Valproic Acid Improves Glucose Homeostasis by Increasing Beta-Cell Proliferation, Function, and Reducing its Apoptosis through HDAC Inhibition in Juvenile Diabetic Rat

Sabbir Khan and Gopabandhu Jena

ABSTRACT: Recent evidence highlighted that there is a link between type-1 diabetes mellitus and histone deacetylases (HDACs) due to their involvement in beta-cell differentiation, proliferation, and function. The present study aimed to investigate the protective role of valproic acid (VPA) on beta-cell proliferation, function, and apoptosis in juvenile diabetic rat. Diabetes was induced in juvenile Sprague–Dawley rats by streptozotocin (75 mg/kg, i.p.) and VPA was administered at the doses of 150 and 300 mg/kg/day for 3 weeks by oral route. Various biochemical parameters, cellular alterations, and protein expression as well as apoptosis were assessed using different assays. VPA treatment significantly decreased plasma glucose, beta-cell damage, and apoptosis as well as increased the beta-cell function, insulin level/expression. The present study demonstrated that VPA improves beta-cell proliferation and function as well as reduces beta-cell apoptosis through HDAC inhibition. Our findings provide evidence that VPA may be useful for the treatment of juvenile diabetes.

INTRODUCTION

Type-1 diabetes mellitus (T1DM) is a chronic autoimmune disorder characterized by hyperglycemia due to compromised insulin secretion from beta-cell and/or reduced beta-cell mass, which generally developed in genetically susceptible individuals by environmental factors [1, 2]. T1DM usually occurs in younger people and termed as juvenile-onset diabetes, even if it can occur at any age [3]. According to International Diabetes Federation (IDF), T1DM has low prevalence (5%–10%), however its incidence increases gradually (2%–3%/year), particularly in younger (<15 years) people [4]. Both genetic and epigenetic factors contributed equally in the pathogenesis of T1DM [5, 6]. Epigenetic alterations such as post-translational modification, DNA methylation and non-coding RNAs play a critical role in the development of pancreas, cellular differentiation, proliferation, and functions [7].

Recent evidence suggested that there is a link between T1DM and histone deacetylases (HDACs) because HDAC inhibitors contribute in the beta-cell proliferation and function [8–10]. Over expression of HDACs can modulate the lineage control of pancreatic islet cells [10, 11]. Further, HDAC inhibitors enhance and maintain the expression profile of the endocrine markers in the pancreas [11, 12]. Moreover, regulation of insulin gene transcription is also modulated by acetylation status of histone, suggesting the essential role of HDACs in insulin synthesis/expression [9]. Both clinical and experimental studies have been shown that apoptosis is the primary cause of beta-cell death in T1DM through various signaling [3]. HDAC inhibitors protect beta-cell from various pathological insults such as cytokine-induced damage and apoptosis in vitro and in vivo [8, 12, 13]. HDAC inhibitors

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also suppress virus-induced inflammatory responses and progression of T1DM in rats [14]. Recently, we have reported that HDAC inhibition by sodium butyrate protects beta-cell damage and apoptosis as well as improve glucose homeostasis in juvenile diabetic rats [15].

Valproic acid (VPA) is a first-line drug used for the treatment of seizures, migraine, and bipolar disorders. VPA has been proven as a HDAC inhibitor and preferably subdued the activities of class I and II HDACs [16, 17]. It is a matter of debate that VPA modulates insulin synthesis/secretion, body weight, and insulin signaling, but the exact mechanism remains unknown [18]. It has been reported that VPA directly enhances the insulin secretion in epilepsy patients [19]. Recently, Manaka et al. [20] have been reported that chronic exposure of VPA promotes insulin release by reducing $\mathrm{K}_{\text{ATP}}$ channel current without affecting $\mathrm{Ca}^{2+}$ signaling in mouse islets. Further, acute VPA treatment also reduces blood glucose by potentiating insulin action in streptozotocin (STZ)-induced T1DM in mice [21]. It has also been reported that VPA exerts hypoglycemic, anti-lipidemic, and anti-oxidant in alloxan-induced type-1 diabetic rat [22]. Interestingly, a clinical study also showed glucose-lowering effect of VPA by single i.v. administration during oral glucose tolerance test in patients with newly diagnosed epilepsy [23]. Recently, we have reported the renoprotective and anti-fibrotic effects of VPA in T1DM through HDAC inhibition [24, 25]. Additionally, VPA also reduced the insulin-resistance, fat accumulation, dyslipidemia, and hepatic-glucose production in type-2 diabetic rat, suggesting its effects on peripheral tissues like liver and adipose tissue [26]. Therefore, here we hypothesized that VPA can modulate the beta-cell mass, insulin synthesis/release, and might improve glucose homeostasis in T1DM through HDAC inhibition and/or associated mechanisms in STZ-induced T1DM in juvenile rat. The present study was planned in juvenile animals to mimic the possible clinical pathogenesis of T1DM, as it generally occurred in younger age people.

