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ARTICLE

Inhibition of histone deacetylases prevents cytokine-induced toxicity in beta cells

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Abstract
Aims/hypothesis The immune-mediated elimination of pancreatic beta cells in type 1 diabetes involves release of cytotoxic cytokines such as IL-1β and IFNγ, which induce beta cell death in vitro by mechanisms that are both dependent and independent of nitric oxide (NO). Nuclear factor kappa B (NFκB) is a critical signalling molecule in inflammation and is required for expression of the gene encoding inducible NO synthase (iNOS) and of pro-apoptotic genes. NFκB has recently been shown to associate with chromatin-modifying enzymes histone acetyltransferases and histone deacetylases (HDAC), and positive effects of HDAC inhibition have been obtained in several inflammatory diseases. Thus, the aim of this study was to investigate whether HDAC inhibition protects beta cells against cytokine-induced toxicity.
Materials and methods The beta cell line, INS-1, or intact rat islets were precultured with HDAC inhibitors suberoylanilide hydroxamic acid or trichostatin A in the absence or presence of IL-1β and IFNγ. Effects on insulin secretion and NO formation were measured by ELISA and Griess reagent, respectively. iNOS levels and NFκB activity were measured by immunoblotting and by immunoblotting combined with electrophoretic mobility shift assay, respectively. Viability was analysed by 3-(4,5-dimethyldiazol-2-yl)-2,5-diphenyl-tetrazolium bromide and apoptosis by terminal deoxynucleotidyl transferase mediated dUTP nick end labeling (TUNEL) assay and histone-DNA complex ELISA.
Results HDAC inhibition reduced cytokine-mediated decrease in insulin secretion and increase in iNOS levels, NO formation and apoptosis. IL-1β induced a bi-phasic phosphorylation of inhibitor protein kappa Bα (IκBα) with the 2nd peak being sensitive to HDAC inhibition. No effect was seen on IκBα degradation and NFκB DNA binding.
Conclusions/interpretation HDAC inhibition prevents cytokine-induced beta cell apoptosis and impaired beta cell function associated with a downregulation of NFκB transactivating activity.

Keywords Autoimmunity · Beta cells · Cell signalling · Histone deacetylases · IFNγ · IL-1β · Nitric oxide · Nuclear factor kappa B · SAHA · TSA

Abbreviations

EMSA	electrophoretic mobility shift assay
HDAC	histone deacetylase
IκBα	inhibitor protein kappa B alpha
iNOS	inducible nitric oxide synthase
MTT	3-(4,5-dimethyldiazol-2-yl)-2,5-diphenyl-tetrazolium bromide
NO	nitric oxide
NFκB	nuclear factor kappa B
SAHA	suberoylanilide hydroxamic acid

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TUNEL terminal deoxynucleotidyl transferase mediated dUTP nick end labeling

Introduction

The double-stranded DNA helix is wrapped around core histones (H2A, H2B, H3 and H4) forming nucleosomes, which are further coiled and compacted into the higher-order chromosomal structure. It is widely accepted that this densely packed DNA structure is maintained by the enzymatic activity of the histone deacetylases (HDAC), which maintain the histones in a deacetylated state. Deacetylated histones prevent the binding of transcription factors and suppress the transcriptional machinery [1–3]. Inhibition of HDACs results in hyper-acetylation and unravelling of DNA permitting gene activation. There are, however, exceptions to this general rule [4–6].

Significant interest has emerged in the blocking of HDAC activity as a possible treatment in neoplasia. Inhibition of HDAC decreases proliferation and induces terminal differentiation or cell death in several cancers, and HDAC inhibitors have been tested in Phase I to II trials for the treatment of various malignancies [1].

In recent years, a potent anti-inflammatory effect of HDAC inhibitors at concentrations lower than those needed to suppress tumour cell growth in vitro and in vivo has been discovered [7]. This has proven to be advantageous in autoimmune diseases such as rheumatoid arthritis or systemic lupus erythematosus, where treatment with HDAC inhibitors blocks production of inflammatory mediators such as nitric oxide (NO) and cytokines in vitro and disease progression in vivo in animal models [8–10]. Although the molecular mode of action of HDAC inhibition is still debated, inhibition of the transcription factor nuclear factor κB (NFκB) seems to be important [1], raising the hypothesis that similar mechanisms could operate in beta cells, where the activation of NFκB and the resulting formation of NO is a critical event.

The NFκB family of proteins encompasses five mammalian members: p65 (RelA), RelB, c-relA, p50 and p52, and these form various homo- and heterodimers, depending on cell type and inducing stimuli [11]. Activation of the NFκB pathway is initiated by stimulus-dependent phosphorylation of the inhibitor κB protein, inhibitor protein kappa B alpha (IκBα), which in the resting cell retains NFκB in the cytoplasm. Phosphorylation triggers ubiquitination and subsequent degradation of IκBα by the 26S proteasome complex and releases NFκB, which can then translocate to the nucleus, bind to κB enhancer elements and activate gene transcription [12]. As part of a negative feed-back loop, NFκB induces production of its own negative regulator, IκBα, which inactivates NFκB and terminates the NFκB response by shuttling the NFκB–IκBα complex back to the cytoplasm [13].

In addition to histones, other proteins, including transcription factors such as NFκB, can be modified by acetylation/deacetylation [12, 14]. NFκB acetylation leads to increased DNA-binding, enhanced transactivation and resistance to assembly with IκBα [12].

