Diabetologia (2010) 53:2569–2578
DOI 10.1007/s00125-010-1892-8

ARTICLE

Lysine deacetylases are produced in pancreatic beta cells and are differentially regulated by proinflammatory cytokines

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Received: 18 March 2010 / Accepted: 13 July 2010 / Published online: 28 September 2010
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Abstract

Aims/hypothesis Cytokine-induced beta cell toxicity is abrogated by non-selective inhibitors of lysine deacetylases (KDACs). The KDAC family consists of 11 members, namely histone deacetylases HDAC1 to HDAC11, but it is not known which KDAC members play a role in cytokine-mediated beta cell death. The aim of the present study was to examine the KDAC gene expression profile of the beta cell and to investigate whether KDAC expression is regulated by cytokines. In addition, the protective effect of the non-selective KDAC inhibitor ITF2357 and interdependent regulation of four selected KDACs were investigated.

Methods The beta cell line INS-1 and intact rat and human islets were exposed to cytokines with or without ITF2357. Expression of mRNA was assessed by real-time PCR and selected targets validated at the protein level by immunoblotting. Effects on cytokine-induced toxicity were investigated by in vitro assays.

Results *Hdac1* to *Hdac11* were expressed and differentially regulated by cytokines in INS-1 cells and rat islets. *HDAC1*, *-2*, *-6* and *-11* were found to be expressed and regulated by cytokines in human islets. ITF2357 protected against cytokine-induced beta cell apoptosis and counteracted cytokine-induced attenuation of basal insulin secretion. In addition, cytokine-induced regulation of *Hdac2* and *Hdac6*, but not *Hdac1* and *Hdac11*, was reduced by KDAC inhibition.

Conclusions/interpretation All classical KDAC genes are expressed by beta cells and differentially regulated by

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Electronic supplementary material The online version of this article (doi:10.1007/s00125-010-1892-8) contains supplementary material, which is available to authorised users.

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cytokines. Based on the relative expression levels and degree of regulation by cytokines, we propose that *HDAC1*, *-2*, *-6* and *-11* are of particular importance for beta cell function. These observations may help in the design of specific KDAC inhibitors to prevent beta cell destruction in situ and in islet grafts.

Keywords HDAC · Histone · IL-1 · KDAC · Lysine deacetylase inhibitors

Abbreviations

HDAC	Histone deacetylase
iNOS	Inducible nitric oxide synthase
KAT	Lysine acetyltransferase
KDAC	Lysine deacetylase
NFκB	Nuclear factor κB
SAHA	Suberoylanilide hydroxamic acid

Introduction

Acetylation of histone and non-histone proteins regulates gene transcription and protein function. Acetylation is catalysed by lysine acetyltransferases (KATs) and deacetylation by lysine deacetylases (KDACs). Recently, Choudhary and co-workers demonstrated more than 3,500 acetylation sites within 1,735 non-histone proteins in addition to 61 sites in histones [1], underlining the importance of KATs and KDACs in the post-translational regulation of non-histone proteins. The conventional wisdom is that hypoacetylation of histones is associated with gene repression [2, 3], but this may be an oversimplification [4, 5]. Histone acetylation is a dynamic process and active gene regions are the site of KAT and KDAC activity [6]. Two protein families with KDAC activity exist; the sirtuins (SIRT1 to SIRT7) [7] and the classical KDACs, i.e. histone deacetylases (HDAC1 to HDAC11) [8]. The classical KDACs are inhibited by a range of synthetic small molecules indicated for or tested in clinical trials for several disorders, including myeloproliferative diseases [9], neurodegenerative disorders [10] and inflammatory conditions such as systemic-onset juvenile idiopathic arthritis (SOJIA) [11]. We have previously shown that two structurally related non-selective KDAC inhibitors, suberoylanilide hydroxamic acid (SAHA) and trichostatin A, inhibit cytokine-induced beta cell damage [12], suggesting that KDAC inhibitors may be exploited therapeutically in type 1 and type 2 diabetes [13]. The novel KDAC inhibitor ITF2357 has anti-inflammatory [14] and neuroprotective properties [15], and is a non-selective KDAC inhibitor as, for example, the IC_{50} of HDAC1, -2, -3, -6, -10 and -11 ranges between 100 and 350 nmol/l [16].

The classical KDACs are divided into three classes based on phylogeny and gene sequence: (1) class I: HDAC1, -2, -3 and -8; (2) class II: HDAC4, -5, -7 and -9 (sub-class IIa), and HDAC6 and -10 (sub-class IIb); and (3) class IV: HDAC11.

Class I and IIb KDACs are ubiquitously produced [17], class IIa is produced in cardiac and skeletal muscle (HDAC5 and -9) [18–20], and in the retina and skeletal growth plates (HDAC4) [21, 22] and thymocytes (HDAC7) [20], whereas *HDAC11* is expressed in the brain, heart, skeletal muscle, kidney and testis [23, 24]. An exhaustive expression profiling of the classical KDACs in the beta cell has not been published. HDAC1, -2, -3 and -6 are produced in three insulinoma cell lines, whereas production of HDAC4, -5 and -7 varies between cell lines [25]. Less is known about their regulation at the expressional level following cytokine exposure. In an exploratory microarray study, the effect of cytokines on primary rat beta cell production of *Hdac1*, *-2*, *-3*, *-4*, *-5*, *-6* and *-10* was assessed [26]; however, the findings were not confirmed by real-time PCR or at the protein level, and there are no published studies of KDAC expression in human islets.

Here we investigated the relative expression profile of the classical KDACs in INS-1 cells and their regulation upon cytokine exposure. Of particular interest, *Hdac1*, *-2* and *-6* were constitutively expressed at high levels, but downregulated by cytokines, whereas *Hdac11* was the only KDAC markedly upregulated upon cytokine exposure. The cytokine-induced downregulation of selected KDAC genes was confirmed in rat and human islets, and upregulation of *Hdac11* was also observed in rat islets. In addition, we identified ITF2357 as a novel inhibitor of cytokine-induced beta cell death and report interdependent regulation of KDACs, since basal and/or cytokine-induced expression of candidate KDACs was inhibited by ITF2357.