**MATERIALS AND METHODS**

**Animals**

Animals were procured from the Central Animal Facility of the institute and experimental protocol was approved by the Institutional Animal Ethics Committee (IAEC). All the experiments were performed on juvenile Sprague–Dawley rats in accordance with the Committee for the Purpose of Control and Supervision of Experimentation on Animals (CPCSEA) guidelines. Animals were kept under controlled environment at room temperature ($22 \pm 2^\circ C$) and humidity ($50 \pm 10\%$) with automatically controlled 12 h light and dark cycle. Feed and water were provided ad libitum. Animals were acclimatized at least for 3 days prior to commencement of the experiment.

**Chemicals and Reagents**

Sodium valproate (CAS No. 1069-66-5, purity >98%), D-glucose, sucrose, tris(hydroxymethyl) aminomethane (tris) base, trichloroacetic acid (TCA), SDS, proteases inhibitor cocktail, and dibutyl phthalate in xylene were purchased from Sigma–Aldrich chemicals (Saint Louis, MO). STZ, EDTA, sodium fluoride, triton-X-100, and glass slides were purchased from Himedia (Mumbai, India). Primary and secondary antibodies, BSA, and luminol reagent were purchased from Santa Cruz Biotechnology (Dallas, TX, USA), while other routine laboratory reagents such as ethanol, NaCl, xylene, and formalin were purchased from local suppliers.

**Experimental Design and Animal Treatment**

All the animals (90–120 g) were randomized into five groups consisting of 10 animals in each group. Group-1, control receiving saline; group-2, VPA control (VPA300), receiving VPA 300 mg/kg/day for 3 weeks by oral route; group-3, diabetic (D) induced by i.p. injection of STZ (75 mg/kg); group-4, (D+VPA150), diabetic rats treated with VPA at the dose of 150 for 3 weeks by oral route; and group-5, (D+VPA300), diabetic rats treated with VPA at dose of 300 mg/kg/day for 3 weeks by oral route. All animal were sacrifice after 24 h of the administration of last dose. The doses of VPA 150 and 300 mg/kg were selected on the basis of previous studies [24, 27–29].

**Quantification of Biochemical Parameters**

The plasma glucose, serum glutamic oxaloacetic (SGOT) or aspartate transaminase (AST) and serum glutamate-pyruvate transaminase (SGPT), or alanine transaminase (ALT) were quantified by commercially available kits (ACCUREX, Mumbai, India), while plasma insulin was quantified by ELISA kit (Mercer Expert Assays, Los Angeles, CA, USA) according to manufacturer’s instructions. The HbA1c was quantified by commercially available kit (Coral Clinical System, Goa, India) according to manufacturer’s instructions and results were expressed as % HbA1c.
TABLE 1. Effect of Diabetes and VPA Treatment on the Body and Organ Weights, Plasma Glucose, and Insulin Levels as well as HbA1c in Juvenile Rat after 21 Days Treatment

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Con</th>
<th>VPA300</th>
<th>Diabetes</th>
<th>D+VPA150</th>
<th>D+VPA300</th>
</tr>
</thead>
<tbody>
<tr>
<td>Change in body weight (g)</td>
<td>82.90 ± 6.37</td>
<td>85.90 ± 8.10</td>
<td>−19.29 ± 6.49ab</td>
<td>−3.57 ± 4.83</td>
<td>5.33 ± 7.72</td>
</tr>
<tr>
<td>Pancreas weight (g)</td>
<td>0.62 ± 0.03</td>
<td>0.61 ± 0.04</td>
<td>0.41 ± 0.01ab</td>
<td>0.47 ± 0.02</td>
<td>0.53 ± 0.05</td>
</tr>
<tr>
<td>Liver weight (g)</td>
<td>9.00 ± 0.35</td>
<td>9.04 ± 0.44</td>
<td>0.41 ± 0.01ab</td>
<td>0.47 ± 0.02</td>
<td>0.53 ± 0.05</td>
</tr>
<tr>
<td>Plasma glucose (mg/dL)</td>
<td>116.06 ± 4.06</td>
<td>110.67 ± 3.80</td>
<td>620.02 ± 20.54ab</td>
<td>610.45 ± 4.02</td>
<td>455.67 ± 69.99c</td>
</tr>
<tr>
<td>Plasma insulin (ng/mL)</td>
<td>2.55 ± 0.16</td>
<td>2.42 ± 0.29</td>
<td>0.45 ± 0.11ab</td>
<td>0.96 ± 0.23</td>
<td>2.25 ± 0.21c</td>
</tr>
<tr>
<td>%HbA1c</td>
<td>4.48 ± 0.25</td>
<td>4.32 ± 0.15</td>
<td>7.92 ± 0.70ab</td>
<td>7.60 ± 0.44</td>
<td>6.88 ± 0.23</td>
</tr>
</tbody>
</table>

