HDAC Inhibitor-Mediated Beta-Cell Protection Against Cytokine-Induced Toxicity Is STAT1 Tyr701 Phosphorylation Independent

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Histone deacetylase (HDAC) inhibition protects pancreatic beta-cells against apoptosis induced by the combination of the proinflammatory cytokines interleukin (IL)-1 β and interferon (IFN)- γ . Decreased expression of cell damage-related genes is observed on the transcriptional level upon HDAC inhibition using either IL-1 β or IFN- γ alone. Whereas HDAC inhibition has been shown to regulate NF κ B-activity, related primarily to IL-1 β signaling, it is unknown whether the inhibition of HDACs affect IFN- γ signaling in beta-cells. Further, in non-beta-cells, there is a dispute whether HDAC inhibition regulates IFN- γ signaling at the level of STAT1 Tyr701 phosphorylation. Using different small molecule HDAC inhibitors with varying class selectivity, INS-1E wild type and stable HDAC1-3 knockdown pancreatic INS-1 cell lines, we show that IFN- γ -induced *Cxcl9* and *iNos* expression as well as *Cxcl9* and GAS reporter activity were decreased by HDAC inhibition in a STAT1 Tyr701 phosphorylation-independent fashion. In fact, knockdown of HDAC1 increased IFN- γ -induced STAT1 phosphorylation.

Introduction

THE PROINFLAMMATORY CYTOKINES interleukin (IL)-1 β and interferon (IFN)- γ induce apoptosis in pancreatic beta-cells, which is believed to contribute to the loss of functional beta-cell mass in both type 1 and type 2 diabetes mellitus (Donath and others 2008; Mandrup-Poulsen and others 2010). IL-1 β signaling is relayed mainly through the mitogen-activated protein kinases (MAPKs) and the transcription factor nuclear factor (NF) κ B (Donath and others 2008), whereas IFN- γ predominantly signals through the signal transducer and activator of transcription (STAT)1 pathway. Binding of dimerized IFN- γ to the IFN receptor causes receptor dimerization and activation of Janus kinase (JAK) 1/2, which subsequently phosphorylates STAT1 at Tyr701. This phosphorylation results in STAT1 dimerization and translocation to the nucleus and induction of gene expression through binding to IFN- γ -activated sites (GAS) and IFN-stimulated response elements (ISRE) in promoter regions of IFN-controlled genes (Platanias 2005). IL-1 β and IFN- γ signaling may converge at the promoter of genes having both GAS/ISRE and NF κ B-binding sites, leading to a potentiation of gene expression (Burke and others 2013a),

thus explaining the synergy in beta-cell toxicity of the combination of these cytokines. Of note, IFN- γ also signals through the MAP kinase ERK1/2 and regulates gene expression independently of STAT1 (Ramana and others 2002).

We have previously shown that the proapoptotic actions on beta-cell lines and isolated murine and human islets of the cytokine combination IL-1 β and IFN- γ with or without the addition of tumor necrosis factor (TNF)- α can be prevented by inhibiting the classical Zn²⁺-dependent histone deacetylases (HDACs) (Larsen and others 2007; Lundh and others 2010, 2012; Chou and others 2012; Christensen and others 2014). Mechanistically, HDAC inhibition reduced the transcription of inflammatory genes possibly through decreased NF κ B DNA binding, and HDAC3 was found to regulate NF κ B in INS-1 cells. Production of iNos and IkB α protein, both encoded by genes dependent on NF κ B-activity, was also reduced. In addition, HDAC inhibition reduced IL-1 β -mediated self-induction in beta-cells (Dahllöf and others 2012). Collectively, these findings demonstrate that HDAC inhibition regulates the IL-1 β -NF κ B signaling pathway. Importantly, HDAC inhibition was also found to blunt IFN- γ -induced *Cxcl10* expression (Dahllöf and others 2012), indicating a role for HDACs in IFN- γ signaling. However, it

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is unknown where HDAC activity exerts its role in the IFN- γ signaling cascade.

