Published in final edited form as:

Exp Cell Res. 2015 January 1; 330(1): 212–221. doi:10.1016/j.yexcr.2014.09.031.

Trichostatin A Affects the Secretion Pathways of Beta and Intestinal Endocrine Cells

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Abstract

Histone deacetylase inhibitors (HDACi) were recently identified as having significant clinical potential in reversing β -cell functional inhibition caused by inflammation, a shared precursor of Type 1 and Type 2 diabetes. However, HDACi are highly complex and little is known of their direct effect on important cell secretion pathways for blood glucose regulation. The aims of the present study were to investigate the effect of HDACi on insulin secretion from β -cells, GLP-1 secretion from L-cells, and recombinant insulin secretion from engineered L-cells. The β -cell line β TC-tet, L-cell line GLUTag, or recombinant insulin-secreting L-cell lines were exposed to Trichostatin A for 24 hours. Effects on insulin or GLP-1 mRNA, intracellular protein content, processing efficiency, and secretion were measured by real-time PCR, ELISA, and radioimmunoassay. HDACi increased secretion per viable cell in a dose-dependent manner for all cell types. Effects on mRNA levels were variable, but enhanced intracellular polypeptide content and secretion were comparable among cell types. Enhanced recombinant insulin secretion was sustained for seven days in alginate microencapsulated L-cells. HDACi enhances β - and L-cell secretion fluxes in a way that could significantly improve blood glucose regulation in diabetes patients and holds potential as a novel method for enhancing insulin-secreting non- β or β -cell grafts.

Keywords

Histone deacetylase inhibitors; Trichostatin A; Regulated secretory pathway; Diabetes; β -cells; Intestinal endocrine cells

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Introduction

The prevalence of diabetes is doubling every 10 years, with 347 million people affected worldwide. Since current treatments place considerable burden on the healthcare system, the need exists for a novel, clinically feasible treatment that cures or prevents diabetes. Recently, exciting new studies have identified histone deacetylase (HDAC) inhibitors (HDACi) as having significant clinical potential in reversing β -cell functional inhibition caused by inflammation, a shared precursor of Type 1 and Type 2 diabetes. Since certain small molecule HDACi drugs are FDA-approved for epilepsy and cancer therapies, repurposing their use for diabetes treatment is a tangible option.

It is now recognized that acetylation plays an important role in diabetes etiology; it regulates the master transcription factor in the inflammation nuclear factor, (NF)- κ B, which is critical to inducing β -cell death when activated (1, 2). Two groups have demonstrated *in vitro* that HDACi treatment helps prevent cytokine-induced β -cell death (3, 4). A recent *in vivo* study reported that low dose HDACi administration to weaning nonobese diabetic mice up to 100-120 days of age reduced diabetes incidence by 38-45% and increased the percentage of islets without infiltration by 15% (5). In addition to this effect, HDACi drugs have shown evidence of alleviating insulin resistance and glucose uptake in skeletal muscle and liver cells (6, 7). HDACi effects on other cells whose functions are critical to β -cell function, such as glucagon-like peptide-1 (GLP-1)-secreting intestinal L-cells, have not been investigated.

Although HDACs are known to play a key role in chromatin remodeling by acting on histone proteins, recent findings show that HDACs also act on 875 other classes of proteins (8). HDACi are therefore inherently non-specific and may have effects on diabetes patients besides anti-inflammation. As β -cell function is critical for proper blood glucose regulation, investigating direct HDACi effects on β -cell function is vital toward developing a clinically acceptable treatment. Some studies have observed HDACi effects on β -cell line or islet function, but as an aside to their primary investigation of anti-inflammatory effects (3, 4) or a pre-culture method for improved islet transplantation (9). So far, the reported effects have been contradictory.

To investigate the cause for increased postprandial insulin levels in epilepsy patients treated with Valproic Acid (VPA), Luef et al. studied the effects of VPA on human islets (10). The group reported a dose-dependent increase in secreted insulin from islets incubated with VPA, but this effect was attributed to the behavior of VPA as a fatty acid derivative rather than as an HDACi. After thorough review of the HDACi literature, Christensen et al. identified a gap in understanding of whether HDACi affects the β -cell secretion pathway (11).

