

Regulatory roles for histone deacetylation in IL-1 β -induced nitric oxide release in pancreatic β -cells

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Abstract

Histone (de)acetylases control gene transcription *via* modification of the chromatin structure. Herein, we investigated potential roles for histone deacetylation (or hypoacetylation) in interleukin-1 β (IL-1 β)-mediated inducible nitric oxide synthase (iNOS) and nitric oxide (NO) release in insulin-secreting INS 832/13 (INS) cells. Western blot analysis suggested localization of members of Class 1 and Class 2 families of histone deacetylases (HDACs) in these cells. Trichostatin A (TSA), a known inhibitor of HDACs, markedly reduced IL-1 β -mediated iNOS expression and NO release from these cells in a concentration-dependent manner. TSA also promoted hyperacetylation of histone H4 under conditions in which it inhibited IL-1 β -mediated effects on isolated β cells. Rottlerin, a known inhibitor of protein kinase C δ , also increased histone H4 acetylation, and inhibited IL-1 β -induced iNOS expression and NO release in these cells. It appears that the putative mechanism underlying the stimulatory effects of rottlerin on acetylation status of histone H4 are distinct from the HDAC inhibitory property of TSA, since rottlerin failed to inhibit HDAC activity in nuclear extracts isolated from INS cells. These data are suggestive of potential regulatory effects of rottlerin at the level of increasing the histone acetyltransferase activity in these cells. Together, our studies present the first evidence to suggest a PKC δ -mediated signalling step, which promotes hypoacetylation of candidate histones culminating in IL-1 β -induced metabolic dysfunction of the isolated β cell.

Keywords: apoptosis • β -cell • histones • histone deacetylases • iNOS, PKC δ

Introduction

Many of the biological processes, including the expression of inflammatory genes, DNA repair, and cell proliferation are governed by epigenetic modifications at the genomic level. Two key mechanisms regulating gene transcription are DNA methylation and histone acetylation. Histone acetyltransferases (HATs) and histone deacetylases (HDACs) represent two classes of enzymes, which control gene transcription by regulating the overall acetylation status of histones, thereby rendering modification of the chromatin structure for either activation or repression of

transcription. The HATs acetylate the ϵ -amino acid groups of lysine residues of histones. Addition of acetyl groups neutralizes the positive charges and increases the hydrophobicity of histones thereby reducing their affinity for DNA, and altering the nucleosome structure, which facilitates the binding of transcriptional factors. HDACs reverse the function of HATs by removing the acetyl groups from the acetylated histones. A growing body of recent evidence suggests that transcriptional dysregulation due to imbalances between the functional activation of HATs and HDACs affects cellular functions, including inflammatory responses during autoimmune diseases such as rheumatoid arthritis [1, 2], systemic lupus erythematosus [3] and diabetes [4].

Recent experimental evidence also suggests that several key cellular events in pancreatic islet β cells, such as controlling the transcription of the insulin gene, genes involved in glucose

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homeostasis and pancreatic differentiation genes, are under tight regulation by histone (de)acetylation [5–7]. These studies convincingly demonstrated that glucose-induced regulation of insulin gene expression involves hyperacetylation of histone H4 at the insulin gene promoter, which is carried out by the specific recruitment of p300, a HAT, in the presence of stimulatory glucose concentrations [6]. Available data also suggest that the β cell-specific transcription factor, pancreatic duodenal homeobox-1 and p300 interact with each other in isolated β cells *in vitro* and *in vivo* [8].

In the context of the current studies, it is well established that the islet β cell is susceptible to autoimmune aggression by immune-mediated cells, which secrete cytokines (*e.g.* interleukin-1 β [IL-1 β]) resulting in their demise *via* apoptotic mechanisms [9–12]. The cytotoxicity of IL-1 β is attributed primarily to the induction of iNOS and the subsequent generation of NO [13]. Several studies have indicated that iNOS expression is under the fine control of the transcription factor NF- κ B [14–18]. Recent studies in multiple cell types, including pancreatic β cells, demonstrated that NF- κ B-induced transcription is tightly regulated by the acetylation–deacetylation cycle of histones [19–23]. In this context, recent studies by Larsen *et al.* in islet β cells demonstrated that inhibition of HDACs by pharmacological intervention using trichostatin A (TSA) prevents cytokine-induced β -cell death [23]. They further demonstrated that such an effect of TSA is due to its ability to down-regulate the NF- κ B transactivating activity, thus inhibiting iNOS expression and subsequent NO production [23].

One important protein that is involved in the early signalling events of IL-1 β -mediated iNOS expression and NO release is the novel isoform of protein kinase C, PKC δ . Previously, Carpenter *et al.* [24] demonstrated that the activation of PKC δ is essential for the activation of NF- κ B. Such conclusions were drawn primarily using adenovirus-mediated over-expression of wild-type and kinase-dead mutant forms of PKC δ . These studies demonstrated that the kinase-dead mutant form of PKC δ significantly attenuated IL-1 β -stimulated iNOS protein expression and NO production, suggesting a key regulatory role for this kinase in IL-1 β -mediated effects on the islet β cell.

Based on this existing evidence, and as a logical extension to our published [25–28] and ongoing studies, we undertook the current investigation to determine the potential role of histone (de)acetylation in IL-1 β -induced iNOS expression and NO release in insulin-secreting INS 832/13 (INS) cells. We accomplished the above objective by two independent approaches; first, through the use of sodium butyrate (NaB) or TSA to impede the functional activation of candidate HDACs, and second, using rottlerin, a known inhibitor of PKC δ , to attenuate the PKC δ activation step in early steps of the signalling cascade, leading to iNOS expression and subsequent NO release. Our current findings implicate key regulatory roles for histone deacetylation (or hypoacetylation) in IL-1 β -mediated iNOS effects on insulin-secreting β cells.

Materials and methods

Materials

IL-1 β was purchased from R&D Systems (Minneapolis, MN, USA), Griess reagent, TSA, NaB and anti-actin serum were purchased from Sigma (St Louis, MO, USA). Cell viability assay (MTT) kit was purchased from Roche (Indianapolis, IN, USA). Affinity purified, monoclonal antibody directed against iNOS was purchased from Transduction Laboratories (Lexington, KY, USA). Rabbit polyclonal antibodies directed against total histone H4 or its acetylated form (acetyl-peptide corresponding to residues surrounding Lys8 of histone H4), and anti-sera against HDAC1, HDAC2, HDAC3, HDAC4, HDAC5, HDAC6 and HDAC7 were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). HDAC assay kit was purchased from Upstate Biotechnology (Lake Placid, NY, USA). Rottlerin was obtained from Biomol (Plymouth Meeting, PA, USA). Cycloheximide was obtained from Calbiochem (San Diego, CA, USA). Trizol, SuperScript III First-Strand Synthesis System, and primers for iNOS and 18S were from Invitrogen (Carlsbad, CA, USA) and SYBRgreen was obtained from Applied Biosystems (Foster City, CA, USA). NE-PER nuclear and cytoplasmic extraction kit was purchased from Pierce Biotechnology (Rockford, IL, USA).