Inhibition of the class I HDACs. HDAC1-3 has been associated with decreased STAT1 activity through regulation of JAK1 phosphorylation (Klampfer 2004) as well as regulation of Tyr701 STAT1 phosphorylation (Krämer and others 2009). Mechanistically, it has been suggested that acetylated STAT1 (presumably on K410 and K413) forms complex with the phosphatase TCP45 causing a dephosphorylation (and thereby inactivation) of STAT1 at Tyr701 (Ginter and others 2012). Accordingly, preincubation, but not coincubation with nonselective HDAC inhibitors led to increased STAT1 acetylation, thereby blocking IFN- γ -induced STAT1 phosphorylation on Tyr701 subsequently reducing STAT1 transcriptional activity (Krämer and others 2009; Ginter and others 2012). Furthermore, knockdown of HDAC3 led to normalization of IFN- α -induced Tyr701 STAT1 phosphorylation. However, these data have been questioned, even with higher concentrations of HDAC inhibitors (Antunes and others 2011).

Given the importance of STAT1 signaling in cytokine-induced beta-cell death (Moore and others 2011), the ability of HDAC inhibition to reduce IFN- γ -induced *Cxcl10* expression (Dahllöf and others 2012) and the debated role of HDAC inhibition in phosphorylation and activation of STAT1, we investigated whether HDACs regulate Tyr701 STAT1 phosphorylation in pancreatic beta-cell lines, a highly sensitive target of IFN- γ -action and synergy.

Materials and Methods

Cells and reagents

INS-1 and INS-1E (kindly provided by P. Maechler and C. Wollheim, University of Geneva, Switzerland) were maintained in a complete medium as described previously (Lundh and others 2010; Chou and others 2012). For INS-1 experiments, rat IFN- γ from R&D Systems was used. For INS-1E experiments, rat IL-1 β and mouse TNF- α were purchased from R&D Systems and mouse IFN- γ from Sigma. Antibodies for pSTAT (Tyr701, #9171), STAT1(#9172), and secondary horseradish-peroxidase-conjugated goat anti-mouse and anti-rabbit antibodies were from Cell Signaling. Acetyl-Histone H3(#DAM1655257) antibody was from Millipore and beta-actin antibody from Sigma or Abcam. HDAC inhibitors were from Selleck Chemicals and BRD0476 was synthesized in-house as previously described (Chou and others 2011).

Real-time PCR

The analyzed RNA originates from our previous study (Dahllöf and others 2012). In short, INS-1 cells were seeded in 6-well plates in 2 mL complete medium. After 2 days, the medium was removed and cells were preincubated with givinostat or vehicle for 1 h. Subsequently, cells were exposed to IFN- γ or vehicle for the indicated time periods. Total RNA was harvested, converted to cDNA, and analyzed on a 7900HT Real-Time PCR machine (Applied Biosystems) using the TaqMan probes (*iNos*) or SYBR Green (*Cxcl9*) (Applied Biosystems). Each condition was analyzed in triplicates for *iNos* (probe ID: Rn00561646_m1) and *Cxcl9* (forward and reverse primers: CAAGGCACAT

TCCACTACAA and CCTTGCTGAATCTGGGTCTA) and a reference gene (*Hprt1* probe ID: Rn01428093_m1; forward and reverse primers: GCAGACTTTGCTTTCTCTGG and CCGCTGTCTTTTAGGCTTTG). The reactions were amplified in the TaqMan Gene Expression or SYBR Green Master Mix (Applied Biosystems).

Immunoblotting

One million INS-1/INS-1E cells were seeded in 6-wells (NUNC) in 2 mL media. After 2 days, media were changed and cytokines and small molecules added for the indicated time periods. INS-1E cells were lysed in the Passive lysis buffer (Promega). Total protein was adjusted for protein concentration, separated by 4%–12% SDS-PAGE and transferred to a PVDF membrane. Blots were developed using the chemiluminescence detection system SuperSignal (Thermo Fisher Scientific), and light emission was captured using an Imaging Station 4000MM (Carestream). INS-1 cells were lysed, adjusted for protein concentration, blotted, and developed as previously described (Lundh and others 2010). Quantifications were performed using ImageJ (<http://rsbweb.nih.gov/ij/>).

Immunoprecipitation

Ten million INS-1 cells were seeded in 10 cm dishes (NUNC) in 10 mL medium. After 3 days, media were changed to 5 mL and givinostat (1 h preincubation) and IFN- γ were added. Cells were washed twice in ice-cold PBS and lysed (HEPES [50 mM], NaCl [150 mM], EDTA [10 mM], Na4P2O7 [10 mM], NaF [100 mM], adjusted to pH 7.5. Triton [1%], Na3VO4 [2 mM], and Protease Inhibitors [Sigma-FAST, Sigma] were added before lysis). Total protein was adjusted for protein concentration and equal amounts of protein (400 μ g) subjected to IP against total STAT1 (5 μ L, #9172) and incubated at 4°C overnight. Dynabeads Protein G (Life Technologies) were washed 3 times in IP buffer and added to IP reaction for 1 h at 4°C. Beads were washed 3 times with IP buffer before adding 6 $\times$ SDS/PAGE loading buffer and heated to 95°C for 5 min. Samples were subjected to immunoblotting as described. Immunoblotting against acetylated lysines (Christensen and others 2014) was done with a 1:500 antibody solution.

