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Chemical hypoxia in human pluripotent NT2 stem cell-derived neurons: Effect of hydroxamic acid and benzamide-based epigenetic drugs

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ABSTRACT: Hypoxia-induced oxidative stress contributes to neuronal damage leading to many neurodegenerative disorders. Hypoxia promotes many downstream effectors such as hypoxia-inducible factor-1α (HIF-1α) in order to restore respiratory homeostasis due to low oxygen availability and increased ROS. Use of histone deacetylase (HDAC) inhibitors may modulate hypoxia-induced neuronal cell damage. In this study, we used two chemically diverse HDAC inhibitors to investigate their effect on hypoxia-exposed neuronal cells. Human pluripotent NT-2 stem cell-derived neuronal differentiated cells were exposed to $CoCl_2$ pre-treatment for 6h to induce hypoxia, prior to supplementation of HDAC inhibitor (SAHA or MGCD0103). Treatment with HDAC inhibitor improved cell viability in hypoxia-induced neuronal cells. The increased HIF1α expression in hypoxia-induced neuronal cells was blunted by these HDAC inhibitors with a concomitant decrease in ROS production. $CoCl_2$ treatment caused an increase in IL-1β, which was significantly inhibited by these HDAC inhibitors. Furthermore, apoptosis induced in these $CoCl_2$ treated neuronal cells was mitigated by SAHA as well MGCD0103 suggesting that these HDAC inhibitors are capable of reducing cellular toxicity, inflammation and apoptosis, and thus, could be beneficial as therapeutic molecules for many neuropathological conditions.

Keywords: pluripotent; neuron; hypoxia; histone deacetylase; gene expression

1.0 INTRODUCTION

Oxygen is an oxidizing agent; its homeostasis is essential for maintaining physiological balance during cell development and growth [1]. Under many pathological conditions, lack of oxygen supply (known as hypoxia condition), interferes with energy metabolism; this eventually causes cell death [2]. Neurological disorders occur due to hypoxia-induced reactive oxygen species (ROS) production in neuronal cells [3]. The prolonged hypoxic condition may lead to neuronal DNA damage, cell death and apoptosis, resulting in brain injury [4,5]. In hypoxic conditions, mitochondria release apoptotic proteins such as cytochrome c (Cyt c) responsible for oxidative phosphorylation and reduced ATP synthesis [6]. Increased Cyt c is associated with increased ROS production due to decrease in antioxidant enzymes activity [7], which may lead to oxidative stress (OS). Hypoxia is regulated by the hypoxia-inducible factor (HIF), a heterodimeric transcription factor [8] that regulates oxygen homeostasis via the presence of an oxygen-regulated subunit, HIF-1α [9]. Heme oxygenase 1 (HMOX1) plays an important role in the antioxidant defense system and iron homeostasis [10]. In general, ROS production in the mitochondria is responsible for stabilizing HIF-1α [11] and regulates HMOX1 [12]. Mitigation of OS can be achieved by altering transcriptional regulation via epigenetic modifiers in order to cause biased induction of cell survival pathways [13,14].

Pharmacological inhibition of these epigenetic modifiers, histone deacetylases (HDACs) is vital for protection against many pathological disorders. Alteration in these epigenetic factors may be a beneficial approach to promote oxidative defense mechanism [15]. Effectiveness of HDAC inhibitors (HDACi) has been shown to control many biological responses, including oxidative stress and inflammatory responses, which in turn, affect downstream signaling and systemic cellular functions. Currently, four FDA-approved HDAC inhibitors are in use for the treatment of different types of cancers and tumors [16]. Also, suberoylanilide hydroxamic acid (SAHA), a class I and II HDACi, has been approved as a treatment for cutaneous T-cell lymphoma [17]. The benzamide-based drug, MGCD 0103 exhibited potent and selective antiproliferative activities against a broad spectrum of human cancer cell lines *in vitro* and *in vivo* [18]. Clinical trials of many of these FDA-approved HDAC inhibitors, however, failed to prove efficacy and effectiveness due to lack of complete understanding of their mechanisms at the cellular level. Experimental evidence suggests that inhibition of HDAC may protect neuronal cells from brain ischemia injury and other neurodegenerative disorders [19-21]. Nevertheless, their effect at the cellular level during many neuropathological conditions such as neuronal ischemic injury and micro-vacuolation of the cerebral cortex needs to be explored.

NT2 cells are differentiated into neuronal cells upon retinoic acid treatment [22]. These differentiated neuronal cells express many structural proteins such as microtubule-associated proteins (MAPs) and neuronal cell adhesion molecules (NCAMs) as well as and functional proteins such as calmodulin and G-protein-coupled receptors (GPCRs) and thus could be used to mimic molecular signaling underlying human neurodegenerative diseases and disorders. Accumulation of the neurotransmitter glutamate around the neurons increases Ca2+ influx that activates many signaling pathways [23,24]. Effect of HDAC inhibitors in the mitigation of oxidative stress, inflammation and apoptosis in these hyper-active differentiated neuronal cells has not been fully elucidated. In our present study, we investigated the effect of two chemically diverse HDAC inhibitors (Vorinostat; SAHA and Mocetinostat; MGCD0103) on different cellular functions in hypoxia-exposed neuronal cells.