All the values are expressed as mean ± SEM (n = 5–10), ***p < 0.001 and **p < 0.01, “a” versus control “b” versus VPA control, and “c” versus diabetic control. “−”show decreased in body weight (difference between final and initial body weight).

Glucose Tolerance Tests

Intraperitoneal glucose tolerance test was performed as described by Khan and Jena with some modifications [15]. In brief, animals were fasted for 6–8 h then D-glucose (2 g/kg) was administered by i.p. injection and blood samples were collected at 0, 15, 30, 60, and 120 min and plasma glucose were measured. Total AUC were calculated using the trapezoidal method [30] and expressed as % of control.

Histology and Immunohistochemistry

The pancreas and liver were fixed in 10% neutral buffer formalin and histological slides were prepared according to standardized protocol in our laboratory and described previously [31] with some modifications using commercially available kit Novolink™ Polymer Detection System (Leica, Milton Keynes, UK) according to manufacturer instruction’s using the primary antibodies against the insulin, proliferating cell nuclear antigen (PCNA), and acetylated histone H3 (Santa Cruz Biotechnology). Slides were examined using Olympus microscope (Model BX 51; Tokyo, Japan). Randomly 15–20 fields or focuses from each animal were observed and quantified by Image J (version 1.46m) software.

Evaluation of Apoptosis in Islets by TUNEL Assay

Pancreas was fixed in the chilled 10% formalin, embedded in paraffin, and cut into 5 μm thick sections. Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay was used to assess the DNA fragmentation (Catalogue No. QIA39; Calbiochem, Oncogene Research Product, La Jolla, CA, USA). The assay was performed according to the manufacturer’s instructions and total cell population and TUNEL positive cells were counted using the image analysis software ‘Isis’ (Carl Zeis, Axioimager M1, Germany) and images were acquired using charged coupled device camera and expressed as % apoptotic cells.

Histone Extraction and Immunoblotting

The histone/nuclear protein extraction was performed as previously described [15] with some modification. Approximately one (g) of tissue was homogenized in 5 mL of low sucrose (12%) buffer (buffer-A) containing protease inhibitors and homogenate was carefully layered on 5 mL high sucrose (15%) buffer (buffer-B) containing inhibitors and centrifuged at 660 × g for 10 min. Cellular pellet was suspended in buffer-A with a detergent and layered on buffer-B then centrifuged at 660 × g for 10 min resulted nuclear pellet was again suspended, layered, and centrifuged. Final pellet was dissolved in low salt buffer (LSB) with NP-40 and sonicated three times for 10 s then centrifuged at 18000 × g for 10 min. The pellet was dissolved in LSB containing 0.25 M hydrochloric acid with sonication and kept for 30 min in ice then centrifuged at 27000 × g for 30 min. The supernatant was precipitated with TCA and centrifuged at 18,000 rpm for 30 min. The pellet was washed with acetone containing HCl and subsequently with pure acetone, then histones was dissolve in water and sample for SDS-PAGE was prepared. Equal amounts of proteins were loaded in 14% SDS-PAGE and transferred onto nitrocellulose membrane. Immunoblotting was performed using anti-acetylated histone H3 and total histone H3 primary antibodies with horseradish peroxidase-conjugated secondary antibodies. Protein signals were detected by enhanced chemiluminescence and quantified with the help of Imagequant TL software (Imagequant 350; GE Healthcare, Hong Kong, China).
FIGURE 1. Effect of diabetes and VPA treatment on (A) survival rate, (B and D) glucose tolerance including area under curve (AUC) after a challenge dose of D-glucose. (C) Representative photomicrographs of histological alterations in islets stained with hematoxylin and eosin (H&E), magnification 1000×. Dashes lines show severely damage islets in diabetic pancreas. All the values are expressed as mean ± SEM, (n = 6–10). ***p < 0.001 and *p < 0.05, “a” versus control, “b” versus VPA control and “c” versus diabetic control.