Promoter reporter assays

The *Cxcl9* reporter construct (fragment –1319/+73, Gene Association NM_145672), containing the promoter reporter Gaussia Luciferase (GLuc) and Secreted Alkaline Phosphatase (SEAP) as internal control, was purchased from Genecopoeia. The GAS reporter, a kind gift from Professor Decio L. Eizirik (Moore and others 2011), was cotransfected (Eugene 6 Transfection Reagent, #11 988 387 001; Roche) with pGL4.74[hRLuc/TK] Vector (#E692A; Promega). Transfected cells were exposed to IFN- γ (1.3 ng/mL) alone or in combination with IL-1 β (150 pg/mL) for 6 h. Luciferase and SEAP signals were measured according to the manufacturer's instructions with the Secrete-Pair Dual Luminescence Assay Kit (#SPDA-D010; Genecopoeia) or the Dual Luciferase Reporter Assay System (#E1910; Promega). *Cxcl9* reporter signal was normalized to SEAP signal and GAS reporter signal was normalized to hRLuc/TK signal for each individual well.

Statistical analysis

Comparisons between groups were carried out either by ANOVA followed by a Tukey-corrected *post-hoc* Student's *t*-test or Student's *t*-test when appropriate. Data were analyzed using either the GraphPad Prism version 6 or SAS 9.1. *P* values less than 0.05 were considered statistically significant.

Results

HDAC inhibition reduces time and dose-dependent IFN- γ -induced *Cxcl9* and *iNos* expression

To study the effect of HDAC inhibition on IFN- γ signaling, we investigated the expression of the monokine induced by IFN- γ (*Mig/Cxcl9*) and *iNos* in INS-1 cells exposed to IFN- γ at 3 time points, with and without the nonselective HDAC inhibitor givinostat (ITF2357) at 125 nM, a dose previously demonstrated to reduce cytokine-induced apoptosis (Lundh and others 2010). Both 0.1 and 1.3 ng/mL of IFN- γ induced the expression of *Cxcl9* and *iNos* after 3 and 6 h, and this induction was significantly reduced by HDAC inhibition (Fig. 1). Although we did not detect a significant increase in NO production after 6 h (data not shown), the HDAC inhibition-mediated regulation of *Cxcl9* and *iNos* expression provides evidence to indicate HDAC regulation of the IFN- γ signaling pathways.

HDAC inhibition reduces cytokine-induced *Cxcl9* and GAS reporter activity

To further investigate the mechanism underlying HDAC inhibition of IFN- γ -driven gene expression, we assessed the ability of givinostat to reduce STAT1-dependent gene transcription through two different promoter reporter assays. As shown in Fig. 2A, givinostat reduced the activity of the *Cxcl9* promoter construct (rat *Cxcl9* promoter -1394/+73), most pronounced when a combination of IFN- γ and IL-1 β was used. An *in silico* analysis of the rat *Cxcl9* promoter sequence (Table 1) used in this assay revealed several binding sites for both STAT1 and NF κ B (mainly driven by IL-1 β), in accordance with the human *Cxcl9* promoter sequence (Ellis and others 2010) indicating that *Cxcl9* is regulated by STAT1 and NF κ B. To isolate the role of HDAC inhibition of IFN- γ -STAT1 signaling, we tested whether givinostat would affect a known STAT1 reporter containing 3 GAS consensus sequences (Moore and others 2011). Figure 2B shows that this was indeed the case and, together with Fig. 2A, suggests that the reduction of IFN- γ -induced gene expression shown in Fig. 1 is likely through a reduction in STAT1-driven transcriptional activity.