Due to their immune suppressive properties, HDACi also have potential in protecting allo- or xeno-geneic cell-based grafts from immune rejection after transplantation, which is often a challenge when using a cell-based therapy (12). Prior to evaluating HDACi for graft immune protection after transplantation, *in vitro* studies must be performed to assess direct effects on cell function. HDACi studies on recombinant insulin secretion from engineered non- β -cell pancreatic substitutes do not currently exist in the literature.

The overall objective of this work was to demonstrate the effects of TSA, a clinically relevant HDACi, on hormone secretion from endocrine cells over a broad range of diabetes therapy research applications. Current research ranges from developing therapeutic drugs to optimizing graft transplantation. For the latter, a major challenge in transplanting engineered non- β cells is in enhancing recombinant insulin secretion to therapeutic levels. Four cell types were therefore tested to investigate how TSA affects natural hormone secretion from β and L-cells as well as recombinant hormone secretion from genetically engineered L-cells developed in our lab. For each cell type, select steps within the secretion pathways were studied for a better understanding of TSA effects. The findings presented in this work provide a foundation for critical research areas to branch off from, including the investigation of 1) the TSA mechanism involved in secretion enhancement, 2) therapeutic HDACi effects on diabetes preclinical systems, and 3) HDACi enhancing effects on β and non- β cell grafts in diabetes preclinical systems.

Materials and methods

Cells

Murine insulinoma β TC-tet and murine GLUTag L-cell lines were used to study HDACi effects on the function of β - and L-cells. The purpose of using the β TC-tet cells rather than islets was to investigate the direct effects of HDACi on only β -cell function. To study the effects of HDACi on non- β -cell graft function, the GLUTag-INS cell line was used. GLUTag-INS cells were previously generated in our lab by stable human B10 insulin transfection (13). A genetic variant of GLUTag-INS, GLUTag-EINS (EINS), was also generated in our lab by lentiviral transduction of GLUTag-INS with the wild-type human insulin transgene (LV-WT-INS; Emory University; Dr. John Shires) to improve the level of secreted insulin. The development and characterization of this cell line are described in (14).

β TC-tet cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma, St. Louis MO) with 25 mM glucose and supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin (P/S), and 6 mM L-glutamine. GLUTag, GLUTag-INS, and EINS cell lines were cultured as in (15) using DMEM with 25 mM glucose, without L-glutamine (Corning cellgro, Manassas, VA, Cat. #15-017), and supplemented with 10% FBS and 1% P/S. All cell cultures were propagated in a humidified incubator at 37°C/5% CO₂.

Relative mRNA quantitation

Cells were harvested for RNA isolation using the E.Z.N.A Total RNA Kit I (OMEGA bio-tek, Norcross, GA) followed by cDNA synthesis using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Grand Island, NY). cDNA was synthesized from 1 μ g of RNA following the manufacturer's protocols. Real-time, relative quantitation of mRNA was accomplished using SYBR Select Master Mix (Applied Biosystems) and StepOnePlus Real-Time PCR System (Life Technologies) for measurement and comparative C_T method analysis. Human insulin, mouse insulin (INS-1), and GLP-1 primers were designed using National Center for Biotechnology Information's Primer-BLAST design software and mouse beta-actin (ACTB) primers were used as endogenous controls. Primer sequences: forward human insulin 5'-CTA CCT AGT GTG CGG GGA AC-3', reverse

human insulin 5'-AGC TGG TAG AGG GAG CAG AT-3'; forward mouse insulin 5'-CTT GTT GGT GCA CTT CCT AC-3', reverse mouse insulin 5'-TGC AGT AGT TCT CCA GCT GG-3'; forward GLP-1 5'-ACA GCA AAT ACC TGG ACT CCC GCC GT-3', reverse GLP-1 5'-CCT CGG CCT TTC ACC AGC CAA GCA A-3'; forward mouse ACTB 5'-GCA CAG CTT CTT TGC AGC TC-3', reverse mouse ACTB 5'-CTT TGC ACA TGC CCG AGC C-3'. Primers were purchased from Eurofins MWG Operon, Huntsville, AL and used at 300 nM concentrations.

