 MBq [γ-^{32}P]ATP and 15 µg GST-ATF-2 (ERK1/2) or 5 µg HSP25 (MAPKAP-K2). Reactions were terminated by addition of 35 µl SDS sample buffer and boiling for 5 min. The samples were then analysed for kinase activity as described above.

Statistical analysis. Results are presented as means ± SEM. Wilcoxon's matched-pair test was used as a non-parametric statistical test and p less than 0.05 was chosen as the level of significance.

Results

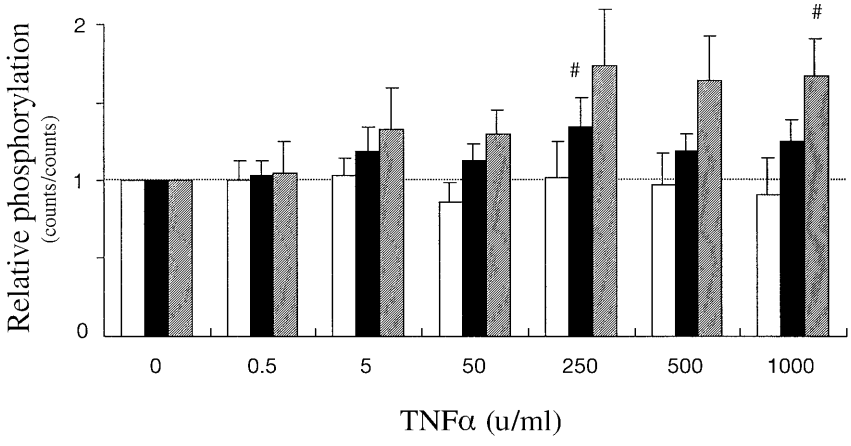
IL-1β and TNFα, but not IFNγ significantly increase MAPK activity. To investigate the effect of increasing concentrations of IL-1β, TNFα or IFNγ on the activity of p38 and ERK1/2 in isolated rat islets of Langerhans the phosphorylation of Elk-1, ATF-2 and HSP25 was measured by kinase assay of whole-islet lysates from concentration-response experiments with each of the three cytokines.

We found IL-1β caused a concentration-dependent increase in the phosphorylation of HSP25, ATF-2 and Elk-1 at and above 4, 20 and 60 U/ml of IL-1β, respectively ($p < 0.05$, Fig. 1). The peak phosphorylation of Elk-1, ATF-2 and HSP25 was approximately 2-fold, 3-fold and 4.5-fold above baseline activity, respectively. The phosphorylation of the three substrates was not further increased above 60 U/ml IL-1β (data not shown).

The TNFα cytokine increased the phosphorylation of ATF-2 and HSP25 1.3-fold and 1.7-fold of the baseline activity at 250 and 1000 U/ml, respectively ($p < 0.05$, Fig. 2), and IFNγ reduced the phosphorylation of Elk-1 and ATF-2 at 2.5 and 10 U/ml, respectively ($p < 0.05$, Fig. 3). Higher concentrations of IFNγ (1000 U/ml) had no statistically significant effect on the substrate phosphorylation (data not shown).

High constitutive phosphorylation of especially ATF-2 and HSP25 was observed in these experi-

Fig. 2. Relative phosphorylation of the substrates Elk-1 (□), ATF-2 (■) and HSP25 (▨) after exposure to TNFα for 20 min. Data are means ± SEM ($n = 6$). # $p < 0.05$ vs control