Statistical Analyses

Results are shown as mean ± standard error of mean (SEM) for each group. Statistical analysis was performed using SigmaStat (Version 3.5) statistical software (Systat Software, San Jose, CA, USA). One way ANOVA was used to determine the level of significance among the difference groups, and post-hoc analysis was performed using Tukey’s test, and p < 0.05 considered to be statistically significant.

RESULTS

Effect of VPA on Body and Organ Weight as well as Mortality/Survival

The progression of diabetes significantly decreased the body weight (weight gain) gradually with time as compared with the non-diabetic animals, while VPA treatment restored the weight gain, but it was statistically insignificant (Table 1 and Supp. Figure S1). Similarly, VPA treatment restored the diabetes-induced decreased liver and pancreas weight as compared with respective controls, but it was statistically insignificant (Table 1). Moreover, VPA treatment decreased/delayed the diabetes-associated mortality rate as compared with respective controls (Figure 1A).

Effect of VPA on Glucose, HbA1c, and Glucose Tolerance

VPA treatment significantly decreased the diabetes-induced plasma glucose as compared to controls (Table 1). VPA treatment failed to decrease diabetes-associated increase in the HbA1c level as compared with respective controls (Table 1). Diabetes led to
FIGURE 2. Effect of diabetes and VPA treatment on the beta-cell proliferation and insulin expression in pancreas of juvenile rat. Representative photomicrographs of IHC showing expression of insulin and PCNA in islets, magnification 1000×. Arrows show the PCNA positive cells. Dashes-line circles show islets. All the values are expressed as mean ± SEM (n = 5). ***p < 0.001 and **p < 0.01, “a” versus control, “b” versus VPA control and “c” versus diabetic control.

significantly impair the glucose tolerance and clearance following single challenge dose of glucose, while VPA (300 mg/kg) significantly improved the impaired glucose tolerance as compared with respective controls (Figures 1B and 1D).

Effect of VPA on Histological Alterations in Islets (Endocrine Pancreas)

Diabetes induced significant histological alterations particularly in the endocrine pancreas as compared with normal control. Approximately 70%–80% obliteration of endocrine cells (beta-cell) in islets as well as decreased size of islets was observed in diabetic animals as compared with control (Figure 1C). VPA treatment significantly ameliorated the above histological alterations such as decreased islets size and beta-cell damage as compared with diabetic control (Figure 1C and Supp. Figure S2).

Effect of VPA on Beta-Cell Proliferation, Function, and Apoptosis

To evaluate the beta-cell proliferation and function the expression of PCNA and insulin were evaluated in the pancreas by immunohistochemistry (IHC).
Results revealed that diabetes significantly decreased the expression of both PCNA and insulin, while VPA treatment significantly increased the same in a dose-dependent manner as compared with respective controls (Figure 2). Further, VPA treatment also significantly reduced the diabetes-associated beta-cell apoptosis as evident by TUNEL assay results (Figure 3). The above results confirmed that VPA treatment restored diabetes-induced decreased plasma insulin level as well as histological alteration in islets as compared with respective controls (Table 1 and Figure 1C).

**DISCUSSION**

The present study demonstrated that VPA treatment significantly decreased the plasma glucose, HbA1c, beta-cell apoptosis, and improved glucose clearance and insulin synthesis/expression as well as delayed mortality rate, which confirmed its anti-diabetic role. The histological evaluation also revealed that VPA treatment significantly ameliorated the beta-cell damage. In the pancreas, HDACs is tightly controlled at normal physiology and modulates its development and cellular differentiation, thereby function [11]. HDAC inhibitors can increase beta-cell mass through modifying the differentiation and proliferation as well as reducing its apoptosis against inflammatory cytokines in STZ-induced diabetes in rodents and INS-1 cells [11, 13, 15]. IHC results indicated that VPA significantly increased the beta-cell proliferation and function as revealed PCNA and insulin expression. Thus, our results indicated that VPA exerts protective effect in T1DM by improving beta-cell mass/function at biochemical and structural level. The possible mechanism for the protective effects of VPA might be HDAC inhibition and subsequent modulation of various genes and transcription factors in non-diabetic animals as compared with untreated animals (Figures 4B and 4C).

**Effect of VPA on HDAC Inhibition and Histone Acetylation**

To evaluate the HDAC inhibition potential of VPA in the present experiment acetylation of histone H3 was evaluated by Western blotting and IHC in pancreas. IHC results also confirmed that VPA treatment significantly restored the acetylation of histone H3 in diabetic rat as compared with respective controls (Figure 4A). Further, VPA treatment significantly increased the diabetes-associated decreased acetylation of histone H3 in the pancreas as compared with respective controls (Figures 4B and 4C). Moreover, VPA *per se* increased the acetylation of histone H3 in non-diabetic animals as compared with untreated animals (Figures 4B and 4C).