Dose-response curves

For β TC-tet, GLUTag, and GLUTag-INS cells, dose-response curves were constructed to determine the effect of varying Trichostatin A (TSA; Sigma) concentrations on secretion and viable cell numbers. Serial dilutions of TSA were made in dimethyl sulfoxide (DMSO; Sigma) and the concentrations tested were: 0, 0.156, 0.313, 0.625, 1.25, and 2.5 μ M. The same volume of DMSO was added to cells treated with 0 μ M TSA. After a 24 hour incubation, wells were washed twice in Dulbecco's Phosphate-Buffered Solution (DPBS; Corning cellgro) to remove any dead cells and changed to stimulating medium (β TC-tet: 16.7 mM glucose for 30 minutes, GLUTag and GLUTag-INS: 5 mM glucose + 2% Meat Hydrolysate (MH) for 2 hours). MH was chosen as a secretagogue for GLUTag cells since L-cells are meal-responsive and directly stimulated by peptones (16). Medium samples were then collected for secreted peptide assay and cells were subsequently trypsinized for trypan blue (Sigma) cell counting. Trypan blue methodology was chosen to determine the number of both viable and dead cells based on cell membrane permeability. Since cells impermeable to trypan blue are considered viable, it is unlikely that the same cells leak hormones like GLP-1 and insulin, which are 4-6 times higher in molecular weight than trypan blue.

Effects on secretion pathways

Cells were seeded in 12-well plates (β TC-tet: 1.8×10^5 cells/cm², GLUTag: 2.1×10^5 cells/cm², GLUTag-INS and EINS: 1.3×10^5 cells/cm²) two days before TSA treatment. On day 0, culture medium was changed to fresh and TSA was added at a concentration of 0.625 μ M. Non-treated control groups were used for direct comparisons. On day 1, insulin or GLP-1 secretion rate tests were performed and cells were harvested for intracellular insulin or GLP-1 and total RNA collection. Intracellular insulin and total RNA were collected from wells run in parallel to those used for secretion rate tests and collection occurred after a one hour basal incubation to capture the initial state of mRNA and intracellular insulin content. To collect intracellular content, TSA and non-treated cell pellets were lysed using the Mammalian Cell Lysis Kit (Sigma). Proinsulin and insulin concentrations were measured from β TC-tet samples by Mouse Insulin and Rat/Mouse Proinsulin ELISA kits (Mercodia Inc., Winston Salem, NC) and from GLUTag-INS and EINS samples by Ultra-Sensitive Human Insulin and Human Proinsulin Radioimmunoassays (RIA; Millipore, Billerica, MA). GLP-1 concentrations were measured from GLUTag samples by GLP-1 (Active) ELISA (Mercodia Inc.). All secretion and intracellular data were normalized to the number of viable cells.

Studies with the HDACi Tubacin (Sigma) were performed to determine if HDAC6 plays a role in secretion enhancement. This is a more specific approach since TSA acts on ten

different HDACs, including HDAC6. The choice of Tubacin was based on reports that inhibition of HDAC6, a cytoplasmic HDAC, increased vesicular transport and secretion of hormones in neuroendocrine cells by increasing acetylated α -tubulin levels (17). In the present study, the Tubacin treatment method was identical to TSA treatment, except a concentration of 4 μ M was used. This concentration was estimated based on cyto blot analyses of TSA and tubacin effects on acetylated tubulin levels in A549 cells (18).

Long-term effects

Long-term effects of TSA were evaluated using microencapsulated EINS cells for 14 days *in vitro*. EINS cells were grown in T-75 tissue culture flasks and treated for 24 hours with 0.625 μ M TSA. Cells were then washed twice with DPBS, trypsinized, microencapsulated in 3.3% (wt/vol) LVM alginate (LVM; Novamatrix, Drammen, Norway), and cross-linked in 30 mM BaCl₂ solution using an electrostatic droplet generator (Nisco Engineering, Zurich, Switzerland) as previously described (19). Insulin secretion tests were performed on 0.1 mL microcapsule volumes 1, 7, and 14 days after microencapsulation. Control groups were run in parallel and treated in the same way, except with 0 mM TSA. Microcapsule volumes were then solubilized in ethylenediaminetetraacetic acid disodium salt dihydrate solution (0.2 M; pH 9) to harvest cells for intracellular protein collection and total RNA isolation.