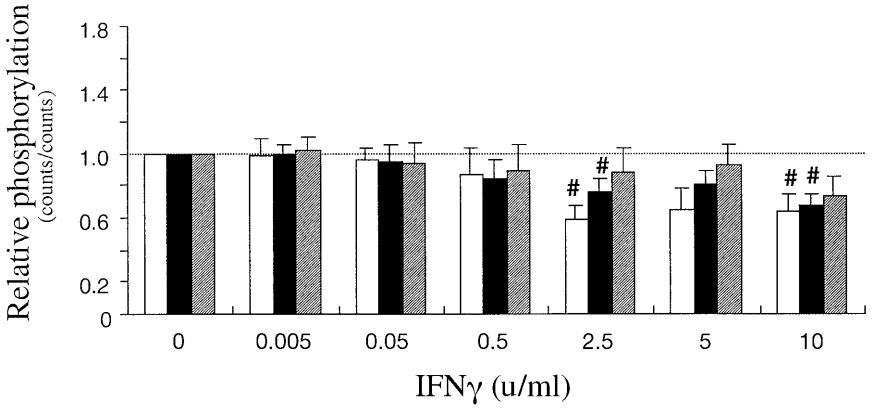


Fig. 3. Relative phosphorylation of the substrates Elk-1 (□), ATF-2 (■) and HSP25 (▨) after exposure to IFNγ for 20 min. Data are means ± SEM ($n = 6$). # $p < 0.05$ vs control

ments. We therefore investigated the effect of pre-incubation of the islets for 0–24 h before addition of IL-1β on the basal phosphotransferase activity. Pre-incubation had no statistically significant effect on baseline phosphorylation of Elk-1 (Fig. 4) but the IL-1β-induced response significantly increased after 3 h of pre-incubation and tended to decline when using preincubation periods longer than 6 h. The baseline phosphorylations of ATF-2 and HSP25 were significantly reduced when using a pre-incubation period (3 to 12 h and 1 to 3 h, respectively) before exposure to cytokines (Fig. 4). The baseline phosphorylations of both substrates increased when using pre-incubation periods longer than 12 h. The relative phosphorylation of the three substrates (cytokine-induced phosphorylation divided by baseline phosphorylation of the substrate) was highest when using 3 h of pre-incubation before exposure to cytokines and this optimum was used in the following experiments.

We next compared the relative phosphorylations of Elk-1, ATF-2 and HSP25 induced by IL-1β, TNFα or IFNγ in the same experiment after 3 h of pre-incubation (Fig. 5). The phosphorylation of the substrates Elk-1, ATF-2 and HSP25, after exposure to 60 U/ml IL-1β, increased 3.3-fold, 7-fold and 10.1-fold above the baseline level, respectively ($p < 0.025$). The phosphorylation of HSP25, ATF-2 and Elk-1 was increased 1.3- to 1.4-fold over baseline by TNFα ($p < 0.05$ for ATF-2). Exposure to 10 U/ml IFNγ tended to reduce the phosphorylation of the Elk-1 and ATF-2 in accordance with data in Fig. 3 ($p < 0.025$ for ATF-2).

To investigate the kinase specificity we immunoprecipitated ERK1/2 and MAPKAP-K2 from lysates after exposure to IL-1β, TNFα and IFNγ for 20 min (Fig. 6) because previous studies showed that ERK 1/2 and p38, but not JNK, phosphorylated Elk-1, ATF-2 and HSP25 in IL-1β exposed rat islets [8]. Due to the large amount of tissue needed for immunoprecipitation only one experiment was done to verify the results from the whole-cell lysate kinase assays.

The activity of ERK1/2 and p38 increased considerably after exposure to IL-1β (Fig. 6) in accordance with the kinase activity measured by whole-cell lysate kinase assay (Fig. 5).

Synergistic effect of combinations of cytokines on MAPK activity. To investigate the effect of combinations of cytokines on the activity of the MAPKs in islets of Langerhans, submaximum concentrations of each cytokine chosen from the concentration-response experiments in Figures 1–3 were used alone or in combinations (Fig. 7).

We found Elk-1 phosphorylation was increased when exposed to 20 U/ml IL-1β or 250 U/ml TNFα ($p < 0.05$ and 0.02, respectively vs control and $p < 0.02$ vs the lower concentration of the same cytokine, Fig. 7). The Elk-1 phosphorylation was decreased ($p < 0.05$) when incubated with 2.5 U/ml IFNγ. The ATF-2 and HSP25 phosphorylations were increased when incubated with 4 or 20 U/ml IL-1β or 250 U/ml TNFα ($p < 0.05$, Fig. 7).