NFκB is a point of convergence in the signal transduction pathways activated by the proinflammatory cytokine IL-1β in beta cells, and plays a pivotal role in the cytokine-induced beta cell death seen in type 1 diabetes [15–17]. NFκB controls induction of several inflammatory genes, one being the gene encoding inducible NO synthase (iNOS), leading to NO formation and beta cell death [17, 18]. Blockade of the NFκB pathway has been shown to inhibit Fas-triggered apoptosis and cytokine-induced suppression of glucose-stimulated insulin secretion in human islets [16], as well as expression of the nitric oxide synthase 2 gene, NO formation and cell death by apoptosis and necrosis in primary rat islets [17].

Using both beta cell lines and primary beta cells, as well as two different HDAC inhibitors, namely trichostatin A (TSA), and suberoylanilide hydroxamic acid (SAHA), we investigated the role of acetylation/deacetylation regulation in cytokine-induced beta cell toxicity.

Materials and methods

Reagents Recombinant mouse IL-1β was from BD Pharmingen (Erembodegen, Belgium), whereas recombinant rat IFNγ was obtained from R&D Systems (Oxford, UK).

The HDAC inhibitor SAHA was synthesised by Italfarmaco (Cinisello Balsamo, Italy) [19], while TSA was purchased from Calbiochem (San Diego, CA, USA). Both inhibitors were dissolved in DMSO (vehicle) to a stock concentration of 1 and 200 μmol/l, respectively.

Cells INS-1 and INS-1E cells were a kind gift from C. Wollheim, Departments of Cell Physiology and Metabolism, University Medical Center, Geneva, Switzerland [20] and were maintained in RPMI-1640 culture medium with glutamax supplemented with 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin (all from Invitrogen/Gibco, Taastrup, Denmark), and 50 μmol/l β-mercaptoethanol (Sigma, St Louis, MO, USA). Cells were cultured under standard cell culture conditions at 37°C in a humidified atmosphere containing 5% CO_2. Cells were passaged weekly and precultured for 2 days prior to cytokine treatment. At the day of experiment medium was changed, and inhibitors were added to the specified conditions 30 min preceding cytokine exposure for various times.

Islet isoloation and preculture Primary neonatal rat islets were isolated from 3- to 6-day-old Wistar Furth Rats (Charles River, Sulzfeldt, Germany) and precultured for 7 days as previously described [21, 22] in complete medium (RPMI 1640 with 100 U/ml penicillin and 100 μg/ml streptomycin) supplemented with 10% newborn calf serum (Invitrogen/Gibco).

Adult rat islets were obtained by the following procedure described by Eizirik et al. [23].

Islet culture Five hundred randomly picked islets per 1 ml of complete medium + 0.5% human serum, or 50 islets per 100 μl complete medium + 0.5% of human serum, were placed in 12-well or 96-well plates (NUNC, Roskilde, Denmark), respectively. Islets were left for 2–3 h before pre-exposure to HDAC inhibitors or control medium for 1 h and subsequently exposed to cytokines for various times.

Monolayer culture Islets for monolayer cultures were prepared as previously described [24].

HDAC activity We analysed 20 ng of nuclear protein, which had been extracted from INS-1 cells, for HDAC activity using an HDAC assay kit (No. 17-374; Upstate, Hampshire, UK) with or without SAHA or TSA in a final concentration of 1 or 200 nmol/l, respectively.

Immunoblotting We seeded 500,000 INS-1E cells or 500 neonatal rat islets in 12-well plates (Nunc) in 1 ml of complete medium. Inhibitors and cytokines were added to the indicated conditions for various time points ranging from 5 min to 6 h of stimulation. Cells were lysed, protein content measured by the Bradford method and lysates subjected to gel electrophoresis as previously described [25]. Antibody to IκBα (sc-371) and β-tubulin (sc-5274) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA), anti phospho-IκBα (#9246) from Cell Signaling (Beverly, MA, USA), anti iNOS (#610332) from BD Pharmingen (San Diego, CA, USA) and anti β-actin (ab6276) from Abcam (Cambridge, UK). Immune complexes were detected by chemiluminescence using LumiGLO (Cell Signaling), and light emission captured digitally by use of the Fuji LAS3000 (Fujifilm, Tokyo, Japan).

Nuclear extracts and electrophoretic mobility shift assay INS-1E cells were cultured in 10 cm dishes to 80–85% confluency. Cells were exposed to inhibitors and cytokines as indicated. Nuclear extracts were isolated and electrophoretic mobility shift assay (EMSA) carried out as described [26]. For detection of NFκB binding a double-stranded oligo was used: AGTCAGCTTCAGAGG GACTTTCCGAGAGG-3′. In supershift experiments, nuclear extracts were preincubated with anti-p65 antibody (sc-37; Santa Cruz) for 30 min at 4°C.

Nitric oxide measurement We seeded 10,000 INS-1 cells or 50 rat islets in 96-well tissue-culture plates (Nunc) in 200 μl complete medium. Following 2 days of cytokine exposure, NO was measured as accumulated nitrite in the medium by mixing equal volumes of cell culture medium and Griess reagent (0.1% naphthylethene diamine hydrochloride; Sigma) in H_2O and 1% sulphanilamide (Bie& Berntsen, Rødovre, Denmark) in 5% H_3PO_4 (Merck, Glostrup, Denmark) [27]. The absorbance was measured at 540 nm and nitrite calculated from a $NaNO_2$ standard curve.

Insulin assay Cells were cultured and handled as described under NO measurement. Accumulated insulin in the incubation media was measured as previously [28, 29], except that the enzyme substrate used here was the TMB^+ ready-to-use-substrate (Kem En Tec, Taastrup, Denmark).