Methods

Cytokines and KDAC inhibitors Mouse IL-1β was from BD Pharmingen (Erembodegem, Belgium) and rat IFNγ was from R&D Systems (Oxford, UK). Human IL-1β was from Sigma (St. Louis, MO, USA), human IFNγ was from BD Pharmingen and human TNF-α was from Endogen (Cambridge, MA, USA). The KDAC inhibitor ITF2357 was donated by Italfarmaco (Cinisello Balsamo, Italy) [14].

Cells INS-1 cells were a gift from C. Wollheim, Department of Cell Physiology and Metabolism, University Medical Center, Geneva, Switzerland [27] and were maintained in complete medium (RPMI 1640 culture

medium with GlutaMAX supplemented with 10% [vol./vol.] FCS, 100 U/ml penicillin, 100 μg/ml streptomycin [all from Invitrogen/Gibco, Taastrup, Denmark] and 50 μmol/l β-mercaptoethanol [Sigma]). Cells were cultured at 37°C in a humidified atmosphere containing 5% CO_2, passaged weekly and precultured for 2 days prior to cytokine exposure. At the initiation of experiments, medium was changed and ITF2357 or vehicle was added 1 h prior to cytokine exposure for the time periods indicated.

Rat islet isolation, preculture and culture Primary neonatal rat islets were isolated from 3- to 6-day-old outbred Wistar Rats (Taconic, Ejby, Denmark) as previously described [28] and precultured at 37°C in humidified atmospheric air for a week in RPMI 1640 with 20 mmol/l HEPES buffer, 2 mmol/l L-glutamine, 0.038% (wt/vol.) $NaHCO_3$, 100 U/ml penicillin and 100 μg/ml streptomycin supplemented with 10% (vol./vol.) newborn calf serum (Invitrogen/Gibco). Before addition of ITF2357 and cytokines, islets were cultured in complete medium supplemented with 2% (vol./vol.) human serum in 55 mm uncoated Petri dishes (VWR, Herlev, Denmark) or 48 well plates (NUNC, Roskilde, Denmark) and left for 2 to 3 h at 37°C to reduce handling stress before exposure to cytokines.

Human islet culture Human islets were obtained from O. Korsgren (Department of Clinical Immunology, Rudbeck Laboratory, Uppsala University Hospital, Sweden) through the Juvenile Diabetes Research Foundation Islet Distribution Program with approval from the Swedish ethics authority. Islets were cultured in RPMI 1640 without glucose (Gibco BRL) and supplemented with 5.6 mmol/l D-glucose (Sigma-Aldrich), 10% (vol./vol.) FCS, 20 mmol/l HEPES buffer, 2 mmol/l L-glutamine, 0.038% (wt/vol.) $NaHCO_3$, and 100 U/ml penicillin and 100 μg/ml streptomycin. Culture was at 37°C in humidified atmospheric air. Before cytokine exposure, islets were transferred to 100 mm Petri dishes (NUNC) in 15 ml RPMI 1640 supplemented with 2% (vol./vol.) human serum (Lonza [BioWhittaker], Basel, Switzerland). Islets were left for 2 to 3 h at 37°C before exposure to cytokines.

Real-time PCR Two and a half million INS-1 cells, 1,000 rat islets or 2,000 human islets were precultured for 1 h in the presence or absence of ITF2357. INS-1 cells were cultured in six well plates (NUNC) in 5 ml complete medium. INS-1 cells and rat islets were exposed to mouse IL-1β and rat IFNγ, and human islets to human IL-1β alone or a combination of human IL-1β, human IFNγ and human TNF-α for various time periods.

Total RNA from INS-1 cells was extracted using a kit (Total RNA and Protein Isolation Kit; Macherey-Nagel, Fischer Scientific, Slangerup, Denmark) and cDNA synthesis was performed using a cDNA synthesis kit (iScript; Bio-Rad, Copenhagen, Denmark). From rat and human islets, total RNA was extracted using the TRIzol method (Invitrogen). cDNA synthesis was performed with a kit (TaqMan Gold RT-PCR; PerkinElmer, Boston, MA, USA). All steps were done according to the manufacturers' guidelines. Assay identification numbers for TaqMan probes used for the real-time PCR are listed in Electronic supplementary material (ESM) Table 1. Real-time PCR was performed as described in the supplier's manual (7900HT Real-Time PCR System; Applied Biosystems, Carlsbad, CA, USA). Each cDNA sample in triplicate was subjected to two individual PCR amplifications using TaqMan probes either for the gene of interest or for the reference gene. Every PCR reaction was amplified in TaqMan Gene Expression Master Mix (Applied Biosystems).

Immunoblotting Five hundred thousand INS-1 cells were seeded in 12 well plates (NUNC) in 1 ml complete medium. Mouse IL-1β and rat IFNγ were added for the indicated time periods. Cells were lysed, protein content measured by the Bradford method, and lysates adjusted for protein concentration and prepared for gel electrophoresis as previously described [29]. A minimum of 8 μg protein was separated by gel electrophoresis. Antibodies against HDAC1 (sc-7872), HDAC2 (sc-7899), HDAC6 (sc-5258) and β-tubulin (sc-5274) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and antibodies against HDAC11 (ab18973) and β-actin (ab6276) from Abcam (Cambridge, UK). Anti-cleaved caspase-3 (9661), anti-rabbit IgG horseradish peroxidase-linked (7074) and anti-mouse IgG horseradish peroxidase-linked (7076) were from Cell Signaling (Medinova, Glostrup, Denmark), and anti-inducible nitric oxide synthase (iNOS) (610332) was from BD Biosciences (Erembodegem, Belgium). Biotin-XX rabbit anti-goat IgG (6517349), biotin-XX goat anti-rabbit IgG (561791), biotin-XX goat anti-mouse IgG (683169), Qdot605 streptavidin-conjugate (676658) and Qdot705 streptavidin-conjugate (563257) were from Invitrogen. After optimisation we found that visualisation by chemiluminescence using LumiGLO (Cell Signaling) [29] was optimal for detection of HDAC1 and HDAC11, cleaved caspase-3, β-actin and β-tubulin immune complexes, and Qdot fluorescence (Invitrogen) was optimal for HDAC2, HDAC6 and β-tubulin immune complexes. In both cases, light emission was captured digitally by FluorChem Q System (Kem-En-Tec, Taastrup, Denmark). Qdot605 and Qdot705 were detected using 605±30 and 705±30 nm band width filters (Kem-En-Tec), respectively.