Addition of low concentrations of TNFα had no effect on the action of the low concentration of IL-1β (Fig. 7). Low concentrations of IFNγ decreased the effect of low concentrations of IL-1β on the phosphorylation of HSP25 ($p < 0.05$, Fig. 7). Incubation with low concentrations of all three cytokines decreased the phosphorylation of ATF-2 and HSP25 compared with the same concentrations of IL-1β alone or IL-1β combined with TNFα ($p < 0.05$).

Low concentrations of IFNγ synergistically increased the effects of high concentrations of IL-1β combined with low concentrations of TNFα on the phosphorylation of all three substrates ($p < 0.05$, Fig. 7).

The phosphorylation of HSP25 induced by the higher concentrations of TNFα was further increased in an additive manner in the presence of low concentrations of IL-1β ($p < 0.05$, Fig. 7).

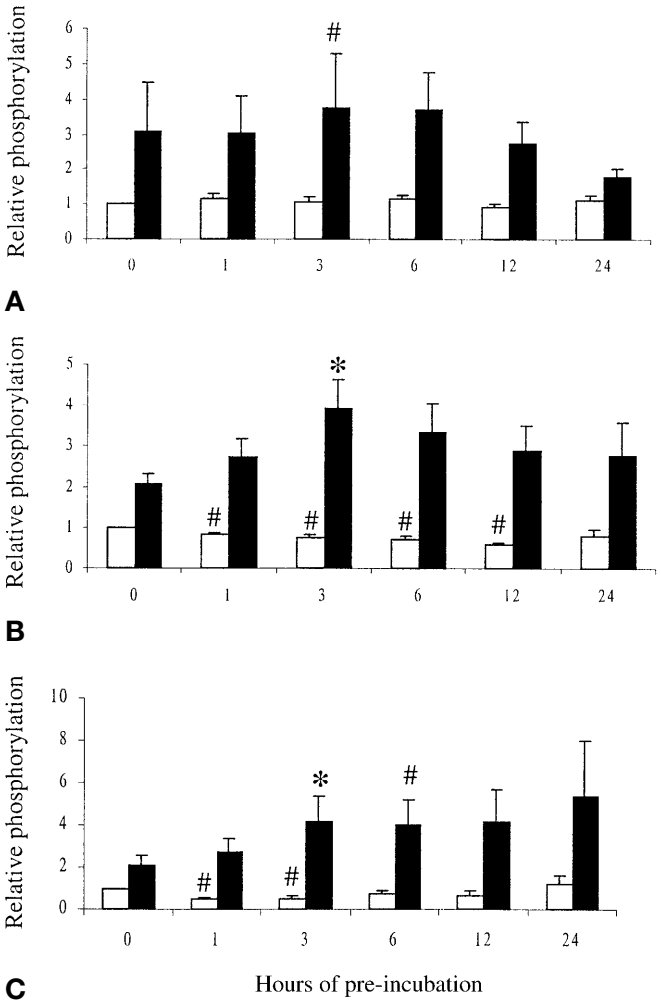


Fig. 4 A–C. Phosphorylation of the substrates Elk-1 (**A**), ATF-2 (**B**) and HSP25 (**C**) after 0–24 h of pre-incubation followed by 20 min incubation with (□) or without (■) 60 U/ml IL-1β relative to the phosphorylation after 0 h of pre-incubation without IL-1β. Data are means ± SEM ($n = 6$). # $p < 0.05$ and * $p < 0.025$ vs 0 h of pre-incubation