The nonselective HDAC inhibitor givinostat does not prevent IFN- γ -induced Tyr701 STAT1 phosphorylation

Since HDACs have been suggested to regulate STAT1 phosphorylation by deacetylating 2 specific lysine residues (Krämer and others 2009; Ginter and others 2012), we decided to investigate whether HDAC inhibition could reduce IFN- γ -induced Tyr701 phosphorylation in INS-1 cells. First, we determined that Tyr701 STAT1 phosphorylation increased after 15-min treatment with either 0.1 ng/mL or

FIG. 1. Interferon (IFN)- γ -induced *Cxcl9* and *iNos* mRNA expression is reduced by histone deacetylase (HDAC) inhibition. INS-1 cells were preincubated with givinostat (125 nM) or vehicle (1 h) before exposure to IFN- γ at either a low (0.1 ng/mL) or high (1.3 ng/mL) concentration. Cells were lysed at the indicated times and the expression of *Cxcl9* or *iNos* mRNA was quantified by qPCR. Data are presented as fold to CTRL + SEM, *n* = 6, analyzed with *t*-test (A) or ANOVA followed by a *post hoc t*-test (B), *P < 0.05 versus CTRL, #*P* < 0.05 versus the respective IFN- γ condition.

1.3 ng/mL of IFN- γ (Fig. 3). Thus, for further experiments, 15- or 30-min exposures to IFN- γ (1.3 ng/mL) were preferred. Next, we assessed the ability of different HDAC inhibitors to reduce this Tyr701 STAT1 phosphorylation. INS-1 cells were preincubated for 1 h with givinostat, followed by exposure to 1.3 ng/mL IFN- γ for 15 or 30 min. Givinostat failed to reduce IFN- γ -induced Tyr701 phosphorylation at either 15 or 30 min (Fig. 4A) or after 6 h (Fig. 4B). Of note, the level of total STAT1 was not affected by the experimental conditions (Supplementary Fig. S1; Supplementary Data are available online at www.liebertpub.com/jir).

Class I selective HDAC inhibitors neither affect cytokine-induced Tyr701 phosphorylation nor acetylation status of STAT1

Givinostat is a nonselective HDAC inhibitor, and we have shown that knockdown of HDAC1 or HDAC 3 reduces cytokine-induced beta-cell death (Chou and others 2012; Lundh and others 2012). Furthermore, Krämer and others (2009) found HDAC3 to mediate the purported phosphorylation-acetylation switch of STAT1 Tyr701. Therefore, we wished to investigate whether inhibition of class I HDACs

FIG. 2. HDAC inhibition reduces cytokine-induced Cxcl9 and GAS promoter activity. INS-1 cells were transfected with either a Cxcl9 (A) or a GAS (B) reporter plasmid. Transfected cells were preincubated with givinostat (125 nM) or vehicle (1 h) before exposure to IFN- γ (1.3 ng/mL) or IFN- γ (1.3 ng/mL) and IL-1 β (150 pg/mL). Luciferase and SEAP signals were measured after 6 h. Data are presented as fold to CTRL + SEM, $n=3-5$, analyzed with ANOVA with *post hoc* *t*-tests. *vs. CTRL, # vs. IFN γ +IL1 β .

reduced Tyr701 STAT phosphorylation. However, neither of the class 1 HDAC-selective inhibitors MS-275 and CI-994 reduced IFN- γ -induced Tyr701 STAT1 phosphorylation (data not shown) in INS-1 cells.

Since we have previously shown that MS-275 reduces cytokine (IL-1 β , IFN- γ , and TNF- α)-induced apoptosis in the INS-1E cell line (Chou and others 2012), we investigated this inhibitor further. Neither preincubation (1 h) nor coincubation with MS-275 affected cytokine-induced pSTAT1 levels after 30 min (Fig. 5A). Increasing the concentration of

FIG. 3. IFN- γ induces Tyr701 STAT1 phosphorylation in a time- and dose-dependent manner. INS-1 cells were exposed to IFN- γ at either a low (0.1 ng/mL) or high (1.3 ng/mL) concentration. Cells were lysed at the indicated times and the phosphorylation of Tyrosine701 on STAT1 was analyzed and quantified. Data are presented as fold to CTRL, $n=1$.

FIG. 4. Nonselective HDAC inhibition does not affect IFN- γ -induced Tyr701 STAT1 phosphorylation. INS-1 cells were preincubated (1 h) with givinostat (125 nM) before exposure to IFN- γ (1.3 ng/mL) for 15 or 30 min (A) or 360 min (B). Phosphorylation of Tyrosine701 on STAT1 was analyzed and quantified. Data are presented as fold to IFN- γ +SEM, $n=3$, analyzed with ANOVA followed by a *post hoc* *t*-test, NS, nonsignificant compared to IFN- γ alone. Representative blots are shown.