Nitric oxide measurement Medium from cells used in the cell-death detection ELISA assay was collected. Nitric

oxide was measured as accumulated nitrite in the medium by mixing equal volumes of cell culture medium and Griess reagent (0.1% [wt/vol.] naphthylethene diamine hydrochloride; Sigma) in H_2O, and 1% (wt/vol.) sulphanilamide (Bie & Berntsen, Rødovre, Denmark) in 5% (vol./vol.) H_3PO_4 (Merck, Glostrup, Denmark). Absorbance was measured at 550 nm and accumulated nitrite calculated from a $NaNO_2$ standard curve.

Insulin assay One hundred rat islets were seeded in 48 well plates containing 300 μl RPMI 1640 with 20 mmol/l HEPES buffer, 2 mmol/l L-glutamine, 0.038% (wt/vol.) $NaHCO_3$, 100 U/ml penicillin and 100 μg/ml streptomycin supplemented with 0.5% (vol./vol.) newborn calf serum (Invitrogen/Gibco). Islets were precultured for 1 h in the presence or absence of ITF2357 (500 nmol/l) and then exposed to mouse IL-1β (150 pg/ml) and rat IFNγ (5 ng/ml) for 24 and 48 h. Accumulated insulin in the incubation medium was measured as previously described [12], except that 0.5% (wt/vol.) BSA was added to the incubation buffer. The anti-guinea pig IgG antibody (8522; Abcam) and the guinea pig anti-porcine insulin (PC2005; H. Kofod, Department of Biomedical Sciences, University of Copenhagen, Denmark) were diluted 1:300,000 in incubation buffer, and the samples were diluted 1:2,000 in incubation buffer before being added to the plates.

Cell death detection by ELISA Fifty thousand INS-1 cells or 25 isolated rat islets were seeded in 48 well plates containing 0.5 ml complete medium. Cells and islets were precultured for 1 h in the presence or absence of ITF2357 and exposed to mouse IL-1β and rat IFNγ for 24 h. Cytokine-induced apoptosis was determined by cell-death detection ELISA (Roche, Basel, Switzerland), which measures the amount of