For glucose-stimulated insulin release, islets were pre-incubated with SAHA and exposed to cytokines for 4 or 18 h. Media was removed and islets were washed for 30 min in KRBH buffer (114.3 mmol/l NaCl, 4.74 mmol/l KCl, 1.15 mmol/l KH_4PO, 1.18 mmol/l $MgSO_4$, 25.0 mmol/l $NaHCO_3$, 10.0 mmol/l HEPES, 4.26 mmol/l NaHO, 2.54 mmol/l $CaCl_2$; pH 7.4) supplemented with 1.7 mmol/l glucose and SAHA and cytokines as previously, followed by 60 min in an identical buffer. Medium was then exchanged for KRBH containing 2 mg/ml BSA and 16.7 mmol/l glucose for another 60 min. Insulin was determined as above.

Viability assay Cells were cultured and handled as described under NO measurement. Following 2 days of cytokine exposure, the proportion of viable/metabolically active cells in control vs cytokine-containing wells was determined by 3-(4,5-dimethyldiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay (Promega, Madison, WI, USA), measuring the conversion of an MTT tetrazolium salt to a coloured formazan product by the mitochondrial enzyme succinate dehydrogenase [30].

Terminal deoxynucleotidyl transferase mediated dUTP nick end labelling assay A detection kit (ApopTag Fluorescein Direct In Situ Apoptosis Detection Kit), no. S7160; Chemicon, Temecula, CA, USA) was used to detect the degree of cytokine-mediated DNA fragmentation in monolayer cultures or INS-1 cells. Monolayer cultures were starved for 24 h in medium without human growth hormone preceding exposure to inhibitor and addition of cytokines to the indicated conditions for 20 h. Some 300,000 INS-1 cells

were cultured in two-well chamber slides (Nunc) in 1.5 ml complete medium and exposed to cytokines for 24 h where indicated. The terminal deoxynucleotidyl transferase mediated dUTP nick end labelling (TUNEL) assay was performed according to the manual of the manufacturer (CHEMICON Europe, Chandlers Ford, Hants, UK).

Cells were subsequently stained with Dapi nuclear stain (Sigma) for 30–60 min at room temperature, washed in PBS and the slides were mounted using drops of Antifade Mounting Media (no. 002627; Dako, Carpintaria, CA, USA).

Cell death detection ELISA Fifty islets were transferred to each well of a 96-well plate containing 200 μl of complete medium. Islets were cultured with or without inhibitors and cytokines for 24 h. The degree of cytokine-induced apoptosis was determined by cell-death detection ELISA (ELISAPLUS; Roche, Basel, Switzerland) measuring the amount of DNA-histone complexes present in the cytoplasmic lysates according to manufacturer's description. To calculate the induction of apoptosis, values were normalised to the total amount of DNA determined by the PicoGreen assay (Roche). This assay is based on a sensitive fluorescent nucleic acid stain facilitating the quantification of double-stranded DNA in solution.

Statistical analyses All data are presented as mean±SEM of *n* independent experiments. Statistical analysis was done using a paired Student's *t* test and a *p* value of less than 0.05 was considered significant.

Results

SAHA and TSA inhibit HDAC activity In order to validate the inhibitory effect of the HDAC inhibitors used, nuclear extracts from INS-1 cells were prepared and the ability of 1 μmol/l of SAHA or 200 nmol/l of TSA to inhibit HDAC activity was tested using an HDAC assay. SAHA and TSA (by 47 and 44%, respectively), significantly inhibited the HDAC activity in insulin secreting cells (Fig. 1).

HDAC inhibition protects against cytokine-induced decrease in beta cell function To investigate whether inhibition of HDACs protects against cytokine-induced toxicity, we first investigated the effect of SAHA and TSA on insulin secretion in INS-1 cells. As expected, exposure to a combination of IL-1β and IFNγ for 2 days markedly reduced accumulated insulin secretion. This was partially prevented by pre-culturing the cells with 1 μmol/l of SAHA or 200 nmol/l of TSA (Fig. 2a). In neonatal rat islets both inhibitors dose-dependently protected against cytokine-induced reduction in accumulated insulin secretion with complete protection seen at 1 and 200 nmol/l, respectively (Fig. 2b and c). In both cell types a significant (17–36%) reduction in insulin secretion was observed with TSA but not with SAHA alone.

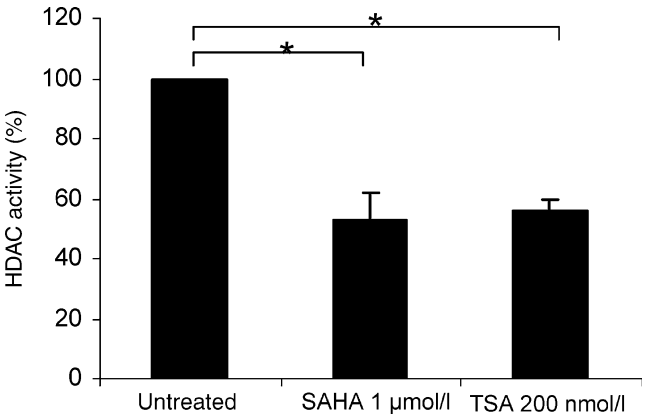


Fig. 1 Inhibitory potential of SAHA and TSA. The ability of SAHA (1 μmol/l) or TSA (200 nmol/l) to inhibit histone deacetylase activity was analysed by the HDAC assay using nuclear extract from INS-1 cells. Results are shown as mean±SEM compared with untreated extracts (n=4). *p<0.05 vs untreated extracts (t test)

To investigate the effect of HDAC inhibition on acute glucose-stimulated insulin release, we subjected primary rat islets pretreated with HDAC inhibitors and exposed to cytokines for a total of 6 or 20 h to low glucose (1.7 mmol/l) and a glucose challenge (16.7 mmol/l) for 1 h. In this case we observed no effect of HDAC inhibition on cytokine-mediated inhibition of glucose-stimulated insulin release (data not shown).