FIG. 5. The class-I selective HDAC inhibitor MS-275 does not affect cytokine-induced Tyr701 STAT1 phosphorylation. (A) INS-1E cells were either preincubated (1 h) with MS-275 (1 μ M) or coincubated (marked with*) with cytokines (IL-1 β , TNF- α , and IFN- γ) for 30 min, $n=4$. (B) Same setup as in (A), except a final concentration of MS-275 of 5 μ M, $n=3$. (C) INS-1E cells were preincubated (1 h) or coincubated (marked with*) with MS-275 (1 μ M) and exposed with cytokines (IL-1 β , TNF- α , and IFN- γ) for 3 h, $n=2$. (D) Same setup as in (A), except MS-275 (5 μ M), BRD0476 (10 μ M), $n=1$. (A-C) Data are presented as fold to IFN- γ +SEM, (A and B) were analyzed with Student's *t*-test, NS, nonsignificant. Representative blots are shown.

MS-275 5-fold (Fig. 5B) or prolonging IFN- γ exposure to 3 h (Fig. 5C) did not alter the results. To show that small molecules can indeed reduce the phosphorylation of Tyr701 in our model system, we applied the novel small molecule BRD0476, which we have shown previously to block STAT1 phosphorylation in beta cells (Scully and others 2013). As expected, BRD0476 blocked the phosphorylation of STAT1 (Fig. 5D). The action of HDAC inhibition had previously been detectable only after a preincubation period of 24 h (Krämer and others 2009). However, preincubation with ITF2357 or MS-275 for 24 h followed by IFN- γ exposure for 30 min did not affect the Tyr701 phosphorylation status (Fig. 6A).

To investigate the reported interplay between acetylated and phosphorylated STAT1, we examined the acetylation status of total STAT1 after 3 h of IFN- γ stimulation with or without preincubation with givinostat (Fig. 6B). Although we detected the phosphorylated form of STAT1 in our pull-down, we failed to detect any acetylation signal using the same acetyl-Lysine antibody as we previously used in pull-down experiments to show acetylation of NF κ B subunit p65 (Christensen and others 2014). This is in contrast to Krämer and others (2009) who showed an increase in IFN- α -stimulated STAT1 acetylation after 3 h, but in accordance with the results reported by Antunes and others (2011).

FIG. 6. HDAC inhibition does not affect IFN- γ -induced Tyr701 STAT1 phosphorylation or STAT1 acetylation, but knockdown of HDAC1 increases IFN- γ -induced Tyr701 STAT1 phosphorylation. (A) INS-1 cells were preincubated (24 h) with givinostat (125 nM) or MS-275 (1 μ M) before exposure to IFN- γ (1.3 ng/mL) for 30 min. (B) INS-1 cells were preincubated (1 h) with givinostat (125 nM) before exposure to IFN- γ (1.3 ng/mL) for 180 min before immunoprecipitation and immunoblotting were performed ($n=4$). (C) Stable INS-1 HDAC1, -2, or -3 knockdown clones [from ref. (Lundh 2012)] were exposed to IFN- γ (1.3 ng/mL) for 30 min. Data are presented as fold change over IFN- γ + SEM, $n=3$ (A) or $n=2-5$ (C), analyzed by ANOVA followed by *post-hoc* *t*-test, * $P<0.01$, NS, nonsignificant. Representative blots are shown.

Knockdown of HDAC1, -2, or -3 does not affect IFN- γ -induced Tyr701 phosphorylation

To further investigate the specific roles of HDAC1-3, we assessed Tyr701 phosphorylation in HDAC1-3 stable knockdown INS-1 cell lines [described in (Lundh and others 2012)]. In line with the previous results, knockdown of HDAC1, HDAC-2, or HDAC-3 did not affect the phosphorylation of STAT1 Tyr701 upon IFN- γ exposure (Fig. 6C). Surprisingly, knockdown of HDAC1 led to an increase in IFN- γ -induced Tyr701 phosphorylation. These results substantiate the finding that preincubation with HDAC inhibitors does not reduce STAT1 Tyr701 phosphorylation in beta cells.

Discussion

In this study, we show that inhibition of HDACs reduces IFN- γ -induced *Cxcl9* and *iNos* expression downstream of Tyr701 STAT1 phosphorylation by reducing STAT1 transcriptional activity.