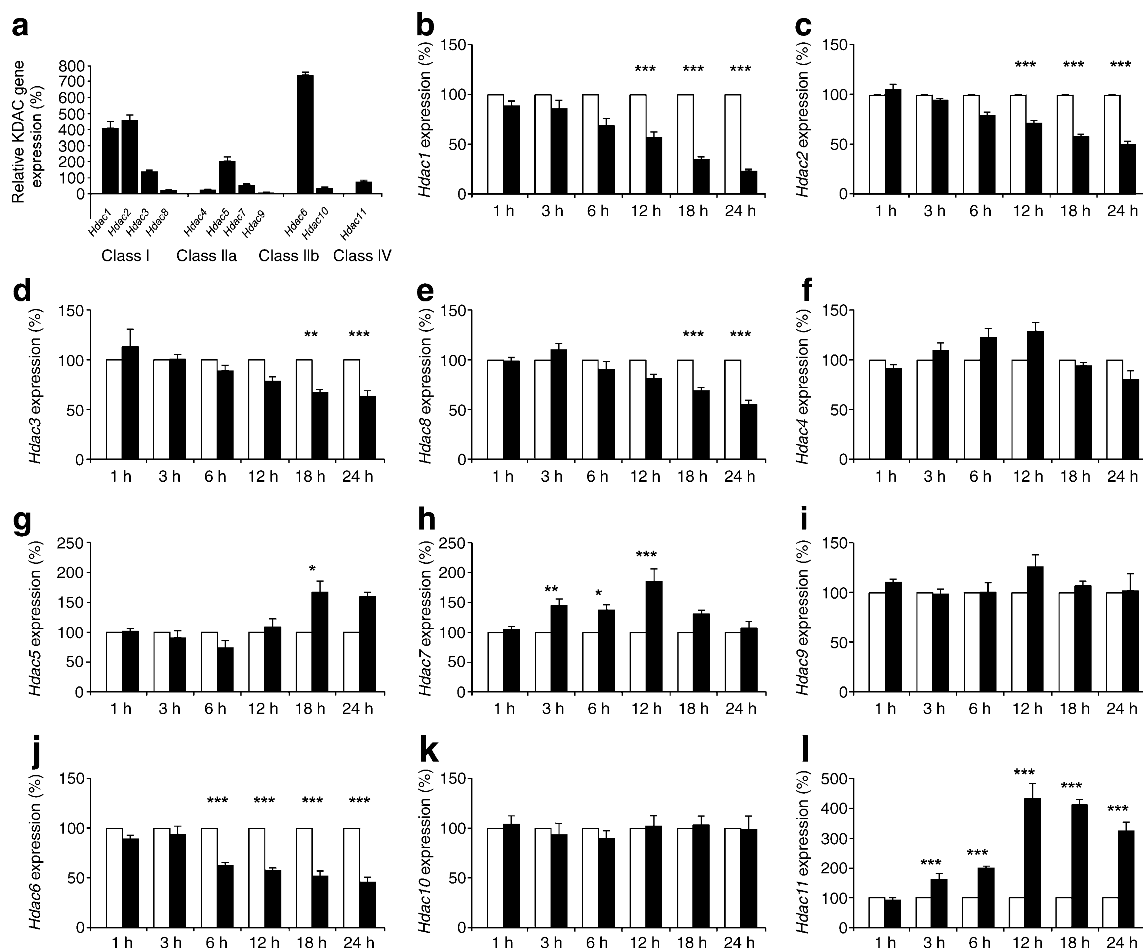


Fig. 1 Basal expression of KDAC mRNA (**a**), and cytokine-regulated expression of *Hdac1* (**b**), *Hdac2* (**c**), *Hdac3* (**d**), *Hdac8* (**e**), *Hdac4* (**f**), *Hdac5* (**g**), *Hdac7* (**h**), *Hdac9* (**i**), *Hdac6* (**j**), *Hdac10* (**k**) and *Hdac11* (**l**) mRNA in INS-1 cells. INS-1 cells were cultured for 1, 3, 6, 12, 18 or 24 h in the presence (black bars) or absence (white bars) of IL-1β (150 pg/ml) + IFNγ (5 ng/ml). Total RNA was isolated, cDNA generated by reverse transcription and expression of KDAC genes quantified by real-time PCR. To correct for variation in input RNA, data were normalised to the expression of the reference gene *Hprt1*. Basal expression was calculated from RNA collected from control cells not exposed to cytokines and normalised to *Hprt1*. Data from six independent experiments are presented as per cent change compared with controls. Results are shown as means ± SEM. *$p<0.05$, **$p<0.01$ and ***$p<0.001$ (ANOVA with Bonferroni-corrected post hoc test)

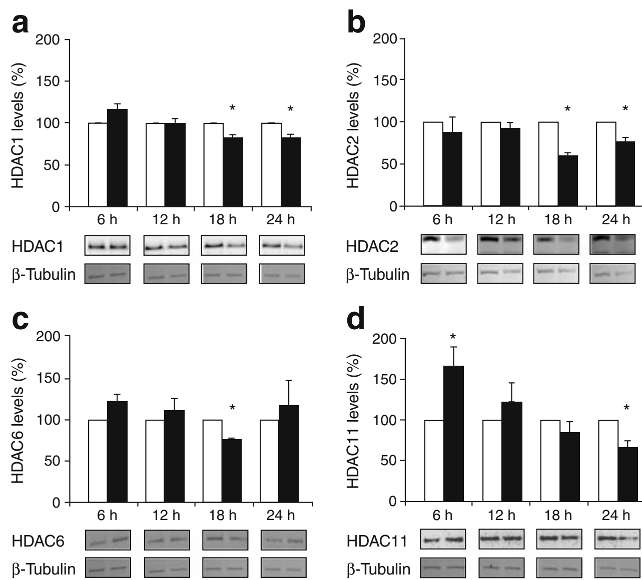


Fig. 2 Cytokine-induced regulation of HDAC1 (**a**), HDAC2 (**b**), HDAC6 (**c**) and HDAC11 (**d**) protein in INS-1 cells. INS-1 cells were cultured for 6, 12, 18 or 24 h in the presence (black bars) or absence (white bars) of IL-1β (150 pg/ml) + IFNγ (5 ng/ml). Lysates were subjected to immunoblotting using antibodies against the individual KDAC proteins and the reference protein β-tubulin. To correct for variation in input protein, data were normalised to β-tubulin. Data from three to five independent experiments are presented as per cent change compared with controls. Results are means ± SEM, representative blots are shown. *$p<0.05$ (Student's paired t test)

DNA–histone complexes present in the cytoplasmic lysates according to the manufacturer's description.

Statistical analysis Comparisons between groups were carried out either by paired t test or by ANOVA followed by Bonferroni-corrected or pre-planned post-hoc tests.

Results

Hdac1 to -11 mRNA is expressed in INS-1 cells and rat islets and differentially regulated by cytokines KDAC expression was investigated in INS-1 cells cultured in the absence of cytokines and the data normalised to the reference gene *Hprt1*, which was unaffected by cytokines and KDAC inhibitors (data not shown). As shown in Fig. 1a, each of the classical KDACs was expressed in INS-1 cells, albeit at different levels. *Hdac6* exhibited the highest expression level followed by *Hdac2* and *Hdac1*, respectively.

Next, we investigated how KDAC expression was affected by cytokines. Cytokines differentially regulated KDACs in a class-specific manner. Thus, cytokines progressively reduced expression of all class I KDAC mRNAs after 18 h ($p<0.01$) and most significantly after 24 h (*Hdac1* by 75%, *Hdac2* by 50%, *Hdac3* by 35% and *Hdac8* by 45%, $p<0.001$; Fig. 1b–e). Class IIa exhibited a more diverse regulation pattern (Fig. 1f–i), with expression of *Hdac4* and *Hdac9* being unaffected by cytokines,