Using the MTT assay, we next tested whether inhibition of HDACs had a positive effect on cell viability. The MTT assay is based on the cellular reduction of formazan and tests mitochondrial function, i.e. the metabolic state of the cell. As is evident from Fig. 2d and e, both SAHA and TSA dose-dependently reduced the IL-1β and IFNγ-mediated reduction in mitochondrial function in neonatal rat islets. Neither of the inhibitors had any effect on basal mitochondrial function. The results indicate that HDAC inhibition can protect against the detrimental effects of cytokines in both primary islets and rat beta cell line.

HDACs increase cytokine-induced beta cell apoptosis Although HDAC inhibitors increase the level of cell death in cancer cells [31, 32], opposite effects have been observed in models of autoimmune diseases, where HDAC inhibition has been shown to downregulate the pro-apoptotic pathways [33].

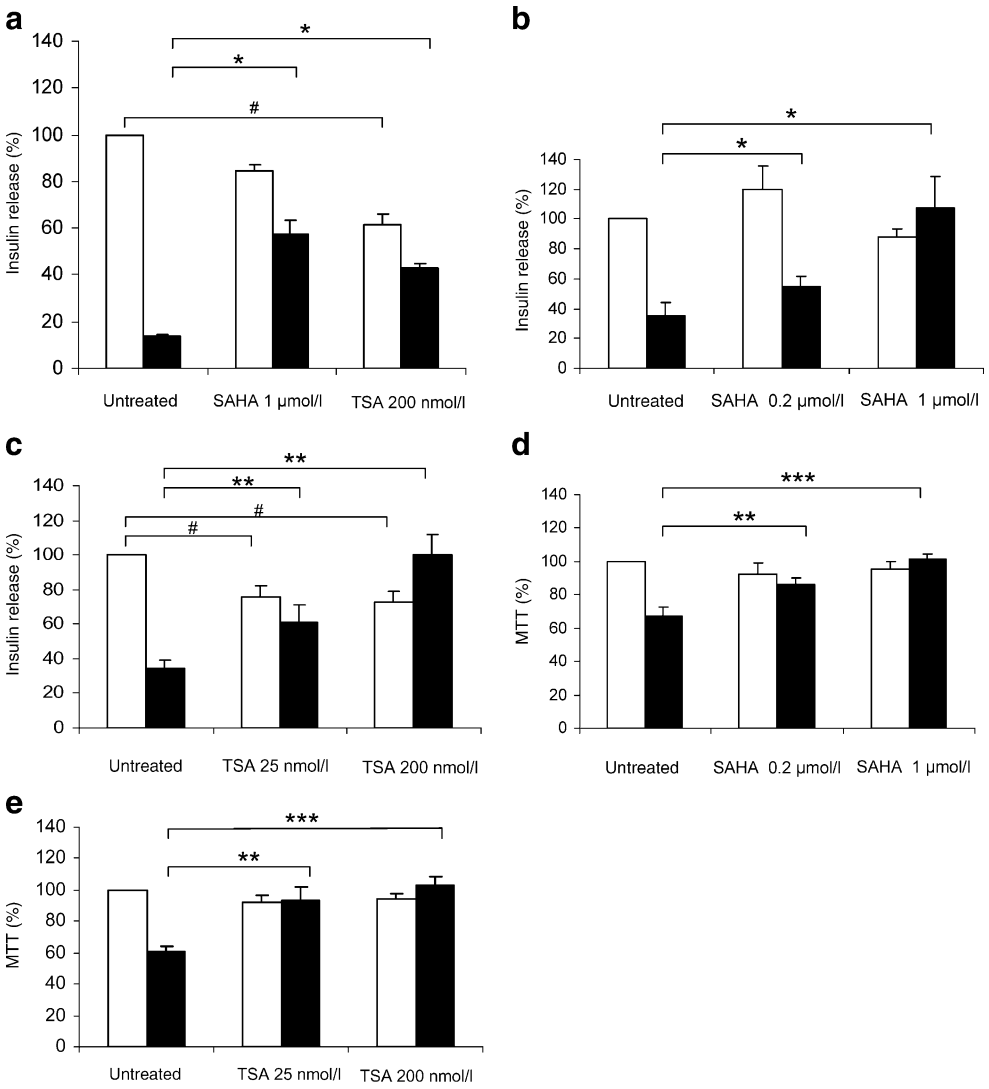


Fig. 2 Effect of HDAC inhibition on insulin secretion and metabolic activity. **a** INS-1 cells were precultured with SAHA (1 μmol/l), TSA (200 nmol/l), or vehicle (*Untreated*) for 30 min followed by exposure to IL-1β 160 pg/ml and IFNγ 10 ng/ml (*black bars*) for 2 days. Medium was collected and insulin content measured by competitive ELISA. Rat islets (**b**, **c**) were precultured with SAHA (0.2 and 1 μmol/l), TSA (25 and 200 nmol/l), or vehicle for 60 min followed by IL-1β 150 pg/ml and IFNγ 2ng/ml treatment (*black bars*) for 2 days. **d**, **e** Neonatal rat islets were precultured and cytokine-treated as described (for **b**, **c**). Culture medium was collected and viability/mitochondrial activity investigated by the 3-(4,5-dimethyldiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay. Results are shown as mean ± SEM compared with unstimulated cells (n=4–6). $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ vs cytokine-stimulated cells; $^{\#}p<0.05$ vs unstimulated cells (t test)

To test whether this was also the case in beta cells, we analysed beta cell apoptosis in response to cytokines using the TUNEL assay. A 40% reduction in cytokine-stimulated apoptosis was observed in INS-1 cells precultured with either SAHA or TSA (Fig. 3a). A small but insignificant increase in basal apoptosis was detected in INS-1 cells exposed to TSA alone. In monolayer cultures isolated from neonatal rats, similar concentrations of SAHA and TSA resulted in a 60% reduction in cytokine-stimulated apoptosis with a similar toxic tendency seen using TSA alone (Fig. 3b), increasing the basal level of apoptosis from 15 to 34%.