Importantly, several studies implicate a role of HDACs in the regulation of STAT1 activity through its acetylation (Krämer and others 2006; Guo and others 2007; Krämer and others 2009; Ginter and others 2012). However, Krämer and others (2009) observed reduced STAT1 phosphorylation in response to HDAC inhibition (Ginter and others 2012), a thorough study by Antunes and others (2011) failed to reproduce these findings despite testing the same constructs, and testing both IFN- α and IFN- γ as well as several non-selective HDAC inhibitors, including VPA and TSA. In line with these results, we did not observe any effects of HDAC inhibition on Tyr701 STAT1 phosphorylation or STAT1 acetylation. Neither 24 h nor 1 h preincubations, nor coinubation with a nonselective HDAC inhibitor (givinostat), nor a class I HDAC inhibitor at different doses (MS-275) affected IFN- γ or cytokine-induced Tyr701 STAT1 phosphorylation. Furthermore, HDAC1-3 knockdown did not reduce IFN- γ -induced Tyr701 STAT1 phosphorylation. In fact, knockdown of HDAC1 increased the phosphorylation approximately 35%. The effect of HDAC inhibition on the level of STAT1 phosphorylation may be cell specific, explaining the lack of HDAC inhibitory effects on Tyr701 phosphorylation in our study. Further arguing for cell specific effects of HDAC inhibition on STAT1 posttranslational modifications, Guo and others (2007) observed an increase in Tyr701 phosphorylation and global STAT1 acetylation in response to HDAC inhibition, which reduced IFN- γ -induced *iNos* expression in RAW264.7 macrophages.

TABLE 1. PREDICTED STAT1 AND NF κ B BINDING SITES IN THE RAT *Cxcl9* PROXIMAL GENE PROMOTER

Sequence	TF	Position
CTGGGA	(STAT1)	-331 & -5
GGGTTTTCC	(NFkappaB)	-134
TCCCCA	(NFkappaB)	-128
AAGTGA	(IRF1/2)	-336

Putative transcription binding sites in the rat *Cxcl9* promoter (-1394/+73) were determined *in silico* using the TRANSFAC Public 6.0-Patch 1.0. Only scores with 100% similarity between reported binding and putative binding sites are shown.

Cxcl9 is an IFN- γ -driven gene (Brice and others 2001), whereas *iNos* is mainly dependent upon NF κ B (typically activated by IL-1 β) but potentiated by IFN- γ (Darville and Eizirik 1998). Although some studies failed to show *iNos* induction by IFN- γ alone (Darville and Eizirik 1998; Sekine and others 2000), these studies were performed using either reporter assay or northern blotting, which may not detect the low induction of *iNos* by IFN- γ observed in our study by qPCR. Burke and others (2013b) recently found a small, albeit statistically insignificant induction of *iNOS* mRNA in INS-1E cells and isolated rat islets of Langerhans. Accordingly, the relative expression of *iNos* we found (20–30-fold) is considerably lower than the expression induced by the combination of IL-1 β and IFN- γ in INS-1 cells (140,000-fold) (Lundh and others 2012), similar to the findings in INS-1E cells (Burke and others 2013b). In line with this observation, and in agreement with the previous results (Darville and Eizirik 1998), we did not detect a significant induction in NO production at the early time point of 6 h. Although the IFN- γ -driven induction of *iNos* shown here may not be physiologically relevant, it serves as a sensitive reporter of IFN- γ -signaling.

By applying a reporter construct containing the proximal rat *Cxcl9* promoter, we show that IL-1 β potentiates the effect of IFN- γ -driven activity and this is reduced by HDAC inhibition. Since the promoter contains several putative NF κ B-binding sites and HDAC inhibition is known to reduce NF κ B transcriptional activity in beta-cells (Christensen and others 2014), the reduction could partly be explained by effects on p65. Nonetheless, a reduction in promoter activity was also seen by HDAC inhibition when only IFN- γ was applied. Furthermore, to address the action of HDAC inhibition on isolated STAT1-signaling, we show that HDAC inhibition indeed reduced the activity of a STAT1 reporter containing 3 GAS sequences. Thus, the reduction in *Cxcl9* mRNA is likely an effect of reduced STAT1 transcriptional activity.