Insulin-secreting cells and culture

INS 832/13 cells were kindly provided by Dr. Chris Newgard (Duke University Medical Center, Durham, NC, USA), and were cultured in RPMI 1640 medium supplemented with 1 mM sodium pyruvate, 50 μ M 2-mercaptoethanol and 10 mM HEPES at pH 7.4. The medium was changed twice weekly, and cells were trypsinized and subcloned weekly [29, 30]. HIT-T15 cells were purchased from American Tissue Culture Collection (Manassas, VA, USA) and MIN6 cells were kindly provided by Dr. John Hutton (University of Colorado Health Sciences Center, Denver, CO, USA).

Quantitation of IL-1 β -induced nitrite release

INS cells were cultured in 24-well plates (3×10^5 cells/well) for 2 days prior to various experimental manipulations (see text). The culture medium was then replaced with medium consisting of either diluent alone or inhibitors with or without IL-1 β . In experiments involving TSA and NaB, β cells were treated with inhibitors for different time periods (see later) prior to the addition of IL-1 β (600 pM; 24 hrs). The medium was collected after the incubation with IL-1 β and centrifuged at 100g for 5 min. Equal volumes of medium and Griess reagent were mixed, and the absorbance was measured at 540 nm as we previously described [25, 26, 28].

Quantitation of IL-1 β -induced iNOS expression

INS cells cultured in 24-well plates were treated with different inhibitors and the cell lysates were separated by SDS-PAGE and then resolved proteins were transferred to a nitrocellulose membrane by wet transfer as in [25, 26, 28]. Blots were then probed with either antibody raised against

iNOS or actin and further incubated with secondary antibody conjugated to horseradish peroxidase (HRP). Immune complexes were detected using an enhanced chemiluminescence (ECL) kit.

Identification of acetylated histones H4

INS cells cultured in 24-well plates were treated with either diluent alone or inhibitors as described in the text. Proteins were extracted in a buffer consisting of 20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β -glycerolphosphate, 1 mM Na_3VO_4 , 1 $\mu\text{g}/\text{ml}$ leupeptin and 1 mM PMSF. Extracted proteins from different conditions were separated by SDS-PAGE, and then resolved proteins were transferred to a nitrocellulose membrane by wet transfer as we described in [25, 26, 28]. Blots were then probed with an anti-serum raised against acetylated-histone H4. Immune complexes were detected using an ECL kit. The same membranes were further stripped and re-probed using rabbit polyclonal antibody raised against total histone H4.

Immunological localization of Class 1 and Class 2 HDACs in insulin-secreting cell lines

INS, HIT-T15 or MIN6 cells were homogenized in the buffer consisting of 20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β -glycerolphosphate, 1 mM Na_3VO_4 , 1 $\mu\text{g}/\text{ml}$ leupeptin and 1 mM PMSF. The lysates were separated by SDS-PAGE, and the resolved proteins were transferred to nitrocellulose membrane. The membranes were then blocked and incubated with anti-HDACs (HDAC 1, 2 and 3; Class 1, and HDAC 4, 5, 6 and 7; Class 2 at 1:1000 dilutions for 1 hr) followed by incubation with HRP-conjugated secondary antibody (1:1000 dilution) for 1 hr. Immune complexes were detected using an ECL kit. Alternatively, nuclear extracts from INS cells were prepared using the NE-PER nuclear and cytoplasmic extraction kit and proteins from those extracts were separated by SDS-PAGE, and the resolved proteins were transferred to nitrocellulose membrane and probed with antibodies raised against Class 1 HDACs (see later).

HDAC activity assays

Nuclear extracts from INS cells (prepared as described earlier) were used to quantitate HDAC activity using a kit from Upstate (Lake Placid, NY, USA) as per the manufacturer's recommendation. In brief, 20 μg of nuclear extracts from INS cells or HeLa cells (used as the positive control) were incubated in the presence or absence of TSA (200 nM) or rottlerin (5 μM) and acetylated substrate at 37°C for 60 min. allowing deacetylation of the substrate. Intensity of the colour developed due to the deacetylation of the substrate was measured at 405 nm in a micro-plate reader. The HDAC activity was calculated as percent of control activity in nuclear extracts prepared from the untreated cells.

Quantitation of relative abundance of IL-1 β -induced iNOS mRNA

INS cells were treated in the absence or presence of cycloheximide (50 μM) and/or TSA (200 nM) for 2 hrs prior to IL-1 β (600 pM) exposure for

an additional 6 hrs. Cells were then washed twice with PBS, and collected using Trizol. Following reconstitution of the RNA, the first-strand cDNA was synthesized using the SuperScript III First-Strand Synthesis System from invitrogen (Carlsbad, CA, USA). In brief, random hexamers, sample RNA and dNTPs were incubated at 65°C for 5 min. to allow annealing. cDNA synthesis mix was then prepared as per the manufacturer's instructions and was added to the sample RNA, which was then incubated at 50°C for 50 min., followed by incubation at 85°C to terminate the reaction. This cDNA was then further used for real-time PCR using SYBR green mix with primers for either 18S (forward sequence 5'-ACGGAAGGGCAC-CACCAGGA-3' and reverse sequence 5'-CACCACCACCCACGGAATCG-3') or iNOS [forward sequence 5'-AGACGCACAGGCAGAGGT' and reverse sequence 5'-GGCACACGCAATGATGG-3']. Abundance of iNOS mRNA was normalized to 18S mRNA and then expressed as fold increase over iNOS mRNA in control cells incubated in the absence of IL-1 β or inhibitors.

Metabolic cell viability determinations

INS cells were seeded at a density of 1×10^6 cells/ml in 96-well plates and then treated with diluent or TSA prior to the addition of IL-1 β . Cell viability was determined by a colourimetric assay (at 550–690 nm), using 3-[4,5-dimethylthiazolyl-2] 2,5-diphenyltetrazolium bromide (MTT), which measures the reduction of MTT into the blue formazan product by metabolically active cells.

Protein assay

Protein concentrations were determined by the dye-binding method of Bradford using bovine serum albumin as the standard [31].

Statistical analyses of data

Statistical significance of differences between the control and experimental groups was determined by ANOVA, and a P value less than 0.05 was considered to be significant.

Results

Localization of HDACs in insulin-secreting cells

Recent evidence from multiple laboratories appears to implicate a role for HDACs in iNOS expression and subsequent NO release during the inflammatory response induced by cytokines [19–23]. The current studies are undertaken to further examine the relative contributory roles for HDACs and histone acetylation in IL-1 β -induced iNOS expression and NO release in pancreatic β cells. At the outset, we determined the immunological localization of HDACs belonging to Class 1 and Class 2 in a variety of insulin-secreting cells, namely INS, HIT-T15 and MIN6 cells. Nuclear extracts from HeLa cells are also used in these experiments as