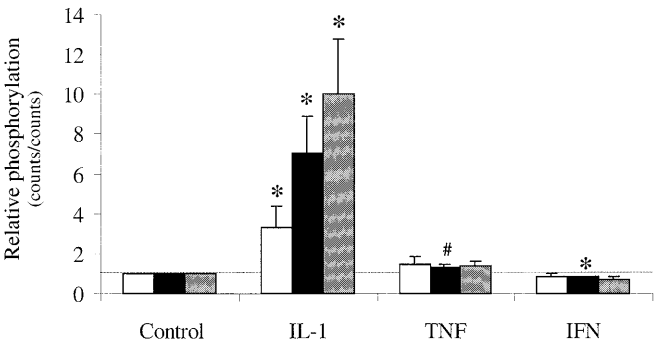


Fig. 5. Relative phosphorylation of the substrates Elk-1 (□), ATF-2 (■) and HSP25 (▨) after 3 h of pre-incubation followed by exposure to 60 U/ml IL-1β, 1000 U/ml TNFα or 10 U/ml IFNγ for 20 min. Data are means ± SEM ($n = 6$). # $p < 0.05$ and * $p < 0.025$ vs control

We found 2.5 U/ml IFNγ reduced IL-1β-induced phosphorylation of all three substrates to the same level of phosphorylation observed with 2.5 U/ml IFNγ alone, in that substrate phosphorylation of 2.5 U/ml IFNγ was not statistically significantly different from that seen at 2.5 U/ml IFNγ + 4 U/ml IL-1β (Fig. 7).

Discussion

Our results show that IL-1β alone and TNFα alone significantly increased ERK1/2 and p38 activity in rat islets of Langerhans whereas IFNγ alone decreased the activity of ERK1/2. Further, in analogy to the potentiating effect of TNFα and IFNγ on IL-1β-induced NO synthesis, inhibition of insulin release and toxicity to islet beta cells, the three cytokines synergised in increasing islet MAPK activity within the same or lower concentration range which has been found to synergise with regard to other variables [2, 3, 7].

We found that TNFα-induced MAPK activity resulted in maximum substrate phosphorylation which was lower than the maximum phosphorylation induced after exposure to IL-1β. The mechanisms of engaging MAPK differ between the IL-1β and TNFα signalling transduction pathways. Binding of IL-1β to the IL-1β receptor type I (IL-1RI) leads to assembly of the IL-1β receptor complex, which include IL-1 receptor-associated protein (IL-1RAcP), MyD88, IL-1β receptor-associated kinase 1 and 2 (IRAK 1/2) [19] and TRAF 6 [20, 21] whereas TNFα binding to TNF receptor type 1 (TNFR1) leads to trimerisation of the TNFR1 and recruitment of TNFR1-associated death domain protein (TRADD) to the death domain of the receptors [22–24]. The TRADD is known to interact with the Fas-associated death domain protein (FADD), TNF receptor-associated factor 2 (TRAF2) and receptor interacting protein (RIP) [25]. Both TRAF 2 and TRAF 6 have been associated with nuclear factor $\varkappa$B (NF-$\varkappa$B) inducing kinase (NIK) induced activation of NF$\varkappa$B [26]. The NIK shares sequence similarity with several MAP kinase kinases and has therefore been suggested to be involved in activation of MAPKs in response to both IL-1β and TNFα [26]. Differences between TRAF 2 and 6 activation of NIK could explain the lower MAPK activity in response to TNFα compared with IL-1β either due to different time-response optima or to differences in the amount of receptor complexes. Combined TRAF 2 and 6 activation of NIK could also explain the additive effect of IL-1β on TNFα-induced MAPK activity.

Interferon gamma alone or when combined in high concentrations with other cytokines reduced the phosphorylation of the ERK substrates. Interferon gamma signalling involves association of homodimeric IFNγ with two type I integral membrane proteins