Mechanistically, we cannot exclude the possibility that the decrease in IFN- γ -induced gene expression by HDAC inhibition may be related to changes in histone acetylation patterns. However, Burke and others (2013a, 2013b) found IL-1 β to increase the recruitment of the histone acetyltransferases CBP and p300 to the *Cxcl10* and *iNos* promoter and an increase in H3 and H4 acetylation in the promoter correlating with increased expression. In this study, we show that HDAC inhibition also increases H3 acetylation (Fig. 6A) arguing for chromatin acetylation-independent effects of HDAC inhibition on gene transcription. In addition, while exogenous plasmid DNA does form nucleosome-like structures in eukaryotic cells (Mladenova 2009), the endogenous functional histone architecture surrounding the *Cxcl9* gene may not be found in exogenous reporter constructs.

Importantly, HDAC1–3 and 6 are known to be required for proper gene transcription. Along with HATs they are recruited to active gene promoter regions by RNA Pol II, where acetylation and deacetylation are essential for repeating gene transcription (Wang and others 2009). Thus, perturbation of this dynamic acetylation/deacetylation process by HDAC inhibition may explain the reduction in *Cxcl9* and *iNos* expression in our system as well as the reduction in reporter assay activities.

We have previously shown that HDAC inhibition causes a similar reduction in the IFN- γ -responsive gene *Cxcl10*

(Dahllöf and others 2012), substantiating a role of HDACs in the expression of IFN- γ -driven genes.

As in the case of *iNos* expression, the induction of *Cxcl10* by IFN- γ was lower compared to that induced by the combination of IL-1 β and IFN- γ (Dahllöf and others 2012; Christensen and others 2014). HDAC inhibition decreases IFN- γ - or IL-1 β -driven *Cxcl10* expression (Dahllöf and others 2012), but decreases only the earliest expression of *Cxcl10* driven by the combination of these cytokines (Christensen and others 2014), apparently because of the more robust signal provided by the combination.

Synergy between IL-1 β and IFN- γ in driving *Cxcl10* expression in insulin-producing cells was found previously (Burke and others 2013a). Although these authors did not report an induction of *Cxcl10* by IFN- γ alone in INS-1 832/13 cells, they did not assess this question in INS-1, INS-1E cells, or rat islets. Importantly, they found that the synergistic expression of *Cxcl10* induced by IL-1 β and IFN- γ was significantly reduced by the removal of an NF κ B-binding site in the *Cxcl10* promoter, and the potentiating effect of IFN- γ was diminished. Furthermore, they found that the presence of p65, STAT1, or Tyr701 STAT1 phosphorylation was required for full *Cxcl10* promoter activity.

We have previously shown that knockdown of HDAC3 protects insulin-producing cells and rat islets against IL-1 β and IFN- γ -induced apoptosis correlating with a decreased activity of p65 (Lundh and others 2012). Furthermore, we found HDAC inhibition to increase the acetylation level of p65, which was associated with a decreased binding of p65 to the *iNos* promoter and diminished *iNos* expression (Christensen and others 2014).

Interestingly, using the *Cxcl1* gene as a model, Burke and others (2014) recently provided evidence that IFN- γ -induced STAT1 Tyr701 phosphorylation causes STAT1 promoter binding, but only induced a robust gene expression when IL-1 β was present, indicating that STAT1-mediated transcription is also regulated downstream of STAT1-promoter binding.

The data provided in this study, together with previous studies, suggest that the gene expression reducing effect of HDAC inhibition is likely mediated at the transcriptional level through: 1) a reduction in NF κ B-activity in the IFN- γ -responsive genes *Cxcl9*, *Cxcl10*, and *iNos* in beta cells and 2) through a reduction in STAT1 transcriptional activity in gene promoters containing STAT1 binding sites as shown.

To summarize, we conclude that the beta-cell protective properties of HDAC inhibitors are partly mediated through a reduction in STAT1-transcriptional activity, but independent of STAT1 Tyr701 phosphorylation. We suggest the effects in this cell type to take place at the promoter level through a reduction in transcription factor-binding, for example, of NF κ B to cell damage-related genes, and perturbation of acetylation/deacetylation dynamics.

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Author Contributions

M.S.D., D.P.C. and M.L. designed, performed, and analyzed experiments and wrote the article. M.H. performed and analyzed experiments. B.K.W. and T.M.P. analyzed data and edited the article. All authors approved the final article. M.L. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Author Disclosure Statement

The authors declare no competing financial interest.

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