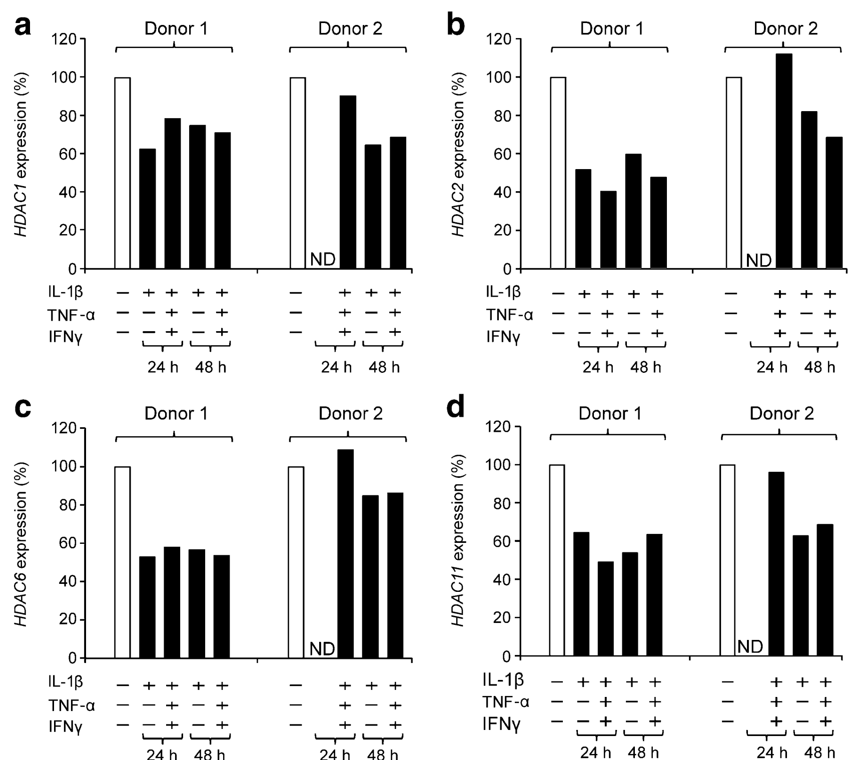


Fig. 3 Cytokine-induced regulation of *HDAC1* (**a**), *HDAC2* (**b**), *HDAC6* (**c**) and *HDAC11* (**d**) mRNA in human islets. Human islets from two donors were cultured for 24 or 48 h in the presence or absence of IL-1β (1 ng/ml) or IL-1β (1 ng/ml) + IFNγ (20 ng/ml) + TNF-α (8 ng/ml). Total RNA was isolated, cDNA generated by reverse transcription and expression of the KDAC genes quantified by real-time PCR. To correct for variation in input RNA, data were normalised to the expression level of *PPIA*. Data are presented as per cent change compared with control (white bars). ND, not determined

whereas *Hdac5* and *Hdac7* were significantly upregulated, reaching peak levels after 24 and 12 h, respectively (*Hdac5* upregulated by 60% and *Hdac7* by 86%). The Class IIb *Hdac6* was time-dependently downregulated after 6 h ($p<0.001$) and was reduced by 55% after 24 h ($p<0.001$), whereas *Hdac10* was unaffected by cytokines (Fig. 1j, k). *Hdac11* (class IV) was upregulated after 3 h ($p<0.001$), with peak expression after 12 h (430%, $p<0.001$; Fig. 1l).

Expression of each KDAC was confirmed in primary rat islets and *Hdac1*, *-2*, *-3*, *-6*, *-7* and *-11* were found to be regulated by cytokines in the same manner as in INS-1 cells (ESM Fig. 1).

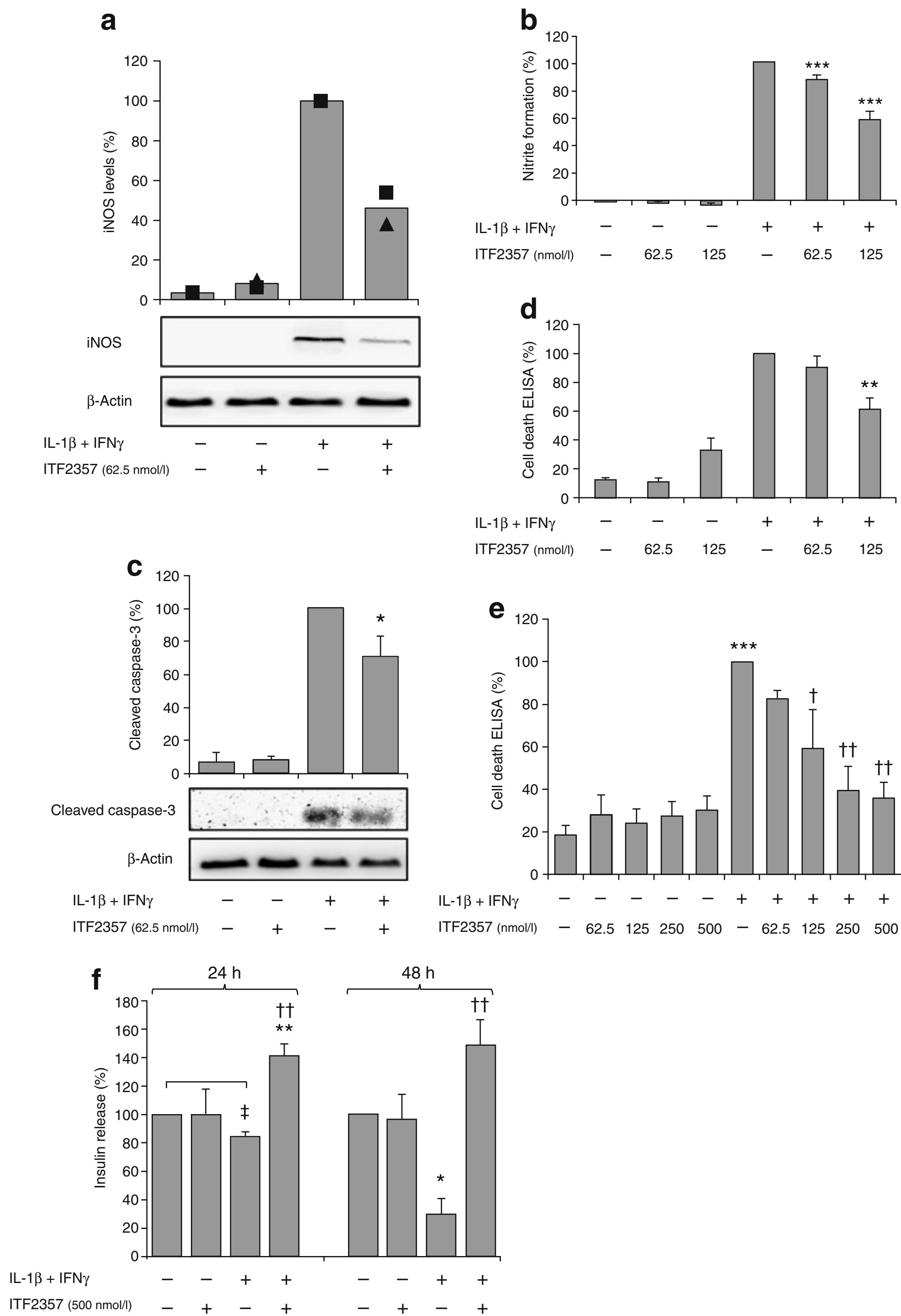
Based on the basal and cytokine-induced expression patterns, *Hdac1*, *Hdac2*, *Hdac6* (high basal expression levels, reduced by cytokines and confirmed in primary rat islets) and *Hdac11* (highest cytokine-induced upregulation in INS-1 cells and primary rat islets) were selected for further analysis.