**FIGURE 3.** Effect of diabetes and VPA treatment on the beta-cell apoptosis in islets of juvenile rat. Representative photomicrographs of IHC showing TUNEL positive cells (apoptotic) in islets, magnification 1000×. Arrows show the TUNEL positive (apoptotic) cells. Dashes-line circles show islets. All the values are expressed as mean ± SEM (n = 5). ***p < 0.001, “a” versus control, “b” versus VPA control and “c” versus diabetic control.
associated with T1DM. These findings supported by previous report that butyrate reduces the beta-cell apoptosis and improves glucose homeostasis by modulating p38/ERK signaling through HDAC inhibition in rat [15]. Although, VPA is a first-line drug for the treatment of epilepsy, but it has adverse effects such as GI disturbances, hepatotoxicity, and reproductive toxicity generally observed during chronic therapy [28, 29]. The present results also confirmed that VPA treatment did not induce any hepatotoxicity as revealed by ALT, AST levels, and histological observation (Supp. Figure S3).

HDAC inhibitors attenuated the expression of pro-apoptotic proteins and beta-cell damage by preventing interleukin-1β (IL-1β)-induced activation of NF-κB and apoptosis signaling in experimental studies [12, 13]. In the present study, VPA treatment significantly reduced the diabetes-associated beta-cell apoptosis, thereby increased its number/mass, which also supported by PCNA expression. The next obvious question is whether the increased beta-cell number/mass is functional or not, insulin expression was evaluated by IHC. Our findings indicated that VPA treatment significantly increased the insulin expression and plasma insulin level, which assured that the increased beta-cells are fully functional (Figure 2 and Table 1). These findings are in agreement with the previous report, which highlighted that VPA directly enhances the insulin secretion in epileptic patients [19]. HDACs play a critical role in the regulation of synthesis/expression of insulin by modulating the histone acetylation [9, 32]. Thus, HDAC inhibition by VPA might contribute toward increase insulin level. It is worthy to mention that chronic exposure of VPA

**FIGURE 4.** (A) Representative photomicrographs of IHC showing the effect of diabetes and VPA treatment on the expression of acetylation of histone H3 in islets, magnification 1000×. Arrows show the acetylated histone H3 positive cells, while dashes-line circles show islets. (A and B) Representative western blots showing the effect of diabetes and VPA treatment on the expression of acetylation of histone H3 in pancreas. All the values are expressed as mean ± SEM (n = 5). ***p < 0.001, **p < 0.01 and *p < 0.05, “a” versus control, “b” versus VPA control and “c” versus diabetic control.
facilitates insulin release by reducing $K_{\text{ATP}}$ channel current in mouse islets [20]. Moreover, VPA administration decreases the glucose level in spontaneously diabetic BB/E rats by modulating carbohydrate and fat metabolism [33]. Further, VPA ameliorated the diabetic nephropathy, renal fibrosis, and pancreatic damage by inhibiting iNOS/NF-κB signaling as well as facilitating autophagy through HDAC inhibition [24, 25, 34]. Together, HDAC inhibition by VPA might be one of the most possible mechanisms for increased beta-cell proliferation and function as well as improved glucose homeostasis. Additionally, VPA is a pleiotropic agent and acts on multiple targets, which might be partly contributed to its protective role in diabetes [35].

The present study demonstrated that VPA increased the beta-cell proliferation, function, and reduced the beta-cell apoptosis. Further, VPA treatment also reduced the plasma glucose, HbA1c, and increased insulin production/expression through HDAC inhibition and might be beneficial for the treatment of juvenile diabetes.

SUPPORTING INFORMATION

**Figure S1.** Effect of diabetes and VPA treatment on body weight of juvenile rats during 3 weeks treatment. All the values are expressed as mean±SEM, (n=6-10). **Figure S2.** Effect of diabetes and VPA treatment on histological alterations in pancreas after 3 weeks treatment. Representative photomicrographs of histological alterations in islets stained with hematoxylin and eosin (H&E), magnification 400x. **Figure S3.** Effect of diabetes and VPA treatment on the histological alterations and liver function marker (ALT and AST). (A) Representative photomicrographs of histological alterations in islets stained with hematoxylin and eosin (H&E), magnification 400x. (B and C) Plasma levels of ALT and AST after 3 week treatment of VPA. All the values are expressed as mean±SEM, (n=6).

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

REFERENCES