Secretion rates

β TC-tet cells were subjected to basal conditions (DMEM with 0 mM glucose) for one hour followed by a thirty minute step-up period under stimulating conditions (DMEM with 16.7 mM glucose). GLUTag, GLUTag-INS, and EINS were subjected to basal conditions (DMEM with 5 mM glucose) for two hours followed by a two hour step-up period under stimulating conditions (basal + 2% MH; Sigma). Media samples were taken before and after each incubation period for rate determinations. Secretion and intracellular data from microcapsules were normalized to metabolic activity measured by alamarBlue™ (Life Technologies, Grand Island, NY) as in (20) using a one hour incubation period.

Statistical analyses

Data were analyzed using Minitab software (Minitab, Inc., State College, PA) and reported as mean $\pm$ SEM; each mean was the average of data from three or more independent experiments. Significance was determined using a one-way analysis of variance (ANOVA) with the general linear model, with significance defined as $p < 0.05$.

Results

Dose-response curves

After treating the cells for 24 hours with varying TSA concentrations, a similar dose-dependent response in secretion per viable cell was observed between the three groups: insulin from β TC-tet cells, GLP-1 from GLUTag cells, and recombinant insulin from GLUTag-INS cells; effects on viable cell numbers also followed comparable trends between groups (Figure 1). At a TSA concentration of 0.313 μ M or higher, all groups secreted significantly more than non-treated controls and appeared to plateau near a TSA concentration of 1.25 μ M (Figure 1A). The inverse was true for TSA effects on viable cell

numbers which were significantly lower than non-treated controls at and above 0.313 μM TSA, plateauing near 1.25 μM TSA (Figure 1B). It is important to note that Figure 1B does not represent cell death, but rather the aggregate effect of both cell death and reduced viable cell growth due to TSA treatment. After TSA treatment and DPBS washes, the percent viability relative to non-treated cells was 80% or higher among all groups (Figure 1C), suggesting that secretion tests were performed on a population consisting of mostly viable cells.

Effects on secretion pathways

EINS was studied for HDACi effects due to its potential to secrete higher quantities of insulin and serve as a therapeutic, non- β -cell pancreatic substitute. To avoid excessive toxicity, an intermediate concentration of 0.625 μM TSA was chosen to specifically assess HDACi effects on endogenous insulin, endogenous GLP-1, and recombinant insulin mRNA levels, intracellular protein content, and secretion rates. TSA-treated β - and L-cells had lower mRNA levels of insulin (78% of control) and GLP-1 (36% of control), respectively, relative to their non-treated controls (Figure 2). On the contrary, insulin transgene mRNA levels were no different after treating GLUTag-INS and 7-fold higher after treating EINS, suggesting that TSA only enhanced lentiviral insulin transgene expression.

TSA treatment of $\beta\text{TC-tet}$ cells caused a 2.5-fold increase in stored insulin and a 2-fold increase in glucose-stimulated insulin secretion, but had no effect on basal insulin secretion (Figure 3; $\beta\text{TC-tet}$). TSA treatment of GLUTag cells increased stored and MH-stimulated GLP-1 secretion by 2 and 1.9-fold, respectively, but had an even greater effect on basal GLP-1 secretion (5 mM glucose) which increased by almost 9-fold (Figure 3; GLUTag); the effect on stimulated secretion was significant with 90% confidence ($p=0.09$). TSA effects on recombinant insulin secretion from GLUTag-INS and EINS cells were similar to those observed on GLP-1 secretion from parental GLUTag cells, but with less of an enhancing effect on basal secretion (Figure 3; GLUTag-INS & GLUTag-EINS). TSA-treated EINS cells secreted 50-70% more recombinant insulin than TSA-treated GLUTag-INS. No significant effects were observed in any cell type after 24 hour, 4 μM Tubacin treatments (Figure 3).