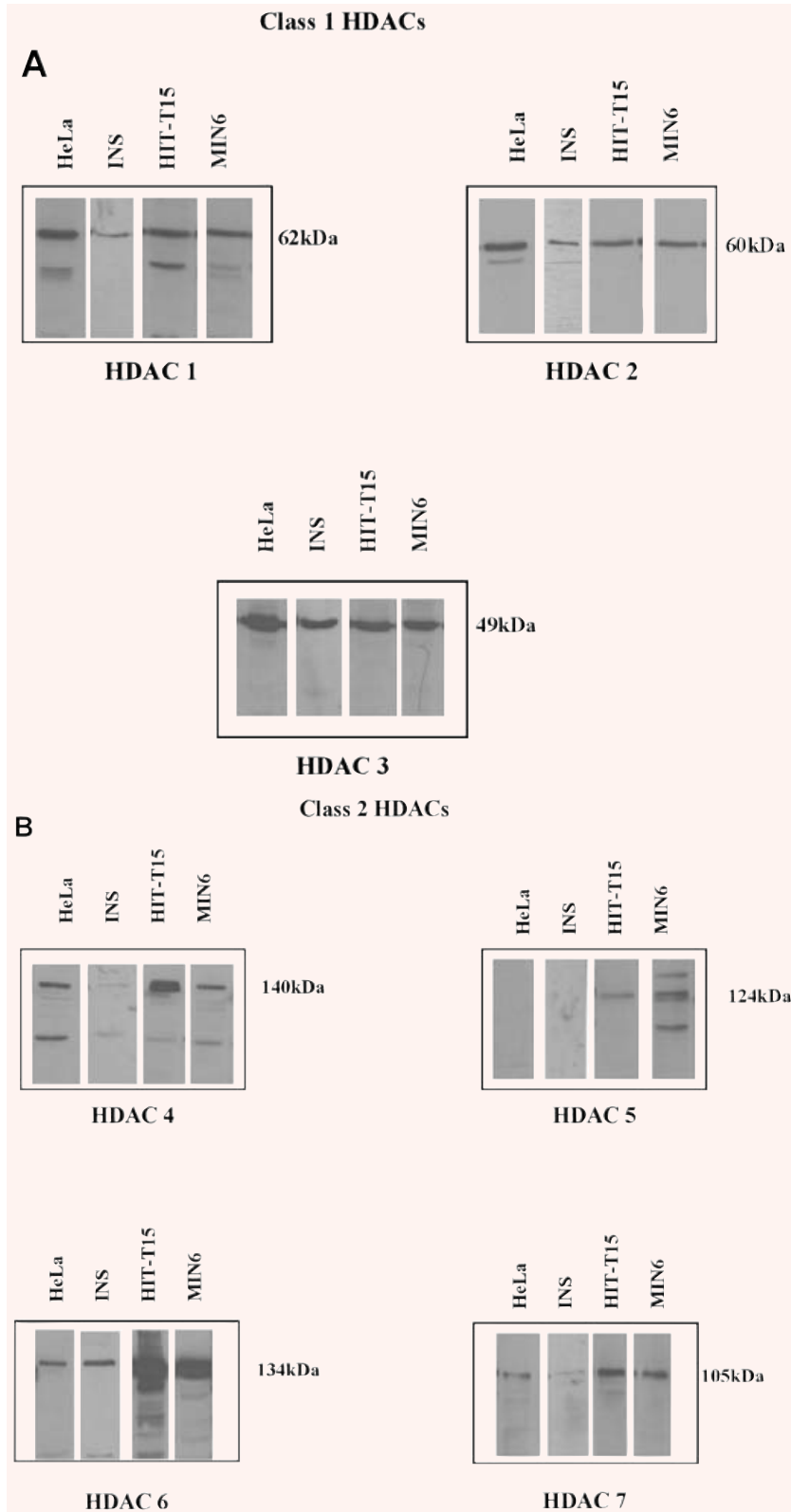
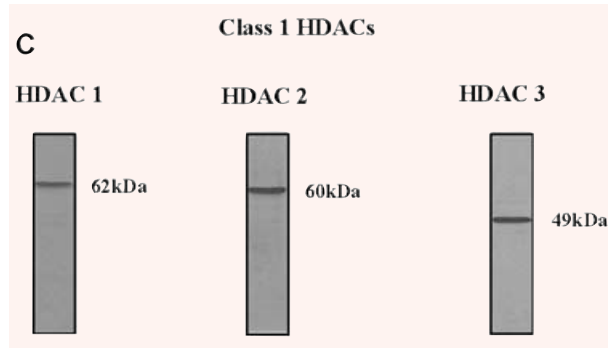


Fig. 1 Immunological localization of the Class 1 and the Class 2 HDACs in insulin-secreting cells. **Panel A:** INS, HIT-T15 or MIN6 cell lysates were separated by SDS-PAGE and transferred to a nitrocellulose membrane. The membranes were then blocked and incubated with anti-sera raised against HDAC 1, 2 or 3 (1:1000 dilutions; 1 hr) followed by incubation with HRP-conjugated secondary antibody (1:1000 dilution) for 1 hr. Immune complexes were detected using an ECL kit. A representative blot from two experiments yielding similar result is shown. (HeLa nuclear extract was used as a positive control in these experiments.) **Panel B:** INS, HIT-T15 or MIN6 cell were separated by SDS-PAGE and transferred to a nitrocellulose membrane. The membranes were then blocked and incubated with anti-sera directed against HDAC 4, 5, 6 or 7 (1:1000 dilutions; 1 hr) followed by incubation with HRP-conjugated secondary antibody (1:1000 dilution; 1 hr). Immune complexes were detected using an ECL kit. A representative blot from two experiments yielding similar result is shown. (As in Panel A, HeLa nuclear extract was used as a positive control in these studies.) **Panel C:** Nuclear extracts from INS cells were prepared using the NE-PER nuclear and cytoplasmic extraction kit (see Methods for additional details). Extracted proteins were separated by SDS-PAGE followed by transfer to a nitrocellulose membrane. The membranes were then blocked and incubated with antibodies raised against HDAC 1, 2 or 3 (1:1000 dilutions; 1 hr) followed by incubation with HRP-conjugated secondary antibody (1:1000 dilution; 1 hr). A representative blot from two experiments yielding similar result is shown.

Continued



positive controls. Data in Fig. 1 (panel A) suggest that all the members of the class 1 HDACs, namely HDAC1, 2 and 3 are localized in all the three insulin-secreting cells, albeit at varying degrees of abundance. Furthermore, data presented in Fig. 1 (panel B) also suggest localization of Class 2 HDACs, namely HDAC 4, 5, 6 and 7 in lysates derived from these cells. Interestingly, however, HDACs 4, 5 and 7 are less abundant in INS cells compared with HIT and MIN6 cells. We further examined localization of Class 1 HDACs in purified nuclear extracts isolated from INS cells (see Methods for additional details). Data in Fig. 1 (panel C) further support our data in Fig. 1 (panel A) to suggest immunological localization of these HDACs in nuclear extracts from INS cells. Intact INS cells and their nuclear extracts were used in all subsequent experiments described later.

HDAC inhibitors markedly attenuate IL-1 β -induced iNOS expression and in NO release in INS cells

Data in Fig. 2 (Panel A) demonstrate that sodium butyrate (NaB), a generic inhibitor of HDACs, modestly, but significantly, inhibited IL-1 β -induced NO release from these cells in a dose-dependent manner (0–1000 μ M). NaB elicited no significant effects on basal NO release (Fig. 2, panel A). NaB also inhibited IL-1 β -induced iNOS expression under the conditions in which it reduced NO release (Fig. 2 B). Together, these data demonstrate that IL-1 β -induced iNOS expression and NO release may require histone deacetylation.