We also investigated the effect of HDAC inhibition on IL-1β and IFNγ-stimulated apoptosis in islets isolated from adult rats, using a cell death detection ELISA, in which the amount of DNA-histone complexes present in cytoplasmic lysates (i.e. apoptotic cells) is measured. Figure 3c and d shows that both SAHA and TSA fully protected against the detrimental effects of cytokine exposure in adult rat islets with no induction in basal apoptosis observed with either of the inhibitors alone.

Inhibition of HDACs affects the NFκB-dependent production of iNOS and formation of NO IL-1β is known to induce expression of the gene encoding iNOS, an effect that is potentiated by IFNγ [18, 34]. The subsequent formation of NO drives cell death by both necrosis and apoptosis, at least in rodent beta cells [35]. Thus, we next examined whether the protective effect of HDAC inhibition against cytokine-induced beta cell death was associated with a reduction in iNOS production. As shown in Fig. 4a and b, both SAHA and TSA dose-dependently reduced iNOS protein production in INS-1 cells. Using neonatal rat islets, a complete prevention of cytokine-stimulated iNOS pro-

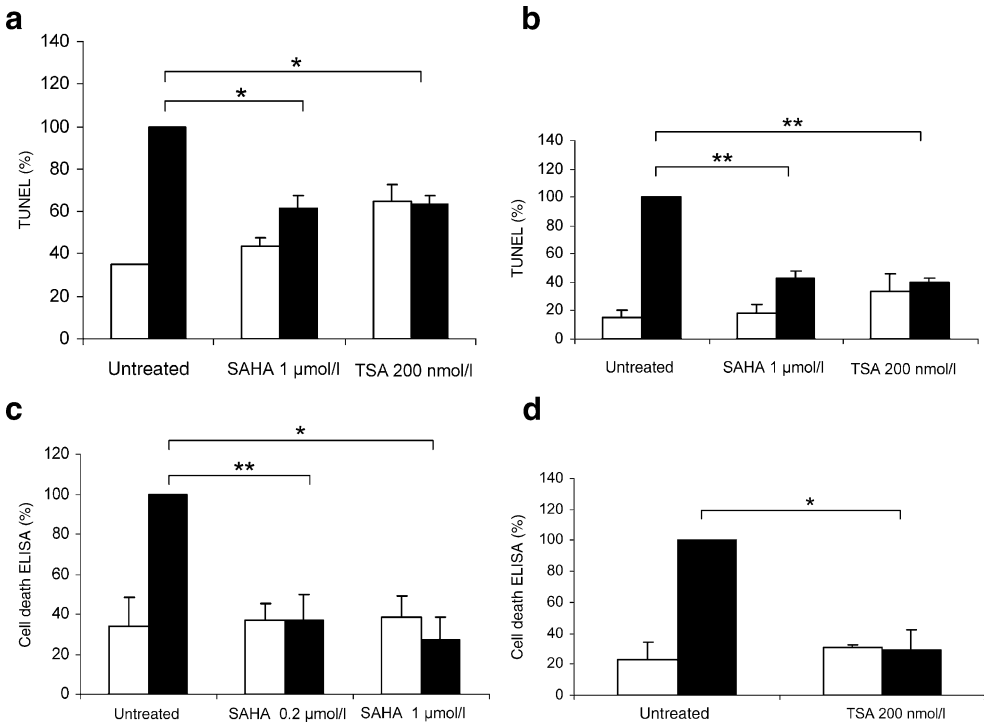


Fig. 3 Effect of HDAC inhibition on cytokine-induced apoptosis. **a** INS-1 cells were pre-cultured with vehicle, SAHA or TSA for 30 min. Subsequently cytokines were added for 20 h (*black bars*). Cells were permeabilised and fixed and enzymatic labelling of DNA strand breaks was performed by use of the TUNEL assay. **b** TUNEL assay performed on monolayer cultures from neonatal rat islets as described for INS-1 cells except that pre-culturing with inhibitors was done for 60 min. **c**, **d** Adult rat islets were pre-cultured as described and exposed to cytokines for 24 h. Amount of DNA-histone complexes present in the cytoplasm (i.e. apoptosis) was determined by cell death detection ELISA and correlated to total amount of DNA. Data are presented as mean ± SEM compared with cytokine-stimulated cells (n=3–4). $^{*}p<0.05$, $^{**}p<0.01$ vs cytokine-stimulated cells (t test)

duction was obtained at 1 and 200 nmol/l of SAHA and TSA, respectively (Fig. 4c). Accordingly, SAHA, and in particular TSA significantly reduced NO formation in INS-1 cells (Fig. 4d). In addition, a 44–46% reduction in NO formation was detected using SAHA (Fig. 4e) or TSA (Fig. 4f) in neonatal rat islets.