Table 1A presents absolute data for secreted insulin or GLP-1 normalized to viable cell number in all four cell types. For insulin-secreting cells, a stimulation index (SI) was maintained after TSA treatment. After TSA treatment of the GLUTag cells, however, there was no stimulation of GLP-1 from 0 to 2% MH. Additional testing of GLP-1 secretion from 0 to 5 mM glucose revealed that TSA-treated GLUTags were stimulated similarly to non-treated GLUTags ($\text{SI}=1.5$). A small, but significant, 10% increase in secreted proinsulin to insulin conversion was found in TSA-treated $\beta\text{TC-tet}$ cells under 16.7 mM glucose conditions (Figure 4A), but no difference was detected in stored insulin processing efficiency relative to non-treated controls (Figure 4B). On the other hand, a 22-28% drop in secreted proinsulin to insulin processing efficiency was observed in TSA-treated GLUTag-INS and EINS cells under 5 mM glucose conditions (Figure 4A); similarly to $\beta\text{TC-tet}$ cells, however, stored insulin processing was relatively constant in treated EINS. These findings suggest that $\beta\text{TC-tet}$ cells were able to sustain secreted proinsulin processing capabilities

upon significant proinsulin augmentation, but recombinant GLUTag-INS and EINS cells were not.

Limiting steps in the secretion pathway of engineered cells

Both GLUTag-INS and EINS stored the same quantities of insulin under non-treated conditions (142 ± 31 v. 157 ± 30 fmol/ $(10^5$ cells); $p=0.75$), but intracellular proinsulin content in EINS was 1.9-fold higher than in GLUTag-INS (7.9 ± 1.1 v. 4.2 ± 0.8 fmol/ $(10^5$ cells), respectively; $p<0.05$). Additionally, basal proinsulin and insulin secretion were not higher in EINS relative to GLUTag-INS under non-treated conditions (Figure 5). These trends observed in the EINS regulated secretory pathway (RSP) perhaps suggest that the intracellular surplus of EINS proinsulin was inefficiently processed into insulin.

TSA treatment appeared to at least partially alleviate this issue, causing a significant increase in EINS proinsulin and insulin secretion rates with 50-70% more secreted relative to treated GLUTag-INS (Figure 5). However, the fold-increase in basal proinsulin secretion was higher than the fold-increase in insulin secretion from both cells, again pointing to a possible limiting step between proinsulin and insulin synthesis. Compared to GLUTag-INS, TSA-treated EINS had the highest insulin secretion rates, proinsulin secretion and intracellular content, and insulin mRNA levels (Table 2). However, TSA-treated GLUTag-INS also secreted more insulin relative to non-treated controls, but in the absence of insulin mRNA enhancement, thus suggesting additional TSA effects on the RSP besides enhanced insulin gene transcription in EINS cells.

Long-term effects

Long-term TSA effects were studied to determine the sustainability of enhanced recombinant L-cell graft function after HDACi treatment. EINS were chosen because they were the most promising insulin secretors. A 3D configuration was chosen because it was better at sustaining viable cell numbers over time than 2D monolayers (data not shown) and alginate microencapsulation has shown promise as a pancreatic substitute configuration in preclinical studies (21). Insulin secretion was 70-80% higher than controls one day post-encapsulation (two days after TSA treatment) and was sustained for seven days in culture (Figure 6). By day 14, however, secretion was no longer enhanced; insulin mRNA and intracellular insulin had also declined. Even on day 7, a dramatic decline in insulin mRNA was observed, but this appeared to have no effect on secretion. It was not until day 14 that intracellular and secreted insulin declined to pre-treatment levels.

Discussion

With the recent presence of HDAC in diabetes literature (11), and the newfound potential for HDACi therapy to protect cytokine-mediated β -cell death (3, 4), there is limited information regarding the impact of HDACi on other important factors that play major roles in diabetes. In this work, we have demonstrated the functional enhancement of β -, parental L-, and recombinant L-cells after HDACi treatment by reporting increased storage and secretion levels of insulin and GLP-1. We have also introduced HDACi treatment as a novel method to enhance recombinant insulin secretion from non- β -cell sources that use an RSP. This

method, in conjunction with additional insulin transgene incorporation, has led to the generation of recombinant L-cells that secrete the highest insulin quantities of previously engineered non- β -cell reported in the literature (Table 1).

TSA was carefully chosen for this investigation due to its similarity in structure to the FDA-approved HDACi drug, Vorinostat (22). In addition, the structure of TSA does not resemble a fatty acid secretagogue, as is the case with another FDA-approved HDACi, VPA. This is beneficial since functional enhancement effects can be attributed only to its action as an HDACi. Lastly, using TSA has allowed for the comparison of this work to the relevant literature since TSA is commonly studied and reported.