We next examined the inhibitory potential of TSA, a more specific inhibitor of HDACs, on IL-1 β -induced NO release and iNOS expression in INS cells. Data in Fig. 3 (panel A) demonstrate that TSA potently attenuated IL-1 β -induced NO release in a concentration-dependent manner, without significantly affecting the basal NO release. Maximal inhibition was seen at 200 nM of TSA (Fig. 3, panel A, bar 2 *versus* bar 10). Compatible with these data are findings in Fig. 3 (panel B), which demonstrate a significant inhibition in IL-1 β -induced iNOS expression by TSA. Together, these data (Figs 2 and 3) implicate that a histone deacetylation (*i.e.* hypoacetylation) step might underlie the IL-1 β -mediated iNOS expression and NO release in pancreatic β cells.

Effects of TSA on IL-1 β -induced loss in metabolic cell viability in INS cells

As indicated earlier, TSA inhibition studies were carried out using a protocol in which INS cells were incubated with TSA for 18 hrs prior to the addition of IL-1 β . Previous data from multiple laboratories have shown that inhibition of HDACs by TSA increases the level of cell death in cancer cells [32–34] and down-regulates the pro-apoptotic pathways in models of autoimmune diseases [35]. To test whether the established inhibitory effects of IL-1 β on metabolic cell viability in isolated β cells are reversed by TSA, we quantitated metabolic cell viability (by MTT assay) in INS cells exposed to IL-1 β in the absence or presence of TSA. Data in Fig. 4 demonstrate a 20% reduction in metabolic viability of these cells following exposure to IL-1 β . Moreover, TSA, by itself, also exerted a significant decrease on the metabolic viability of the INS cells. These data are compatible with more recent findings by Larsen *et al.* [23], demonstrating a similar response (~34%) in basal apoptotic cell death in response to 200 nM of TSA alone. Together, these data warrant potential cytotoxic effects of TSA under prolonged incubation conditions, and therefore, we carried out further studies to determine its effects against IL-1 β -mediated effects following shorter incubation periods.

Protective effects of TSA against IL-1 β -induced NO release at shorter time points in INS cells

We next examined the inhibitory effects of TSA on IL-1 β -induced NO release following shorter exposure periods (*i.e.* 6–8 hrs); a time point at which IL-1 β has been shown to induce iNOS expression and NO release. Data in Fig. 5 demonstrate that IL-1 β -induced NO release was significantly attenuated by TSA as early as 6 hrs. TSA completely abolished IL-1 β -induced increase in NO levels following an 8-hrs incubation (Fig. 5). Taken together, our findings implicate the potential regulation of IL-1 β -induced iNOS expression and subsequent NO release by a histone deacetylation step. It is germane to point out that during this short-term incubation conditions (as above), we observed a decrease (~10%) in the metabolic viability of INS cells treated with IL-1 β alone (100.0 ± 0.00 *versus* 91.0 ± 1.60), when compared to INS cells treated with diluent alone. The metabolic viability of INS cells treated with TSA alone or with TSA and IL-1 β where similar to that of INS cells treated with IL-1 β alone (90.0 ± 1.84 *versus* 88.9 ± 1.90).

Effects of TSA on the relative abundance of IL-1 β -induced iNOS mRNA

In the next series of studies, we determined if the down-regulation of IL-1 β -induced iNOS expression by TSA involves direct histone deacetylation. We also examined if reduction in iNOS gene expression by TSA is an ‘immediate early response’

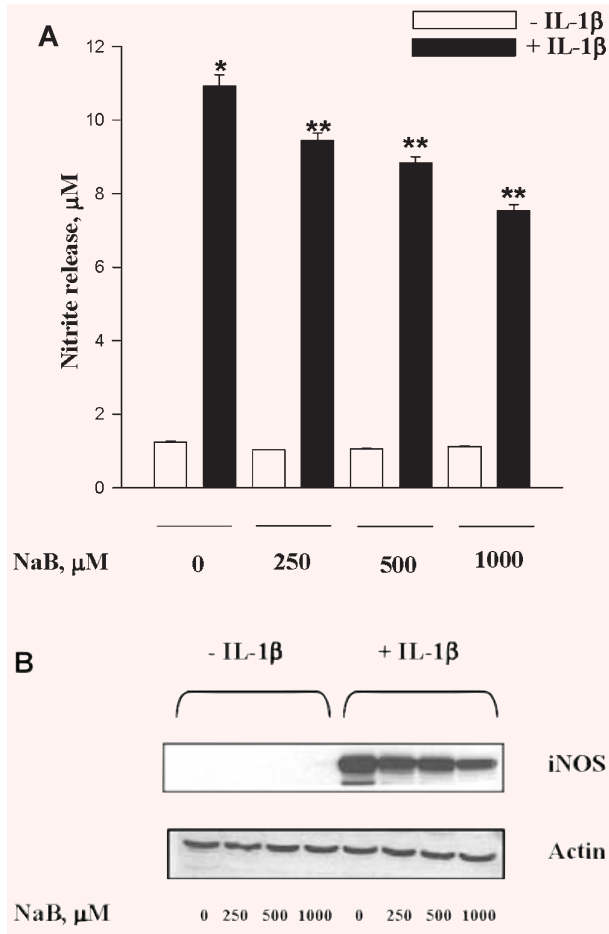


Fig. 2 Sodium butyrate attenuates IL-1 β -induced NO release and iNOS expression in pancreatic β -cells. Panel **A**: INS cells were incubated in the absence or presence of IL-1 β (600 pM; 24 hrs) as indicated. NaB (0–1000 μ M) was also present in the incubation medium (18 hrs prior to the addition of IL-1 β) as indicated in the figure. NO released into the medium was quantitated using Griess reagent. Data, which are expressed as NO released into the medium, are mean \pm SEM from three independent experiments carried out in triplicate. * P < 0.001 compared with basal NO release in the absence of IL-1 β . ** P < 0.001 compared to NO release in the presence of IL-1 β alone. Panel **B**: INS cell lysates incubated in the absence or presence of IL-1 β (600 pM; 24 hrs) and NaB (0–1 mM) were separated by SDS-PAGE, transferred onto nitrocellulose membranes and subjected to immunoblot analysis with an antibody directed against iNOS or actin as described under Methods. Data are representative of three separate experiments with comparable results.