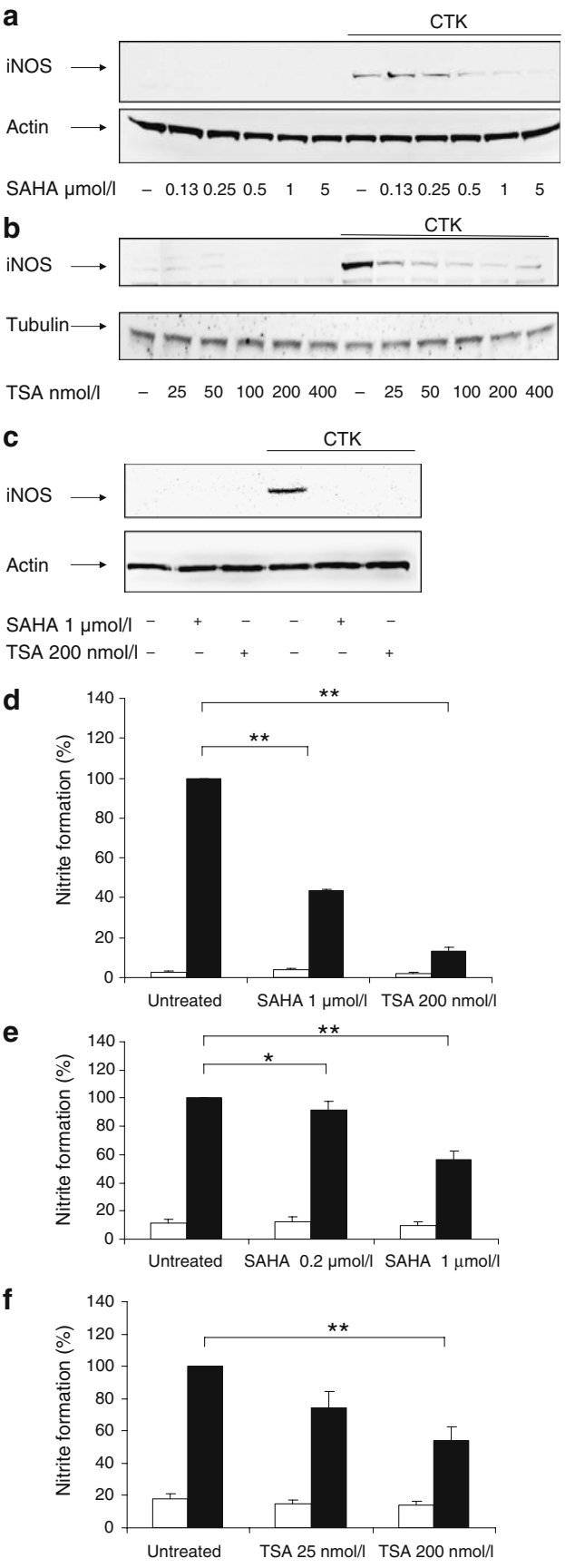
HDAC inhibitors affect the NFκB signalling pathway As NFκB is the key transcription factor responsible for cytokine-induced iNOS production, the results presented above imply that HDACs are involved in the regulation of NFκB transcriptional activity.

To further clarify the potential modulating effect of HDAC inhibition on NFκB activity, we next dissected the level of inhibition by analysing upstream components of the NFκB signalling cascade.

A time-response experiment revealed that IL-1β triggered a bi-phasic phosphorylation of the NFκB inhibitor protein, IκBα, the first peak in phosphorylation being evident within 5 min and the second peak after 30–60 min of cytokine exposure in INS-1 cells (Fig. 5a) and neonatal rat islets (Fig. 5b). Following phosphorylation, IκBα is ubiquitinated and degraded, as seen in the immunoblot showing total-IκBα after 10 to 15 min of IL-1β exposure (Fig. 5a). After 60 min of stimulation, total IκBα re-appeared, coinciding with the second peak of phosphorylation, which indicates that the NFκB pathway is still activated after 60 min of cytokine exposure. The re-appearance of IκBα is probably due to NFκB-mediated expression of its own inhibitor, as part of a negative feedback mechanism.

To investigate whether NFκB activity was influenced by HDAC inhibition at this level in the signalling cascade, a similar experiment was performed, in which INS-1 cells were precultured with SAHA or TSA before IL-1β exposure. From Fig. 5c it can be concluded that the second peak in phosphorylation at 60 min of stimulation is inhibited by SAHA or TSA. This effect is associated with a lack of re-synthesis of total IκBα as evident from the greater level of IκBα following 60 min of IL-1β stimulation alone as compared with islets co-exposed to HDAC inhibitors and IL-1β (Fig. 5c, lanes 4, 7 and 10). Similar effects were seen with SAHA using primary rat islets (Fig. 5d). These data support the notion that inhibition of HDACs reduces NFκB-dependent gene expression.

As IκBα is degraded, NFκB translocates to the nuclear compartment and binds to DNA. To ascertain whether NFκB translocation and DNA binding were reduced by the HDAC inhibitors, nuclear extracts from INS-1E cells pre-incubated with or without the inhibitor alone or in combination with cytokines were isolated and examined by EMSA (Fig. 5e). Although IL-1β and IFNγ clearly induced NFκB DNA binding after 60 min of



◀ **Fig. 4** Effect of HDAC inhibition on iNOS production and NO formation. **a**, **b** INS-1E cells were pre-cultured for 30 min with increasing concentrations either of SAHA (**a**) followed by cytokines (CTK) IL-1β 160 pg/ml and IFNγ 5 ng/ml for 6 h, or of TSA (**b**) followed by IL-1β 160 pg/ml (CTK) exposure for 6 h. Lysates were subjected to immunoblotting using antibodies against iNOS or the house-keeping proteins actin or tubulin. Representative blots are shown (n=4). **c** Neonatal rat islets were pre-cultured with SAHA (1 μmol/l) or TSA (200 nmol/l) for 60 min followed by IL-1β 150 pg/ml + IFNγ (2 ng/ml) (CTK) for 6 h. iNOS production was analysed as above (n=3). **d–f** INS-1 cells or neonatal rat islets were pre-cultured with vehicle or inhibitors and exposed to cytokines (*black bars*) for 2 days. Medium was collected and nitrite content determined by Griess reagent. Data are presented as mean±SEM compared with cytokine-exposed cells (n=3–6). $^{*}p<0.05$, $^{**}p<0.01$ vs cytokine-stimulated cells (t test)

stimulation, no difference in band intensity was observed when cells had been precultured with the HDAC inhibitors.