Enhancing effects of TSA on β TC-tet cells in this study are in accordance with reports of a dose-dependent increase in insulin secretion from human islets treated with VPA (10) and increased insulin secretion after the use of a culture technique on rat islets involving 0.1 μ M TSA treatment for 24 hours (9). The effects of TSA alone on rat islets in the latter study, however, were not assessed and results from the former study were attributed to the structural similarity of VPA to free fatty acid secretagogues. As in the present study, Larsen et al. observed a reduction in insulin release from INS-1 β -cells treated with 0.2 μ M TSA under low glucose conditions for two days (3). However, effects were not studied under glucose-stimulated conditions and insulin secretion was not normalized to viable cell numbers for directly comparing the per-cell functional effects between treated and non-treated populations. Additionally, the long timescale for the experiment was designed to test the effects of TSA on cytokine-mediated apoptosis rather than the direct effects of TSA on β -cell function.

On average across all cell types, the percentage of viable cells from the total cell count after a 0.625 μ M TSA treatment and DPBS washes was only $10 \pm 3\%$ lower than non-treated controls. These data suggest that secretion tests were performed on a population consisting of mostly viable cells and it is unlikely that the majority of heightened secretion was due to the presence of dead cells. Additionally, β -cell basal secretion was comparable to non-treated controls. If these cells were dying, lysing, and leaking insulin due to TSA treatment, basal secretion rates would have appeared higher relative to controls. Instead, the enhancement effect was only seen when β -cells were stimulated.

The observed 21% reduction in insulin mRNA levels in TSA-treated β TC-tet cells and 64% reduction in GLP-1 mRNA levels in TSA-treated GLUtag cells are not well understood. Possible explanations are cellular mRNA degradation due to TSA-induced apoptosis (23), direct effects of TSA on mRNA stability (24), or repressed gene transcription (25). The least likely are the two former reasons since global mRNA degradation due to early-stage apoptosis or decline in mRNA stability should cause the endogenous control (actin-beta) mRNA to also decline. There was no evidence of this from the PCR results (mean (SD) raw C_T values for β TC-tet: ACTB- 25.4 (3.9), ACTB+ 25.7 (3.9); $p=0.9$; $n=3$ and GLUtag: ACTB- 19.1 (0.5), ACTB+ 19.5 (1.1); $p=0.4$; $n=5$). Apoptosis therefore was not measured, especially since cell death and apoptotic effects of HDACi have been previously reported (3, 4).

The purpose of using the β TC-tet cell line as opposed to islets in this study was to investigate the direct effects of HDACi on β -cell function. Limitations, however, are that β -cell lines lack the same clinical relevance as islets and Larsen et al. have suggested that TSA has different toxicity towards cancer cell lines versus primary β -cells or islets (3). It is therefore suggested that preclinical diabetes mouse model experiments be performed in which TSA or Vorinostat doses are optimized and inflammatory as well as secretion effects are studied in relation to both β - and L-cells.

Oral HDACi administration could impact various cells within the body. In diabetes patients, proper GLP-1 secretion from intestinal L-cells is critical to glucose-stimulated β -cell function. Interestingly, GLP-1 increases *in vitro* global acetylation of histone H3 in β -cells (26) and may therefore be affecting processes similar to the ones which HDACi act on. As expected, based on secretion pathway similarities between β - and L-cells, TSA had similar enhancing effects on GLUTag cells. Additionally, higher GLP-1 secretion from HDACi-treated L-cells within the range of 0 to 5 mM glucose may be a potential advantage for achieving glycemic restoration in diabetes patients.

Based on the beneficial effects of HDACi observed on GLUTag cells and the potential immune protective effects of HDACi on transplanted grafts (12), we investigated TSA effects on the secretion pathway of recombinant GLUTag-INS and EINS cells. Use of non- β -cells such as these may alleviate donor cell availability and autoimmune rejection issues associated with islet transplantation (27). In particular, enteroendocrine cells are promising for diabetes therapy, but novel engineering methods need to be developed to generate cells that are capable of secreting therapeutic quantities of recombinant insulin. In this work, TSA-treated EINS cells have come the closest to murine β -cell line secretion, significantly surpassing previously engineered enteroendocrine cells, as indicated in Table 1 (13, 28, 29).