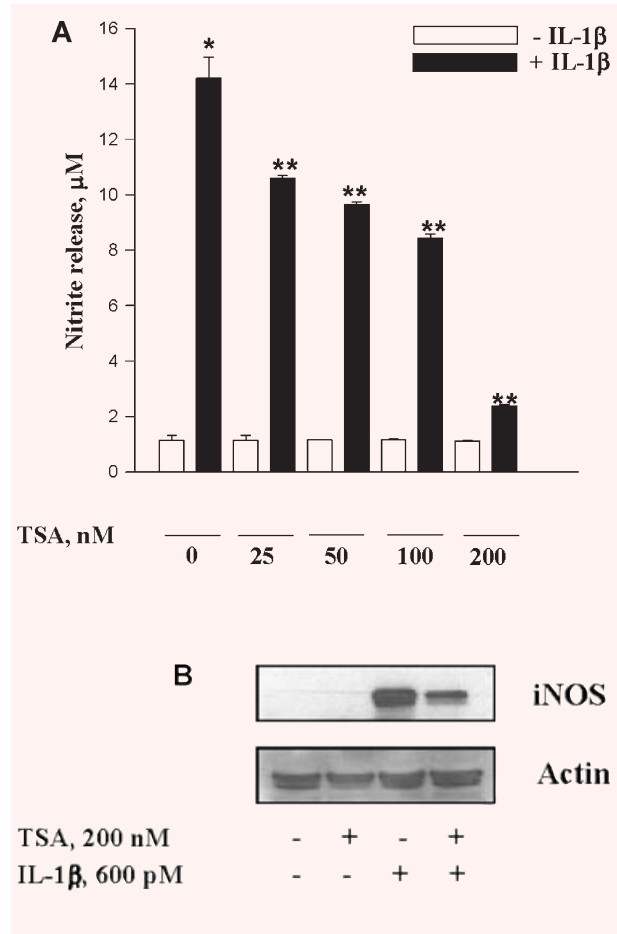


Fig. 3 TSA significantly attenuates IL-1 β -induced NO release and iNOS expression in INS cells. Panel **A**: INS cells were incubated in the absence or presence of IL-1 β (600 pM; 24 hrs) as indicated in the figure. TSA (0–200 nM) was also present in the incubation medium (18 hrs prior to the addition of IL-1 β) as indicated in the figure. NO released into the medium was quantitated using Griess reagent. Data, which are expressed as NO released into the medium, are mean \pm SEM from three independent experiments carried out in triplicate. * P < 0.001 compared with basal NO release in the absence of IL-1 β . ** P < 0.001 compared with NO release in the presence of IL-1 β alone. Panel **B**: INS cell lysates incubated in the absence or presence of IL-1 β (600 pM; 24 hrs), and TSA (200 nM) were separated by SDS-PAGE, transferred onto nitrocellulose membranes and subjected to immunoblot analysis with an antibody directed against iNOS or actin. A representative blot from three experiments yielding similar results is shown.

through the use of cycloheximide to prevent *de novo* protein synthesis. To test this, we quantitated the levels of iNOS mRNA in INS cells treated with or without IL-1 β (6 hrs) and in the presence of either cycloheximide or TSA (singly or in combination).

In these studies, as expected, exposure of INS cells to IL-1 β led to a significant increase in the abundance of iNOS mRNA (Table 1); data compatible with increased iNOS protein expression in the presence of IL-1 β are provided in Figs. 2–5 (control *versus*

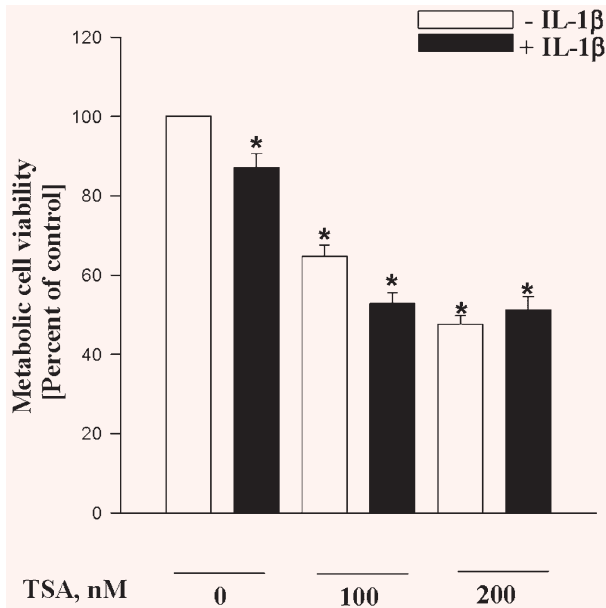


Fig. 4 TSA induces a modest loss in metabolic cell viability in INS cells. INS cells were incubated in the absence or presence of IL-1 β (600 pM; 24 hrs) as indicated in the figure. TSA (200 nM) was also present in the incubation medium as indicated in the figure. Metabolic cell viability was determined using MTT assay as described in Methods. These data indicated a modest, but significant inhibition in metabolic cell viability in INS cells. A similar degree of inhibition in metabolic cell viability was also demonstrable in these cells following incubation with IL-1 β . Loss in metabolic cell viability in these cells was not significantly different in the combined presence of IL-1 β and TSA. Data are expressed as percent of control [mean \pm SEM]. * P < 0.001 compared with control, untreated cells.

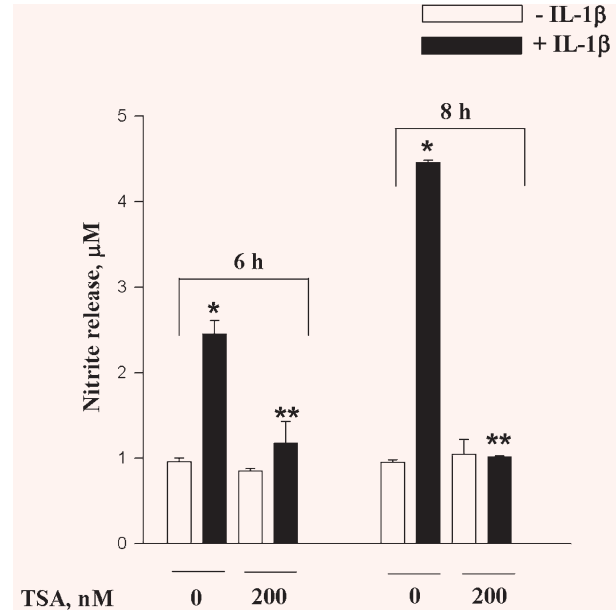


Fig. 5 TSA significantly attenuates IL-1 β -induced NO release during shorter incubation periods of exposure. INS cells were incubated in the absence or presence of IL-1 β (600 pM; 6 or 8 hrs) as indicated in the figure. TSA (200 nM) was also present in the incubation medium (2 hrs prior to the addition of IL-1 β). NO released into the medium was quantitated using Griess reagent. Data, which are expressed as NO released into the medium, were mean \pm SEM from three independent experiments carried out in triplicate. * P < 0.001 compared with basal NO release in the absence of IL-1 β . ** P < 0.001 compared with NO release in the presence of IL-1 β .

IL-1 β -treated). Under such conditions, TSA treatment markedly attenuated IL-1 β -induced iNOS mRNA levels (Table 1); data compatible with iNOS protein expression profiles are provided in Fig. 5. Together, these data provide evidence for a direct role for histone deacetylation (or hypoacetylation) in IL-1 β -mediated increase in iNOS expression (both at the mRNA and protein levels) and subsequent NO release (see above). We also noticed (Table 1) that cycloheximide-treatment completely abolished IL-1 β -induced iNOS mRNA levels within 6 hrs. The degree of inhibition seen in the presence of cycloheximide was much more pronounced compared to that seen in the presence of TSA alone (Table 1). For these reasons, it was not possible herein to study the ability of cycloheximide to block the down-regulation of iNOS mRNA induced by TSA (Table 1). Additional experiments, which examine the iNOS promoter directly using ChIP assay for acetylated histones (*e.g.* histone H4), will be needed to examine direct changes in the iNOS promoter. These studies are currently in progress.