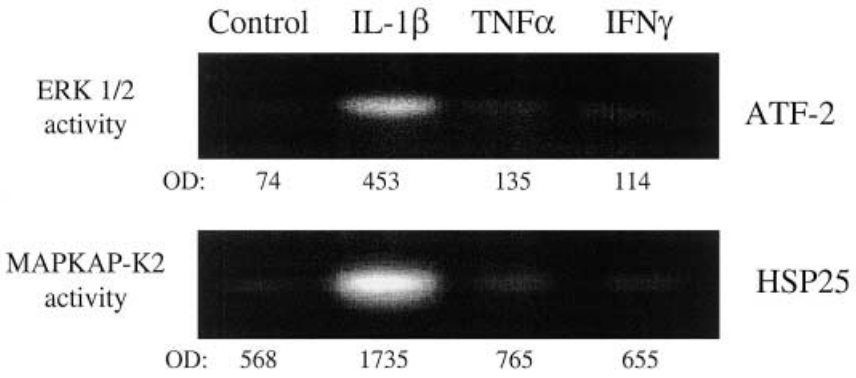
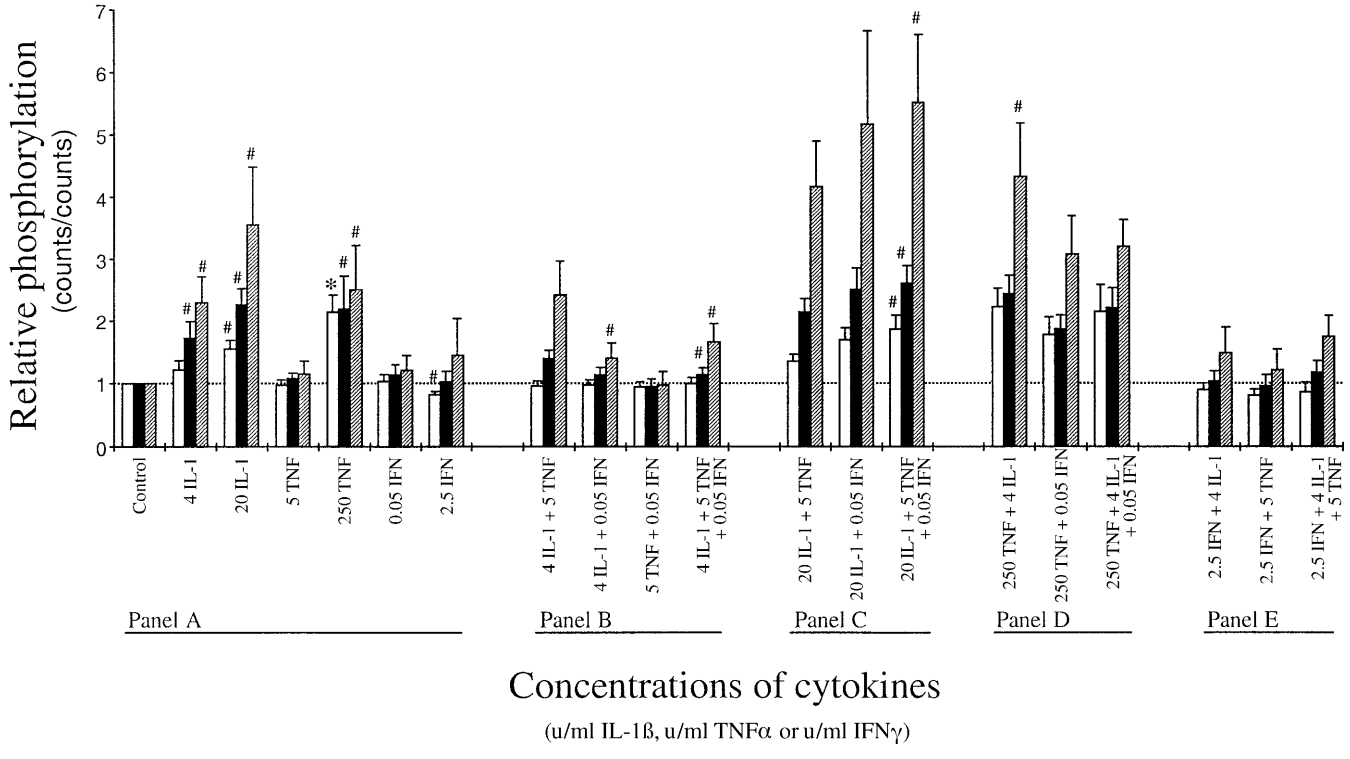