Lentiviral-mediated transgene delivery resulted in unstable insulin gene expression in EINS cells and secretion enhancement was eventually lost over passaging (data not shown). Gene silencing can be an issue when using viruses for transgene delivery (30-32) and inhibiting HDACs in virally transduced cells has in some cases reactivated transgene expression (33, 34). This may explain the 7-fold increase in insulin mRNA levels within EINS cells after TSA treatment. Interestingly, after TSA treatment, EINS cells contained 23 times more insulin mRNA than GLUTag-INS, yet secreted only 70-80% more recombinant insulin. High levels of intracellular and secreted proinsulin suggest insulin processing as a possible limitation to high insulin secretion from engineered L-cells. It may therefore be beneficial to investigate the cause for proinsulin buildup and improve processing capabilities so that the full potential of TSA therapy may be reached.

Although HDACs were originally named for their action on histone proteins, recent studies have shown that they also play a role in 875 other classes of proteins (8). Our results demonstrated that TSA treatment affects the RSPs of β - and L-cells, independently of insulin and GLP-1 mRNA levels. This was most clearly seen after TSA treatment, when stored and secreted insulin and GLP-1 levels increased in β TC-tet, GLUTag, and GLUTag-INS cells even though mRNA levels were either lower or comparable. Additionally, long-term studies of microencapsulated EINS cells confirmed that secretion enhancement could

still be present even after insulin mRNA declined back to original levels. Since tubacin did not cause an increase in secretion, HDAC6 inhibition does not likely play a role in the effects observed with TSA. To further clarify the molecular mechanisms behind TSA effects on secretion, in-depth HDAC knock down studies will be needed.

Evidence from this study supports the potential role of HDACi therapy in preventing or treating diabetes mellitus via β - and L-cell functional enhancement. For type 2 diabetes patients, this approach may address insufficient insulin secretion from still-functioning β -cells by both increasing endogenous GLP-1 secretion from L-cells for indirect β -cell stimulation and directly increasing insulin secretion from β -cells. In the case of insulin-dependent diabetics who have received insulin-secreting cell grafts, the long-term sustained effects of HDACi indicate the promise of HDACi treatment in providing functional enhancement of the graft and possibly graft immune protection as reported in the literature (12). Repurposing FDA-approved HDACi for diabetes treatment and pursuing the suggested methods from this work may benefit the diabetes community by providing treatment options for tighter glycemic control and improved patient compliance.

Acknowledgements

We gratefully acknowledge our funding source NIH (R01 DK076801), José Vasquez and Derrius Anderson (Undergraduate Research Assistants, GT) for their assistance in the PCR and HDACi studies, and Drs. Brubaker and Drucker (University of Toronto Ontario, Canada) for their donation of the GLUTag cells.

Abbreviations

EINS	GLUTag-EINS
HDAC	Histone Deacetylase
HDACi	Histone Deacetylase Inhibitor
RSP	Regulated Secretory Pathway
TSA	Trichostatin A
VPA	Valproic Acid

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Highlights

- We investigate the effects of TSA on the secretion pathways of β and L-cells
- TSA treatment increases secretion from β and L-cells in a dose-dependent manner
- Enhanced recombinant insulin secretion is sustained for seven days in microcapsules
- HDACi could significantly improve blood glucose regulation in diabetes patients
- HDACi may serve as a novel method to enhance non- β or β -cell graft function

Figure 1.

TSA dose-response curves for insulin-secreting β TC-tet cells, GLP-1-secreting GLUTag L-cells, and recombinant insulin-secreting GLUTag-INS cells. Effects of a 24 hour TSA treatment on A) secretion, B) viable cell numbers, and C) percent viability. Secreted hormone data were normalized to viable cell numbers prior to normalization of all data to 0 μ M TSA controls. * $p < 0.05$ versus controls, $n = 3$.

Figure 2.

Quantitation of mouse insulin mRNA in TSA-treated β TC-tet cells, GLP-1 mRNA in TSA-treated GLUTag cells, and human insulin mRNA in TSA-treated GLUTag-INS and EINS cells, all relative to their non-treated controls set equal to one. * $p < 0.05$ versus controls.

Figure 3.

TSA (0.625 μ M) and tubacin (4 μ M) effects on stored and secreted mouse insulin in β TC-tet cells, GLP-1 in GLUTag cells, and recombinant human insulin in GLUTag-INS and EINS cells. All data were normalized to viable cell number prior to normalization to non-treated controls. *p<0.05 versus controls under each condition.