Potential involvement of a PKC δ -dependent step on histone (de)acetylation in the β cell

A recent study by Yuan *et al.* [36] demonstrated a role for PKC δ in the inhibition of the HAT activity of the transcriptional coactivator CBP/p300 and further implicated a role for this signalling step in the inhibition of cell growth. Interestingly, in the context of IL-1 β -induced iNOS expression and NO release, Carpenter *et al.* [24] demonstrated that PKC δ plays a positive modulatory role in iNOS mRNA stability and cell death in islet β cells [24]. Based on these interesting findings and to further decipher potential cross-talk between these pathways, we determined the contributory roles for a PKC δ -mediated histone deacetylation step in the regulation of IL-1 β -induced iNOS expression and NO release in INS cells. For this purpose, we used rottlerin, a known inhibitor of PKC δ , to quantitate its effects on IL-1 β -induced iNOS expression and subsequent NO release in INS cells. Data in Fig. 6 (panel A) demonstrate that rottlerin significantly attenuated IL-1 β -induced NO release in a concentration-dependent manner without significantly affecting basal NO release; these findings are compatible with

Table 1 Relative abundance of IL-1 β -induced iNOS mRNA in INS cells treated with TSA and/or cycloheximide

Treatments	Fold increase in the relative abundance of iNOS mRNA
IL-1 β	125,027 \pm 4561
Cycloheximide	9.90 \pm 1.7
Cycloheximide + IL-1 β	43 \pm 7
TSA	2.43 \pm 0.7
TSA + IL-1 β	3385.24 \pm 115.8
Cycloheximide + TSA	6.04 \pm 1.1
Cycloheximide + TSA + IL-1 β	3.85 \pm 1.0

INS cells were incubated in the absence or presence of cycloheximide and/or TSA for 2 hrs prior to the addition of IL-1 β (see text for additional details). Cells were harvested following 6-hrs incubation with IL-1 β (600 pM). Total RNA was isolated using Trizol reagent, and abundance of iNOS mRNA was assessed by real-time RT-PCR (see Methods for additional details). Values shown are fold increase \pm SEM ($n = 3$ determinations) over iNOS mRNA in control cells incubated in the absence of IL-1 β and/or inhibitors ($P < 0.001$ versus IL-1 β for all the treatment groups).

observations from multiple laboratories [24, 37, 38]. Under these conditions, rottlerin also inhibited IL-1 β -induced iNOS expression (Fig. 6, panel B; ~36% and 51%, respectively, with 2.5 and 5 μ M rottlerin). Together, these data demonstrate that IL-1 β -induced iNOS expression and NO release require a PKC δ -dependent step.

Effects of TSA or rottlerin on the acetylation status of histone H4 in INS cells

Recent studies from the laboratory of Kone [20] have identified novel signalling pathways to further demonstrate potential roles for histone deacetylation in IL-1 β -induced iNOS expression and NO release in mesangial cells. They demonstrated that TSA attenuates IL-1 β -induced iNOS expression in these cells *via* phosphoinositide 3-kinase and p70s6 kinase-dependent signalling steps to increase site-specific histone H4 acetylation at the -978 to -710 region of the iNOS promoter [20]. With these observations in mind, in the next series of studies, we determined the acetylation status of histone H4, in cells treated with diluent alone or IL-1 β in the absence or presence of TSA and/or rottlerin. Data accrued from these studies are provided in Fig. 7, which demonstrate a marked stimulation in the relative abundance of acetylated histone H4 in cells treated with TSA (Fig. 7; bands in lane 1 *versus* lane 5). Furthermore, rottlerin alone induces a significant increase in the

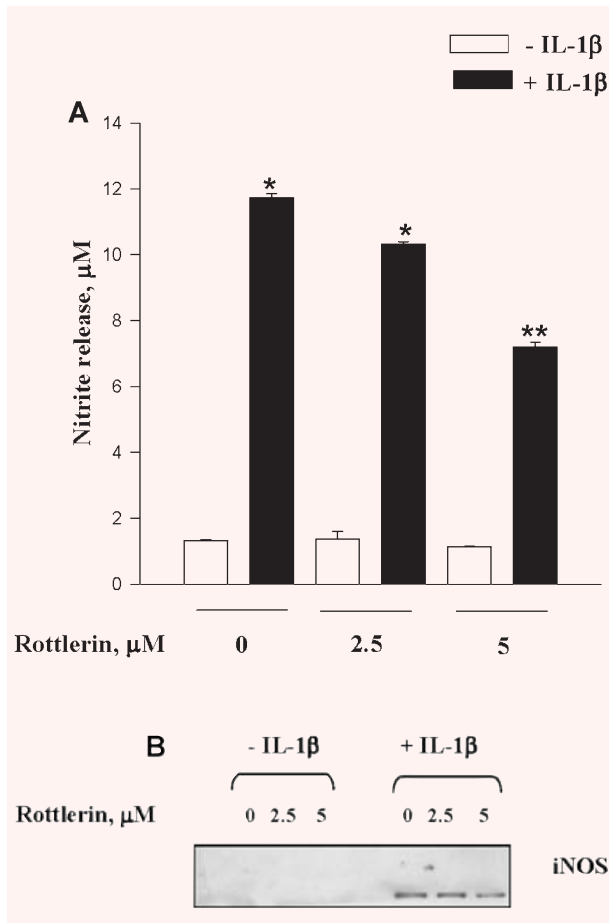


Fig. 6 Rottlerin significantly decreases IL-1 β -induced NO release and iNOS expression in INS cells. **Panel A:** INS cells were incubated in the absence or presence of IL-1 β (600 pM; 24 hrs) as indicated in the figure. Rottlerin (0–5 μ M) was also present in the incubation medium (30 min. prior to the addition of IL-1 β). NO released into the medium was quantitated using Griess reagent. Data, which are expressed as NO released into the medium, are mean \pm SEM from three independent experiments carried out in triplicate. * $P < 0.001$ compared with basal NO release in the absence of IL-1 β . ** $P < 0.001$ compared with NO release in the presence of IL-1 β alone. **Panel B:** Lysates from INS cells incubated in the absence or presence of IL-1 β (600 pM; 24 hrs) and rottlerin (0–5 μ M) were separated by SDS-PAGE, transferred onto nitrocellulose membranes and subjected to immunoblot analysis using an antibody directed against iNOS. Data are representative of three separate experiments with comparable results.

acetylation of histone H4 (lane 3), whereas cells treated with rottlerin and IL-1 β (lane 4) showed a further increase in acetylation of histone H4. Quantitative data from multiple experiments are provided in Fig. 7 (panel B). Also, please note that under these conditions, we observed inhibition of IL-1 β -induced NO release (Fig. 6, panel A, bar 2 *versus* bar 6). Together these data (Figs. 6 and 7)