Cytokine-induced regulation of HDAC1, HDAC2, HDAC6 and HDAC11 at the protein level in INS-1 cells To validate the mRNA expression data, immunoblotting experiments were performed on total protein lysate from INS-1 cells. As shown in Fig. 2, levels of HDAC1 and HDAC2 were significantly reduced by cytokines after 18 and 24 h (Fig. 2a, b). Cytokines significantly downregulated HDAC6 after 18 h (Fig. 2c). HDAC11 was significantly upregulated by the combination of IL-1β + IFNγ after 6 h, but in contrast to findings at the mRNA level, HDAC11 protein levels were significantly reduced after 24 h (Fig. 2d).

Cytokine-induced regulation of HDAC1, HDAC2, HDAC6 and HDAC11 mRNA in human islets We next investigated the regulation of the four selected KDACs in human islets exposed to IL-1β alone or to a combination of IL-1β, IFNγ and TNF-α, selected because this combination potentiates the deleterious effect of IL-1β on human islets [30]. Cytokine-induced KDAC expression was analysed in islets from two donors after 24 and 48 h (Fig. 3), as human islets in general have a delayed cytokine response compared with rat islets [31]. Each of the four KDAC genes was expressed in human islets. At 24 h there was no constant pattern of KDAC expression. After 48 h, *HDAC1*, *HDAC2*, *HDAC6* and *HDAC11* were all found to be downregulated by both cytokine conditions (IL-1β alone or cytokine combination) in both donors, supporting the initial expression signature of *Hdac1*, *Hdac2* and *Hdac6* in INS-1 cells and rat islets. The kinetics of *HDAC11* expression differed between human islets and INS-1 cells and rat islets.

The KDAC inhibitor ITF2357 protects against cytokine-induced toxicity in beta cells To investigate interdependent KDAC regulation, we took advantage of the novel non-selective KDAC inhibitor ITF2357. First we examined the effect of ITF2357 on cytokine-induced toxicity. Preventive effects of ITF2357 on cytokine-induced iNOS levels (Fig. 4a), nitric oxide formation measured by nitrite detection (Fig. 4b), cleavage of caspase-3 (Fig. 4c) and apoptosis (Fig. 4d) in INS-1 cells were demonstrated. The protective effect of ITF2357 against cytokine-induced apoptosis was verified in rat islets (Fig. 4e). In rat islets, ITF2357 was further found to attenuate cytokine-induced impairment of insulin secretion after 24 h and counteracted it after 48 h, as assessed by determining accumulated insulin in the incubation medium (Fig. 4f). Whereas ITF2357 itself had no effect on insulin secretion, co-exposure of rat islets to ITF2357 and cytokines increased insulin secretion significantly after 24 h

Fig. 4 ITF2357 reduces cytokine-induced toxicity in beta cells. ▶ **a** ITF2357 inhibits cytokine-induced iNOS levels in INS-1 cells. INS-1 cells were pre-exposed to ITF2357 (62.5 mmol/l) for 1 h prior to IL-1β (150 pg/ml) + IFNγ (5 ng/ml) exposure for 6 h. Lysates were subjected to immunoblotting using antibodies against iNOS and the reference protein β-actin. To correct for variation in input protein, data were normalised to β-actin. Bars represent mean values of two independent experiments compared with controls. Results from the individual experiments are indicated (squares and triangles). Representative blots are shown. **b** ITF2357 inhibits cytokine-induced nitric oxide production in INS-1 cells. INS-1 cells were pre-exposed to ITF2357 (62.5 mmol/l or 125 mmol/l) for 1 h prior to IL-1β (150 pg/ml) + IFNγ (5 ng/ml) exposure for 24 h. Medium was collected and nitrite content determined by the Griess reaction. Data are presented as means ± SEM compared with cells exposed to cytokines (n=6); $^{***}p<0.001$ (ANOVA, pre-planned post hoc test). **c** ITF2357 inhibits cytokine-induced cleavage of caspase-3 in INS-1 cells. INS-1 cells were pre-exposed to ITF2357 (62.5 mmol/l) for 1 h prior to IL-1β (150 pg/ml) + IFNγ (5 ng/ml) exposure for 24 h. Lysates were subjected to immunoblotting using antibodies against cleaved caspase-3 and the reference protein β-actin. To correct for variation in input protein, data were normalised to β-actin. Values are presented as means ± SEM compared with control (n=3). Representative blots are shown. $^{*}p<0.05$ (Student's paired t test). **d** ITF2357 inhibits cytokine-induced apoptosis in INS-1 cells. INS-1 cells were pre-exposed to ITF2357 (62.5 nmol/l or 125 nmol/l) for 1 h prior to IL-1β (150 pg/ml) + IFNγ (5 ng/ml) exposure for 24 h. Amount of DNA–histone complexes present in the cytoplasm (i.e. apoptosis) was determined by cell death detection ELISA. Data are presented as means ± SEM compared with cells exposed to cytokines (n=6); $^{**}p<0.01$ (ANOVA, pre-planned post hoc test). **e** ITF2357 inhibits cytokine-induced apoptosis in rat islets. Rat islets were pre-exposed to ITF2357 (62.5, 125, 250 or 500 nmol/l) for 1 h followed by IL-1β (150 pg/ml) + IFNγ (5 ng/ml) or vehicle exposure for 24 h. The amount of DNA–histone complexes present in the cytoplasm (i.e. apoptosis) was determined by cell death detection ELISA. Data are presented as means ± SEM compared with rat islets exposed to cytokines (n=3); $^{***}p<0.001$ vs unexposed islets; $^{\dagger}p<0.05$, $^{\dagger\dagger}p<0.01$ vs islets exposed to cytokines only (ANOVA, pre-planned post hoc test). **f** ITF2357 attenuates cytokine-mediated suppression of accumulated insulin release. Rat islets were pre-exposed to ITF2357 (500 nmol/l) for 1 h prior to IL-1β (150 pg/ml) + IFNγ (5 ng/ml) exposure for 24 and 48 h. Accumulated insulin in the medium was assessed by competitive ELISA. Data are presented as means ± SEM compared with control (n=3); $^{*}p<0.05$, $^{**}p<0.01$, $^{\ddagger}p=0.05$ vs unexposed islets; $^{\dagger\dagger}p<0.01$ vs islets exposed to cytokines only (ANOVA, pre-planned post hoc test)