Taken together, these results indicate that HDACs downregulate NFκB-dependent gene transcription downstream of NFκB DNA binding.

Discussion

HDAC inhibitors have been used in the treatment of various malignancies based on their ability to promote terminal differentiation and apoptosis in cancer cells. In addition, the anti-inflammatory potential of these compounds has also been demonstrated in animal models of autoimmune diseases such as rheumatoid arthritis and systemic lupus erythematosus [4, 7–10].

The beta cell destruction and insulin deficiency characterising type 1 diabetes mellitus is at least partially believed to be mediated by cytokines, such as IL-1β and IFNγ. Here we demonstrate that inhibition of HDACs by two different HDAC inhibitors, SAHA and TSA, each exhibited a protective effect against the detrimental effects of cytokines, preventing cytokine-mediated reduction in accumulated insulin secretion, inhibition of mitochondrial function and increase in iNOS production, NO formation and apoptosis. This was shown in a beta cell line (INS-1) and in primary rat islets. Similar results have been obtained using the novel HDAC inhibitor, ITF (data not shown. For information on ITF, see [19]).

TSA and SAHA are two structurally related reversible inhibitors of HDACs in vitro and in vivo. By binding to the zinc-containing pockets of HDAC, they inhibit the activity of class I and II HDACs [36]. Of the ten HDACs, HDAC 1–3, 5, 6 and 8 are produced to various degrees in the pancreas [37], and as shown in this study, both SAHA and TSA are capable of inhibiting HDAC activity in insulin-secreting cells (see Fig. 1). Inhibition of HDAC results in increased acetylated nuclear histones [8], although global

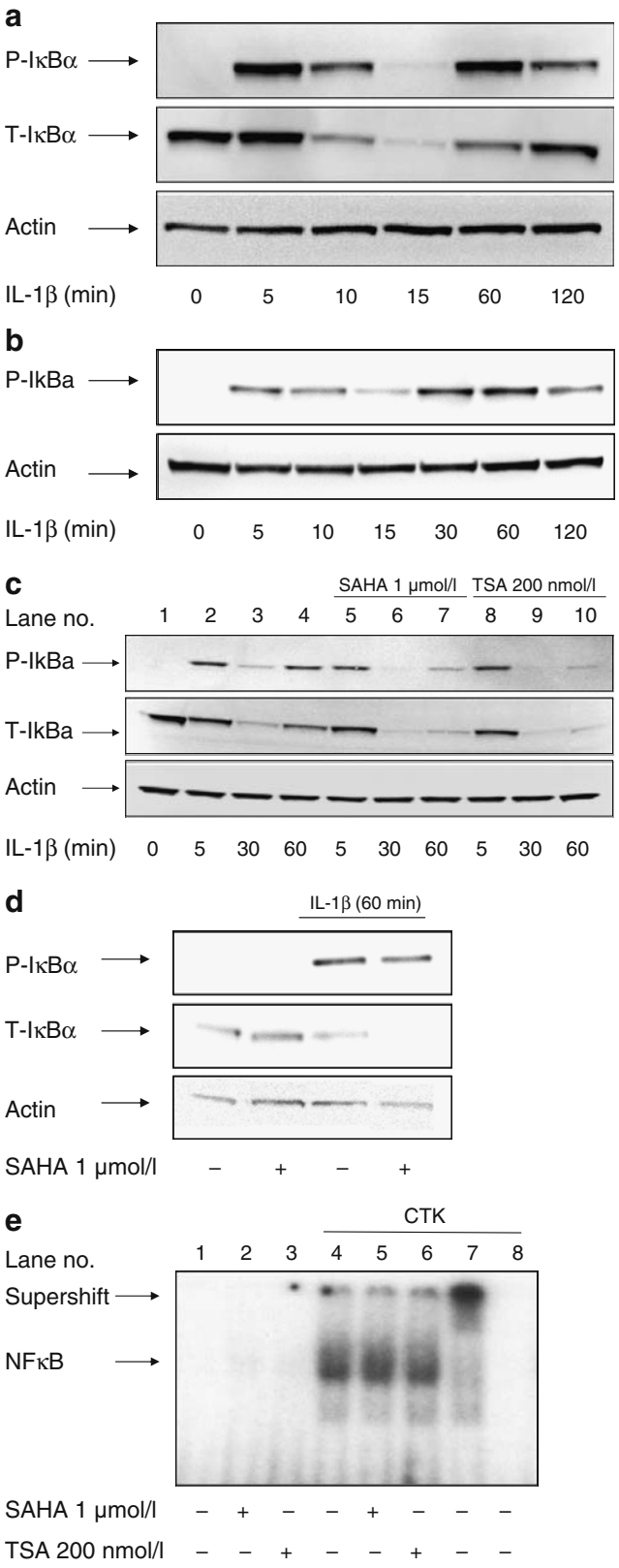


Fig. 5 Effects of HDAC inhibition on inhibitor protein kappa B alpha (*IκBα*.). **a** INS-1E cells were treated with IL-1β (160 pg/ml) for various time points. Lysates were subjected to immunoblotting using antibodies towards phosphorylated (*P*) IκBα, total (*T*) IκBα and actin. **b** Neonatal rat islets were exposed to IL-1β (150 pg/ml) and lysates subjected to immunoblotting as described (for **a**). **c** INS-1E cells were pre-cultured with SAHA (1 μmol/l) or TSA (200 nmol/l) for 30 min followed by IL-1β (160 pg/ml) exposure for various time points and analysed as described above (n=4). **d** Neonatal rat islets were pre-cultured with SAHA (1 μmol/l) for 60 min followed by IL-1β (150 pg/ml) exposure for an additional 1 h. Representative blots are shown for each experiment (n=4). **e** EMSA was performed using nuclear extracts from INS-1E cells pre-cultured with SAHA (1 μmol/l), TSA (200 nmol/l) or vehicle for 30 min, followed by IL-1β 160 pg/ml and IFNγ 5 ng/ml (*CTK*) for 1 h. An NFκB-binding oligonucleotide was used as probe and super-shift performed using an anti p65 antibody (*lane 7*). Competition was performed using 1,000-fold excess of unlabelled oligonucleotide (*lane 8*)

analysis of gene expression following SAHA exposure in cancer cell lines estimated that a very low percentage (approximately 2%) of the genome is regulated by HDAC inhibitors causing both up- and downregulation of genes [38]. SAHA has been used effectively in human primary cells and without overt toxicity in animal models and human trials [8, 32, 39, 40].