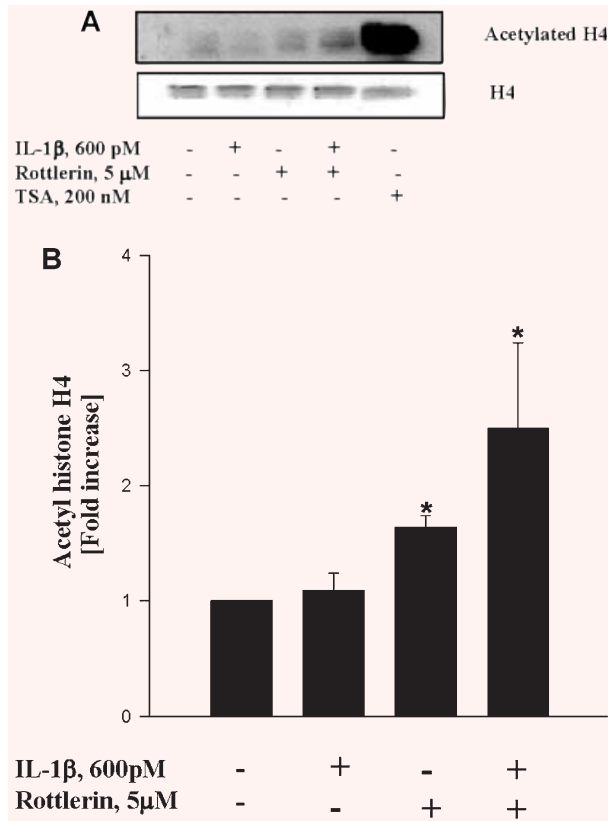


Fig. 7 Rottlerin significantly promotes hyperacetylation of histone H4 in INS cells. **Panel A:** INS cells were incubated in the absence or presence of IL-1 β (600 pM; 24 hrs) as indicated in the figure. Rottlerin (5 μ M) was also present in the incubation medium (30 min. prior to the addition of IL-1 β) as indicated in the figure. Lysates were separated by SDS-PAGE, transferred onto nitrocellulose membranes and subjected to immunoblot analysis with an antibody directed against acetylated histone H4 followed by incubation with HRP-conjugated secondary antibody for 1 hr. The same membrane was stripped and re-probed for total histone H4. Immune complexes were detected using an ECL kit. A representative blot from three experiments yielding similar results is shown. **Panel B:** Relative intensities of the protein bands were quantitated by densitometry. Data, which are represented as fold increase over control, untreated cells, are mean \pm SEM from three independent experiments. * P < 0.05 versus control untreated cells.

are suggestive of additional regulatory roles for PKC δ in IL-1 β -induced effects on islet β -cell metabolism leading to iNOS expression and NO release (see later). In the last set of experiments, as a logical extension to the findings described under Fig. 7, we determined if rottlerin acts as an HDAC inhibitor thereby increasing the acetylation status of specific histones (*e.g.* histone H4) in a manner akin to TSA.

Determination of potential HDAC inhibitory properties of rottlerin in INS cells

To test this, we quantitated HDAC activity in nuclear extracts purified from INS cells (as in Fig. 1, panel C) in the absence or presence of rottlerin or TSA. In addition, HDAC activity was quantitated in nuclear extracts from HeLa cells to determine if the effects of these inhibitors, specifically rottlerin, are global. The data in Fig. 8 demonstrate a marked inhibition of HDAC activity in INS cell nuclear extracts by TSA (200 nM). A comparable degree of inhibition of HDAC activity by TSA was also seen in HeLa nuclear extracts (Fig. 8, bar 4 *versus* 5). However, rottlerin (5 μ M) had no effect on HDAC activity in nuclear extracts from either cell preparation. Based on these findings, we conclude that the effects of rottlerin on histone H4 acetylation (Fig. 7) may be distinct from TSA. They might include, but are not limited to, its inhibitory effects on PKC δ -mediated inhibition of specific HATs, which in turn would retain histone H4 in a hyperacetylated state, thereby inhibiting the induction of iNOS in the presence of IL-1 β (see below).

Discussion

Several lines of evidence implicate chromatin structure re-modeling in gene transcription. One such structural modification of chromatin is through the acetylation of histones, the major protein components of chromatin. This type of modification facilitates the transcription of otherwise restricted genes. Several lines of recent evidence also suggest that a number of transcriptional factors are tightly regulated by histone modification [39–41], including NF- κ B [42–44], which promotes the induction of iNOS in multiple cells lines, including pancreatic β cells, in response to various inflammatory stimuli. However, the precise roles of the acetylation of histones and/or the regulation of acetylated histones in pancreatic β cells in the context of IL-1 β -induced iNOS expression and subsequent NO release still remain only partially understood [23].

In the present study, we attempted to further examine putative regulatory roles for histone acetylation–deacetylation steps in IL-1 β -induced NO release in isolated β cells. Our findings demonstrate that (i) members of Class 1 and Class 2 families of HDACs are localized in a variety of insulin-secreting cells, including INS cells, HIT-T15 cells and MIN6 cells; (ii) two structurally dissimilar inhibitors of HDAC (*e.g.* NaB and TSA) attenuated IL-1 β -induced iNOS expression and NO release; (iii) TSA elicits inhibitory effects on IL-1 β -induced iNOS mRNA levels (within 6 hrs of incubation) in these cells; (iv) in a manner similar to TSA, rottlerin, a specific inhibitor of PKC δ , also significantly attenuated IL-1 β -induced iNOS expression and NO release, while significantly augmenting histone H4 acetylation and (v) in contrast to TSA, rottlerin's effects on histone H4 acetylation are not due to its ability to inhibit HDAC activity in INS cells, at least in broken cell preparations. Based on

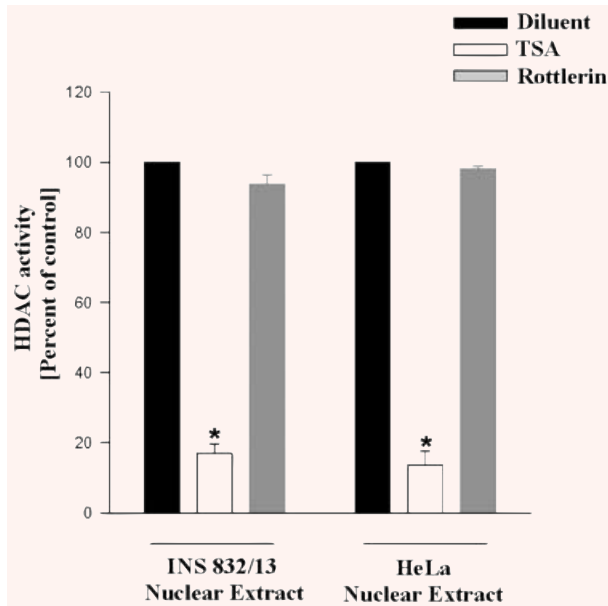


Fig. 8 TSA, but not rottlerin, significantly inhibits HDAC activity in INS cell nuclear extracts. Nuclear extracts from INS cells were prepared using the NE-PER nuclear and cytoplasmic extraction kit (see Methods for additional details). In brief, 20 µg of nuclear extracts from INS cells or HeLa cells (used as a positive control) were incubated in the presence or absence of TSA [200 nM] or rottlerin [5 µM], and the degree of deacetylation of the substrate was measured colourimetrically at 405 nm using a commercially available kit (see Methods for additional details). The data, which are expressed as percent of HDAC activity in untreated controls, are mean ± SEM from four independent experiments. **P* < 0.05 versus control untreated nuclear extracts purified from either INS or HeLa cells.

these findings, we conclude that histone deacetylation (or hypoacetylation) is favourable for IL-1β-induced metabolic effects on isolated β cells.