(141% vs control) and the same tendency was found after 48 h of exposure (149% vs control, $p=0.08$).

KDAC-dependent regulation of basal and cytokine-induced expression of Hdac1, Hdac2, Hdac6 and Hdac11 mRNA In non-beta cell systems, KDAC inhibitors were found to modulate KDAC expression [32]. To investigate whether KDAC inhibition is associated with alterations in basal and cytokine-induced expression of selected KDAC genes in beta cells, INS-1 cells were pre-exposed to ITF2357 for 1 h and then to IL-1β and IFNγ for 24 h. ITF2357 did not affect the basal expression of *Hdac1* and *Hdac2*, whereas a minor but significant inhibition was observed on *Hdac6* and *Hdac11* expression (Fig. 5). ITF2357 did not influence cytokine-induced *Hdac1* and *Hdac11* regulation, but cytokine-induced inhibition of *Hdac2* and *Hdac6* expres-

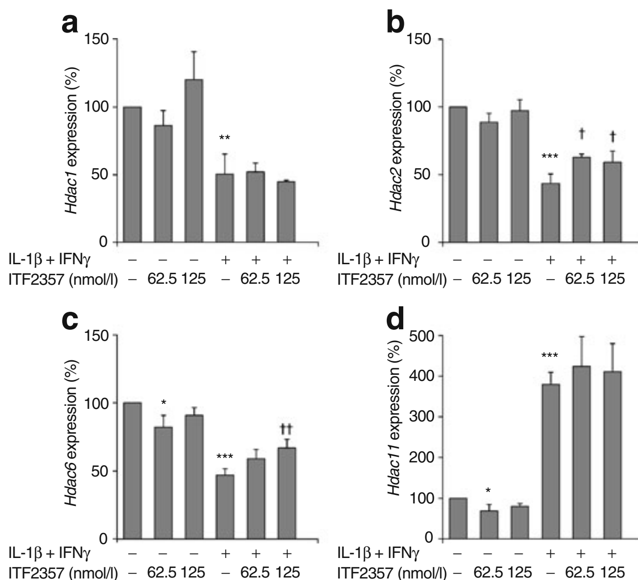


Fig. 5 Effect of ITF2357 on basal and cytokine-induced *Hdac1* (**a**), *Hdac2* (**b**), *Hdac6* (**c**) and *Hdac11* (**d**) mRNA expression. INS-1 cells were pre-cultured with ITF2357 (62.5 or 125 nmol/l as indicated) for 1 h, followed by culturing for 24 h in the presence or absence of IL-1β (150 pg/ml) + IFNγ (5 ng/ml). Total RNA was isolated, cDNA generated by reverse transcription and expression quantified by real-time PCR. To correct for variation in input RNA, data were normalised to the expression level of *Hprt1*. Data from four to six independent experiments are presented as per cent change compared with controls. Results are shown as means ± SEM; **p*<0.05, ***p*<0.01 and ****p*<0.001 vs unexposed cells; †*p*<0.05 and ††*p*<0.01 vs cells exposed to cytokines only (ANOVA, pre-planned post hoc test)

sion was partially reversed ($p<0.05$ and $p<0.01$, respectively). Thus, KDAC activity regulates expression patterns of specific KDACs both under basal conditions and following cytokine exposure for 24 h. ITF2357 did not abolish the cytokine-induced regulation of *Hdac1* and *Hdac11*, suggesting that this regulation is independent of KDAC activity, whereas effects after 24 h of exposure on *Hdac2* and *Hdac6* may be at least in part secondary to effects of cytokines on activity of other KDACs.

Discussion

Cytokines, and in particular IL-1β, have been implicated in the pathogenesis of type 1 and type 2 diabetes mellitus [13]. The protective effect of non-selective KDAC inhibitors on cytokine-induced beta cell toxicity in vitro implies that the classical KDACs contribute to beta cell destruction leading to type 1 and type 2 diabetes mellitus. It remains to be determined which KDAC(s) is (are) responsible for the deleterious effects. We believe that this first comprehensive expression analysis contributes to the identification of the important KDAC subtypes involved in inflammatory beta cell destruction in vitro. Surprisingly, all classical KDACs were expressed in insulin-producing cells and intact rat islets. Although we did not perform complete expression profiling of all KDACs in intact human islets, we did demonstrate similar expression patterns of KDACs selected to be representative of the most interesting candidates, based on expression levels and literature review. Thus, HDAC1 and HDAC2 are involved in nuclear factor κB (NFκB) regulation [33], HDAC6 in stress surveillance [34] and HDAC11 in regulation of IL-10 expression [35], functions all relevant to inflammatory islet responses.