In this study we used islets from newborn and adult rats. One could argue that effects seen in islets isolated from neonatal rats might not be analogous to the outcome in adult islets, as gene expression and sensitivity to cytotoxicity are known to change during the postnatal maturation of islets [41]. However, as protection against apoptosis was observed in both types of islets, the HDAC inhibitors used by us seem equally potent in their ability to protect primary islets against the toxic effect of cytokines, regardless of maturational stage.

Although we generally observed similar positive effects of HDAC inhibition in cytokine-exposed cells, irrespective of cell source, minor toxicity on insulin secretion and apoptotic rate was observed in beta cell lines, especially with TSA. This discrepancy between tumour beta cell lines and primary rat islets may be explained by the fact that tumour cell lines such as INS-1 cells respond to HDAC inhibition similarly to other cancer cells, in which HDAC inhibition is known to induce cell death. This observation further substantiates the importance of using primary beta cells when investigating apoptotic signalling.

Nitric oxide is known to be toxic to beta cells and the prevention of cytokine-induced cell death by HDAC inhibition could at least partly be associated with a decrease in iNOS production and NO formation. The apparent paradox that HDAC inhibition prevents cytokine-induced iNOS production, but only partially inhibits NO-formation (Fig. 4c,e and f) may be explained by the difference in exposure time to cytokines (6 h versus 2 days for iNOS production and NO assay, respectively). Although the protective effect against

cytokine-mediated NO formation was less pronounced in islets than in INS-1 cells, the effect on cytokine-induced apoptosis was more prominent in primary cells. As NO is known to cause cell death by necrosis and apoptosis [35], this may indicate that the proportion of cytokine-induced cell death by necrosis is greater in INS-1 cells than in primary rat islets. Another explanation may be that SAHA induced increased levels of thioredoxin proteins and activity, an induction seen in normal cells but not in transformed cells [32]. Thioredoxin is an active reactive oxygen species (ROS) scavenger and the ROS pathway has been implicated in impaired insulin secretion and cell death in beta cells [42, 43]. Beta cells seem to have inadequate defence mechanisms against oxidative stress, making them particularly vulnerable to this phenomenon compared with non-beta cell counterparts.

A common active form of NFκB is a heterodimer of p65 and p50. Through its activation domain, p65 has been shown to interact with co-activators [12] and more recent studies indicate that the phosphorylation status of p65 is a decisive factor for such interaction. Stimulus-induced phosphorylation of p65 at serine residue 276 determines whether it associates with co-activators or co-repressors, as phosphorylation facilitates CREB binding protein/p300 association, while affinity of HDAC-1 for p65 is decreased [44]. This phosphorylation precedes NFκB acetylation, leading to increased expression of NFκB-responsive genes [45]. We have previously shown that serine 276 does indeed play an important role in cytokine-stimulated NFκB-mediated gene expression in beta cells [25] setting the stage for cytokine-induced NFκB acetylation.

Previous studies in other cell systems have suggested a link between TSA- and SAHA-mediated downregulation of proinflammatory gene expression and alterations in the NFκB signalling pathway. However, the underlying mechanisms seem to depend on the cell type investigated and include on the one hand an HDAC-inhibitor-mediated continual degradation of de novo synthesised IκBα protein (possibly by prolonged activation of the upstream IκB-kinase) causing prolonged presence and DNA-binding of NFκB in the nucleus and enhancing NFκB-dependent transactivation, or on the other hand a reduction in proteasomal degradation of IκBα, preventing NFκB nuclear translocation and DNA binding [4, 8, 46–49].

The lack of reappearance of total-IκBa after HDAC inhibitor exposure in our study was associated with a decrease in IκBα phosphorylation and no change in NFκB DNA binding activity after 1 h of stimulation, suggesting that HDAC influences NFκB-dependent gene expression down-stream of DNA binding in beta cells, probably by modulating the chromatin structure of NFκB-dependent genes, leading to increased NFκB transactivation, promoter activity and iNOS protein production, as has been demonstrated in cultured mesangial cells [4, 50].

While we observed a complete prevention of cytokine-induced decrease in accumulated insulin secretion when pretreating with HDAC inhibitors (see Fig. 2b and c), no effect was seen on acute glucose-stimulated insulin-release after 6 or 20 h of cytokine-exposure (data not shown). These data show that HDAC inhibition protects against cytokine-induced reduction in accumulated insulin secretion by affecting pathways in the secretory response that are independent of the stimulus–secretion coupling of glucose. There are several possibilities of how changes in HDAC activity could affect these pathways, including posttranscriptional modifications of transcription factors or histone modifications induced in (pre)proinsulin gene expression or expression of genes involved in non-glucose-induced signaling of insulin secretion.

The present study provides evidence that changes in the balance of acetylation/deacetylation have a significant impact on the fate of cytokine-exposed beta cells. Our data prompt further analyses exploring the potential use of HDAC inhibitors to protect pancreatic islets from inflammatory attack in the development of type 1 diabetes, as well as in islet grafting.

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