Previous studies from multiple laboratories have demonstrated potential roles for histone acetylation–deacetylation reactions in signalling steps leading to gene transcription [45–47]. Indeed, availability of specific pharmacological inhibitors enabled researchers to further assess the potential relevance of these steps in cellular function. For example, Van Lint *et al.* [48] demonstrated that TSA promotes acetylation of histones thereby affecting the expression of 2% of cellular genes. Our current studies demonstrate that TSA significantly promoted histone acetylation under the conditions in which it inhibited IL-1β-induced iNOS expression and subsequent NO release. In this context, several recent studies have provided convincing evidence to suggest that hyperacetylation limits iNOS activation [19, 49, 50]. Several mechanisms have been put forth by which acetylation of histones alter the functional regulation of transcriptional factors [39–44]. In the light of the fact that NF-κB plays a key regulatory role in

IL-1β-mediated iNOS gene expression and NO release in the islet β cell (see above), the following three studies, among others, have direct relevance to our current observations.

First, using cultured murine mesangial cells and RAW 264.7 cells, Yu *et al.* [19] provided convincing experimental evidence to implicate a positive modulatory role(s) for HDACs in cytokine-mediated induction of the iNOS gene. Through the use of pharmacological (*e.g.* TSA) and molecular biological approaches (*e.g.* over-expression of HDAC isoforms), these investigators demonstrated a direct impact of acetylation status on iNOS and NF-κB promoter activity. In these studies, TSA markedly reduced the ability of a variety of cytokines to induce iNOS and NF-κB promoter activity. Compatible with these observations are findings by these investigators to indicate that over-expression of certain HDAC isoforms resulted in a significant increase in these promoter activities elicited by cytokines. These researchers have also demonstrated specific interaction(s) between HDAC2 and NF-κB. Based on these data, it was concluded that such an interaction at target promoters could contribute to regulation of synthesis and functional activation of various inflammatory genes (*e.g.* iNOS) resulting in cellular dysfunction. Second, more recent studies from the above investigators [20] have provided additional insights into cytokine-induced iNOS gene expression and NO release in mesangial cells. For example, using ChIP assays, these investigators have shown that acetylated histone H4 is associated with –978 to –710 of the iNOS promoter under basal conditions, which was further enhanced in an IL-1β-stimulated cell. Based on data accrued from additional studies, these investigators concluded that HDAC inhibitors (*e.g.* TSA) reduce IL-1β-induced iNOS expression *via* phosphoinositide 3-kinase and p70s6 kinase-dependent pathways, which promote site-specific targeting of acetylated histone H4 at the iNOS promoter. Such a mechanism appears to be distinct from the one involving direct interaction between HDAC2 and NF-κB interaction also leading to iNOS gene expression and NO release (see above). Third, in the context of potential roles of histone (de)acetylation steps in IL-1β-induced iNOS expression and NO release in the islet β-cell, Larsen *et al.* [23] have provided evidence to suggest that inhibition of HDAC activity leads to prevention of cytokine-induced metabolic dysfunction of the β cell primarily at the level of down-regulation of NF-κB transactivating function.

It should be noted that we used two structurally dissimilar inhibitors of HDACs (*i.e.* NaB and TSA) to arrive at the conclusion that histone deacetylation (*i.e.* hypoacetylation) contributes to IL-1β-induced iNOS protein expression and NO release. Our current findings are also important in the light of more recent studies by Larsen *et al.* [23], who demonstrated that TSA exerted significant inhibitory effects on IL-1β-induced iNOS expression and NO release without significantly affecting glucose-stimulated insulin release from primary islets and clonal β cells. Moreover, using clonal MIN6 cells, Mosley and Ozcan [7] have reported glucose-induced hyperacetylation of histone H4 at the insulin gene promoter region, facilitating insulin gene transcription. This study further demonstrated that inhibition of histone deacetylation by TSA achieved hyperacetylation of histone H4 at the insulin

promoter region under basal glucose concentrations. Taken together, findings from the above studies suggest that hyperacetylation of histones (*e.g.* histone H4) promotes glucose-dependent insulin gene transcription, whereas hypoacetylation is favourable for transcription of pro-apoptotic proteins, such as iNOS, under the duress of cytokines.

In addition to data derived from the TSA experiments, we have been able to demonstrate that activation of PKC δ might contribute towards iNOS expression and NO release, specifically at the level of histone H4 (de)acetylation. We observed that rottlerin, a known inhibitor of PKC δ , significantly increased histone H4 acetylation while inducing inhibition of iNOS expression and NO release in IL-1 β -treated INS cells. We observed that effects of rottlerin on histone H4 acetylation are distinct from those of TSA, since we have been unable to demonstrate any HDAC inhibitory property of rottlerin in INS cell nuclear extracts at least under conditions in which TSA significantly inhibited such an activity in INS cell nuclear extracts. Therefore, it is likely that rottlerin inhibits PKC δ -mediated phosphorylation-mediated inactivation of relevant HATs in the islet β cell, leading to hyperacetylation of histones. Indeed, in support of such a formulation, Yuan *et al.* [36] recently reported that PKC δ specifically phosphorylates p300 at serine 89 *in vitro* and *in vivo*. This phosphorylation causes inhibition of HAT activity intrinsic to p300 and induces subsequent hypoacetylation of genes requiring p300 transcriptional activation. Under such a scenario, rottlerin treatment should inhibit PKC δ -mediated inactivation of β cell endogenous HAT activity leading to hyperacetylation of candidate histones. Additional studies are needed to substantiate this viewpoint, which are being carried out in our laboratory currently. However, we cannot rule out the possibility for additional regulatory mechanisms involving PKC δ -independent effects of rottlerin as reviewed recently by Soltoff in [51].

Based on our current data, we propose the following model to implicate a positive modulatory role(s) for histone deacetylation

(or hypoacetylation) in IL-1 β -induced iNOS expression and NO release. Exposure of isolated β cells to IL-1 β results in hypoacetylation of histones. TSA, a known HDAC inhibitor, prevents such a signalling step by inhibiting the candidate HDAC, thereby retaining specific histones in a hyperacetylated state. This, in turn, prevents IL-1 β -induced iNOS expression and NO release. In addition, although not proven herein, exposure of isolated β cells to IL-1 β results in the activation of PKC δ , which in turn phosphorylates and inactivates specific HATs, leading to hypoacetylation of histones, thus favouring iNOS expression and NO release. This viewpoint is supported by our current observations to indicate rottlerin-induced hyperacetylation of histones, followed by inhibition of iNOS expression and NO release in IL-1 β -treated cells. In summary, we provide the first evidence to implicate a PKC δ -dependent signalling mechanism involving hypoacetylation of histones in the cascade of events leading to IL-1 β -mediated iNOS expression and NO release. This pathway may be distinct from the one proposed by Carpenter *et al.* [24] demonstrating a novel role in maintaining the stability of iNOS gene. Our findings, in addition to those reported recently by Larsen *et al.* [23], raise a potential possibility for the use of HDAC inhibitors as novel therapeutics to prevent IL-1 β -mediated metabolic dysfunction of the islet β cell. Future studies will need to focus on the development of novel inhibitors with minimal cytotoxic effects but with a significant inhibitory potency against candidate HDACs.

Acknowledgements

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