Previous studies of KDAC expression in beta cells have been limited by incompleteness, demonstrating only the presence of a limited number of KDACs in insulinoma cells by immunoblotting [25], or by the use of microarrays for hypothesis generation without real-time PCR verification or protein validation [26]. In the latter study, IL-1β + IFNγ exposure of beta cells (6 and 24 h) decreased expression of *Hdac1*, *Hdac2*, *Hdac3* and *Hdac6*, whereas *Hdac4*, *Hdac5* and *Hdac10* were unaffected, in agreement with our observations. Finally, to the best of our knowledge there are no reports of KDAC expression in human islets. The importance of the present report is: (1) the comprehensive combination of basal expression profiling and mapping of the cytokine-induced expression signature; (2) the demonstration of how this is regulated by KDAC inhibitors; and (3) confirmation of expression and regulation of four KDACs in human islets.

The molecular pathways by which cytokines regulate KDAC expression in beta cells remain to be examined. In non-beta cells, information about the regulation of KDAC expression is limited. *HDAC1* and *HDAC6* were expressed at higher levels in pancreatic exocrine tumour cells than in non-transformed acinar cells [36]. The *Hdac1* promoter lacks a TATA box consensus site, but has *cis*-binding sites for the transcription factors specificity protein-1 and nuclear transcription factor-Y. HDAC1 is recruited to its own promoter through these two transcription factors, consequently repressing its own transcription, an effect reversed by the non-selective KDAC inhibitor trichostatin A or by overexpression of KATs [37]. Furthermore, knockdown of HDAC1 results in a compensatory increase of HDAC2 [38]. Bioinformatically, a *cis*-element has been assigned to the *HDAC2* promoter, but functional studies are lacking [39]. Little is known about the expressional regulation of *HDAC6* and *HDAC11*. Interestingly, HDAC6 protein levels but not mRNA expression depend on c-Jun N-terminal kinase 1 activity [40], and furthermore, *Hdac11* expression increases upon cell maturation in oligodendrocytes [23].

In non-beta cells, the expression of *Hdac1* and *Hdac6* has been found to be increased upon non-selective KDAC inhibitor exposure [32]. We were not able to reproduce this finding, possibly due to the use of a different experimental model (neuroblastoma cell line vs INS-1 cell line) and a

different non-selective KDAC inhibitor in non-comparable concentrations (trichostatin A 0.5 μmol/l vs ITF2357 62.5 and 125 nmol/l). We found an apparent discrepancy in cytokine-induced *Hdac11* mRNA and protein regulation at 24 h. This may be explained by microRNA-mediated repression of translation and a high protein turnover rate. In support of this, cytokines have recently been shown to upregulate target microRNAs in beta cells [41]. Results from cytokine-induced expression of *HDAC11* mRNA and protein between human islets and the rodent models were inconsistent, probably due to species differences in cytokine sensitivity and/or the use of different cytokine combinations.

We recently reported that the KDAC inhibitors SAHA and trichostatin A markedly reduced cytokine-induced beta cell functional failure and death in vitro, a finding confirmed by others [12, 25, 42]. Here we show that ITF2357 abrogated beta cell toxicity as measured by iNOS levels, nitric oxide formation, cleavage of caspase-3 and apoptosis, and reduced cytokine-mediated suppression of accumulated insulin release, in line with the previous studies. Interestingly, ITF2357 in combination with cytokines increased insulin release compared with control. As ITF2357 alone had no effect on insulin release or apoptosis, the increased insulin response is likely to be independent of hyperacetylation or passive leakage of insulin due to toxicity of the inhibitor itself. We have also previously reported that the effect of KDAC inhibitors is not likely to be mediated through an increase in insulin exocytosis [12]. Cytokines at low concentrations have been shown to stimulate insulin secretion in rat islets [43] in a manner dependent upon protein kinase C signalling [44]. It is therefore possible that KDAC inhibitors prevent proapoptotic cytokine signalling, for example via NFκB, mitogen-activated protein kinase and signal transducer and activator of transcription 1, but that they spare regulatory pathways affecting insulin secretion. The mechanism explaining this novel action of KDAC inhibitors is unknown and should be investigated further.

Class IIa KDACs (HDAC4, -5, -7 and -9) are unlikely to be mediators of cytokine-induced beta cell death, as SAHA, a selective inhibitor of class I, IIb and IV [16], abrogates cytokine-induced toxicity [12]. The low abundance of class IIa KDACs in the beta cell, described in this study, further supports this notion. The transcription factor NFκB is a central mediator of cytokine signalling in the beta cell [13] and KDAC inhibitors reduce cytokine-induced NFκB-dependent inhibitor protein kappa beta alpha and iNOS production at the protein level in beta [12, 25] and in non-beta cells [45, 46], suggesting that deactivation of NFκB is a crucial step in the mechanism of KDAC inhibition of cytokine signalling in beta cells.

In conclusion, all classical KDACs were found to be expressed by insulin-producing cells and several were regulated by inflammatory cytokines. Interestingly, KDAC inhibitors reversed cytokine-induced inhibition of *Hdac2* and *Hdac6* expression, suggesting an intricate interplay between KDACs and cytokines in beta cells. Further research into the mechanisms of action and importance of the KDAC subtypes in vitro and in vivo is required to judge the relevance of these finding for the development of novel glucose-lowering drugs based on these observations.

Acknowledgements We thank G. K. Singh and H. Fjordvang for excellent technical assistance. This work was supported by grants from the Juvenile Diabetes Research Foundation (grant number 26-2008-893), the Novo Nordisk Foundation and Oticon.

Duality of interest P. Mascagni is employed by Italfarmaco and C. A. Dinarello has received consultancy fees from Italfarmaco. All other authors declare that there is no duality of interest associated with this manuscript.

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