Fig. 6. Lysates were prepared from islets that had been pre-incubated for 3 h followed by exposure to 60 U/ml IL-1β, 1000 U/ml TNFα or 10 U/ml IFNγ for 20 min. ERK 1/2 (upper panel) and MAPKAP-K2 (lower panel) were immunoprecipitated from the lysates and their activities were measured in immunocomplex kinase assay with ATF-2 and HSP25 as substrates ($n = 1$). After SDS-PAGE phosphorylated proteins were visualised by autoradiography. Optical density quantified using ImageQuant is indicated below the autoradiograms

IFNGR1 (IFNγ receptor subunit 1) each connected to a Janus kinase 1 (JAK1). This lead to recruitment of two IFNGR2 (IFNγ receptor subunit 2) each associated with a JAK2 and resulting in activation of the JAKs by autophosphorylation and transphosphorylation. The signal transducer and activator of transcription 1 (STAT1) is then recruited to two binding sites in the receptor complex. The receptor-associated STATs are subsequently phosphorylated by the receptor-bound JAKs leading to dissociation and dimerisation of the STATs [27–29]. The STAT1-homodimers translocate to the nucleus where they bind to specific gamma-activated sequence (GAS) elements of IFNγ-inducible genes. The activity of the STAT proteins can be controlled by phosphorylation of a serine phosphorylation site, which is a possible site of ERK or p38 phosphorylation or both [27, 30]. The increased amount of STAT-dimers after stimulation with IFNγ could recruit and associate with activated ERK and p38 and thereby inhibit the phosphoryla-

Fig. 7. Relative phosphorylation of the substrates Elk-1 (□), ATF-2 (■) and HSP25 (▨) after 3 h of pre-incubation followed by exposure to cytokines for 20 min. Data are means ± SEM ($n = 7$). # $p < 0.05$ and * $p < 0.02$ vs control (Panel A), 4 U/ml IL-1 + or – 5 U/ml TNF (Panel B), 20 U/ml IL-1 + 5 U/ml TNF (Panel C) or 250 U/ml TNF (Panel D)