Figure 4. Effects of TSA on the efficiency of proinsulin to insulin processing measured for A) secreted and B) intracellular proteins in β TC-tet, GLUTag-INS, and EINS cells. * $p < 0.05$ versus non-treated controls.

Figure 5.

TSA effects on GLUTag-INS and EINS insulin and proinsulin secretion rates normalized to viable cell number. Statistical comparisons are indicated between groups under each condition (insulin-basal, insulin-stimulated, proinsulin-basal) and bars sharing symbols are statistically different (*, # $p < 0.05$). Statistical comparisons were also made between basal and stimulated insulin secretion groups, indicating that stimulated secretion was significantly higher than the corresponding basal secretion ($p < 0.05$).

Figure 6.

Long-term effects of TSA on human insulin basal secretion, stimulated secretion, intracellular storage, and mRNA of microencapsulated EINS cells 1, 7, and 14 days after encapsulation. Cells were treated with TSA in monolayers for 24 h prior to microencapsulation. Secretion and intracellular data were normalized to metabolic activity measured by alamarBlue™. All data were normalized to parallel, non-treated control groups.

*p<0.05 versus controls.

Reported secretion rates, stimulation indices (SI), and % proinsulin conversion from A) TSA-treated and non-treated cells in the present study and from B) engineered non-b-cells reported in the literature [engineered mouse pituitary AIT20 cells (35), engineered human NCIH716 L-cells (29), and engineered mouse STC-1 K-cells (28)].

Table 1

		Data Reported as Mean (SD); n 3				
		Secretion rate in fmol(10 ⁶ cells · h)		Stimulation Index (Stimulated/Basal)	% Proinsulin Conversion ((Insulin/Total) × 100)	
A		-TSA	+TSA	-TSA	+TSA	+TSA
βTC-tet Insulinomas	0 mM Glucose	4,581 (140)	3,243 (325)	5.5 (1.3)	13.3 (4.5)	87.6(1.9)%
	16.7 mM Glucose	26,387 (3,356)	41,955 (6,072)			96.1(1.4)%
GLUTag L-Cells (GLP-1 secretion)	5 mM Glucose	23 (11)	196 (72)	1.6 (0.16)	0.34 (0.03)	N/A
	mM Glucose + 2% MH	39 (23)	66 (26)			N/A
GLUTag-INS	5 mM Glucose	153 (31)	375(57)	1.8 (0.08)	1.6 (0.17)	67.4 (3.0) %
	5 mM Glucose + 2% MH	282 (56)	599 (105)			52.2 (6.2)%
GLUTag-EINS*	5 mM Glucose	108(31)	655 (67)	1.7 (0.19)	1.4 (0.09)	70.4 (3.6) %
	5 mM Glucose + 2% MH	193 (52)	888 (86)			50.3 (6.2)%
B						
Engineered Mouse Pituitary AIT20 Cells	0 mM BrCAMP	60		6		N/A
	5 mM BrCAMP		360			
Engineered Human NCI-H716 L-Cells	5 mM Glucose	79 (29)		2.6		80%
	5 mM Glucose + 2% MH	204 (44)				
Engineered Mouse STC-1 K-Cells	1 mM Glucose	32		2.2		N/A
	10 mM Glucose	70				

Effects of TSA on insulin mRNA levels and various stages of the RSP of GLUTag-INS and EINS, represented as a fold-difference from non-treated GLUTag-INS. Basal secretion results are represented under the proinsulin and insulin secretion column. Average non-treated GLUTag-INS values were mRNA-1.0, intracellular proinsulin-4.2 fmol/10⁵ cells, intracellular insulin-142 fmol/10⁵ cells, basally secreted proinsulin-8.6 fmol/(10⁵ cells·h), and basally secreted insulin-15.3 fmol/(10⁵ cells·h).

				Intracellular Content		Secreted	
TSA	Cells	mRNA		Proinsulin	Insulin	Proinsulin	Insulin
(-)	GLUTag-EINS	3.5		1.9	1.1	0.5	0.6
(+)	GLUTag-INS	1.1		2.1	2.6	4.8	2.4
	GLUTag-EINS	23		10.9	3.0	8.0	3.8