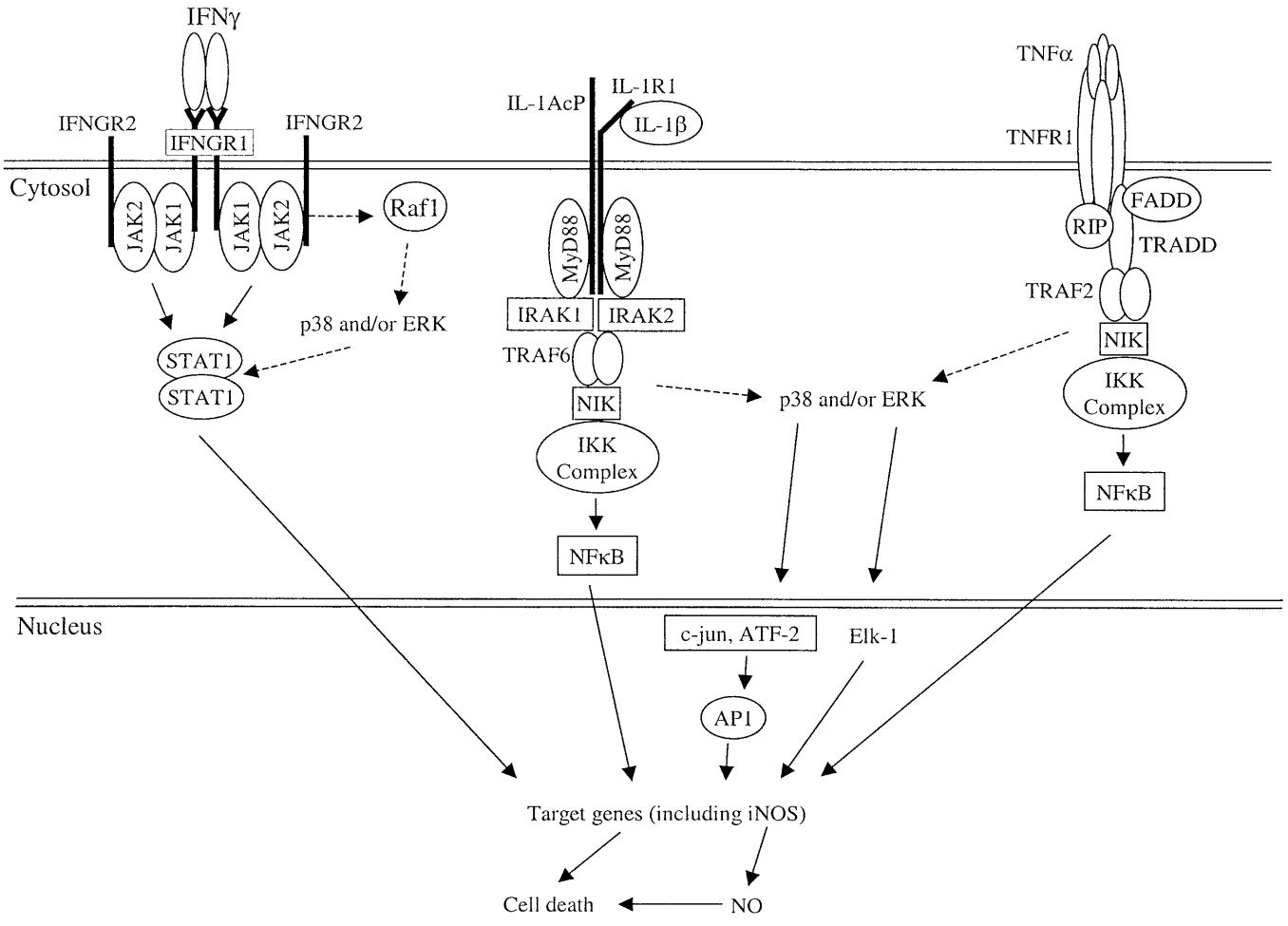


Fig. 8. Overview of suggested key interactions between cytokines, ERK and p38 MAPK and further signalling events. AP-1, activating protein 1; FADD, FAS-associated death domain protein; IFNGR, IFNγ receptor subunit 1; IKK, inhibitory kappa B kinase; IL-1AcP, IL-1 receptor associated protein; IL-1R1, IL-1 receptor type 1; IRAK, IL-1β receptor associated kinase; NFκB, nuclear factor kappa B; RIP, receptor interacting protein

tion of the MAPK substrates Elk-1, ATF-2 and HSP25 in a competitive manner. It is also possible that phosphatases of MAPKs are activated by unknown factors in the IFNγ signal transduction pathway.

The net effect of serine phosphorylation of STAT proteins has been reported to both potentiate and inhibit tyrosine phosphorylation and transcriptional activity. Thus the net effect could depend on the type of extracellular stimulus, the cell type and the activation state of the cell in question [31–38].

The synergistic effect of low concentrations of IFNγ on MAPK activity induced by IL-1β and TNFα could be mediated by JAK2 (and possibly also by JAK1) activation of MAPKs through activation of Raf1 [27, 39].

Cytokine-induced MAPK activity in the islets of Langerhans mainly originates from beta cells because MAPKs are strongly activated in rat insulinoma cells (RIN cells) in response to cytokines [8]. Further cytokine-induced MAPK activation is higher in both transformed and primary sorted beta cells than in alpha cells [40, 41].

In conclusion, TNFα and IFNγ were found to synergise with IL-1β for MAP/SAPK activation. We hypothesise that the synergistic effect of IL-1β, TNFα and IFNγ on pancreatic beta-cell NO synthesis, functional inhibition and apoptosis is related to cross-talk between cytokine signalling cascades involving MAPK.

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