2.0 MATERIALS AND METHODS

2.1 Cell culture and viability assay

Human pluripotent stem cell-derived NTERA-2 cl.D1 (NT2/D1; ATCC) neuronal precursor cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin-glutamine at 37°C with 5% CO_2. Differentiation of NT2 precursor cells into neurons was

performed following standard protocols [22,25]. NT2 cells were treated with 10 µM retinoic acid every 4 days for 4 weeks, followed by replating of these cells in poly-D-lysine (Sigma, India; P8920) coated culture vessels for another 7 days before being used for experiments. Hypoxic incubation was performed by exposing differentiated neuronal cells to 300 µM cobalt chloride ($CoCl_2$; Sigma, India; C8661) for 6 h at 37°C. Following hypoxia, cells were treated with two chemically different HDAC inhibitors for an additional 18 hours-Vorinostat (SAHA; Sigma India SML0061; 50µM final concentration) or Mocetinostat (MGCD0103; Selleckchem India S1122; 1µM final concentration). Cell viability was determined at indicated time points using the trypan blue exclusion assay.

2.2 Cellular ROS assay

$1x10^4$ differentiated neuronal cells were seeded in 96-well tissue culture plates and exposed to normoxic or hypoxic conditions with chemical inhibitors as described above. Cellular oxidative stress was determined via measurement of ROS generation. Briefly, at the end of the experiment at 24 hours, cells were incubated with 25µM 2',7'-dichlorofluorescein diacetate (DCFDA; Invitrogen D399) for 30 minutes. The fluorescence signal was measured on a plate reader (Ex484/Em535).

2.3 Enzyme-linked immunosorbent assay (ELISA)

96-well plates were coated with 250ng of capture antibody (HIF-1α, Novus NB100-105) and incubated overnight at 4°C. Next day, the plates were acclimated at room temperature (RT) and washed twice with washing buffer (PBS containing 0.1% Tween 20 and 1% BSA). Non-specific sites were blocked with 5% BSA for 30min at RT. The plates were washed and incubated for 2h at RT with 100µl of 1:10 dilution of antigen (1mg/ml of HDAC inhibitor-treated or control protein samples). Unbound antibody was removed by washing thrice with washing buffer. After washing, 100µl of 1:500 dilution of horseradish peroxidase-conjugated IgG specific secondary antibody was added to each well. Color was developed by adding 50µl of 0.1% substrate ABTS [2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)] (Sigma, A9941) in 0.1M sodium citrate buffer (pH4.5) and 0.1% H_2O_2 for 15 min at RT in dark. Finally, the reaction was stopped by adding 20ul of 10% SDS to each well. OD was recorded at 492nm in ELISA reader (BioRad, USA). Graphs were plotted and calculation was done to estimate the concentration of protein present in the samples using standard recombinant protein plot.

2.4 Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Total RNA was extracted using Trizol Reagent (Invitrogen, 15596026) following the manufacturer's instructions. qPCR analysis was performed using the qScript One-Step RT-qPCR Kit (Quantabio, 95057) and specific primers (Table 1). The mRNA levels were normalized to β-actin and expressed as fold change from controls using the $2^{-\Delta\Delta Ct}$ method.

Table 1. Primer sequences for qRT-PCR analysis

Gene target	5' primer	3' primer
HMOX-1	CAAAGTGCAAGATTCTGCCC	CAACTGTCGCCACCAGAAAG
TAU	GTAAAAGCAAAGACGGGACTGG	ATGATGGATGTTGCCTAATGAG
GluR	AACCTGCAGAACCGCAAG	GCTTGATGAGCAGGTCTATGC
BAX	TGATGGACGGGTCCGGG	TCCTGGATGAAACCCTGAAGC
BCL-xL	AGGCGGATTTGAATCTCTTTCTCT	GGGCTCAACCAGTCCATTGT
IL-1β	GCCAATCTTCATTGCTCAAGT	AGCCATCATTTCACTGGCGA
β-*Actin*	TCATTCCAAATATGAGATGCG	TAGAGAGAAGTGGGGTGGCT

2.5 Statistical analysis

Data were statistically analyzed in GraphPad Prism and presented as mean ± SEM. One-way or two-way ANOVA or t-test was performed, and $p < 0.05$ was considered significant. Results are presented from at least three independent experiments.

3.0 RESULTS

3.1 HDAC inhibitors mitigate oxidative stress and improve NT2-N survival after hypoxic injury

As demonstrated by several groups, four weeks of retinoic acid treatment renders NT-2 cells to differentiate into neuronal cells (NT2-N) (Figure 1A-1C). Hypoxia was induced in these differentiated neuronal cells by using $CoCl_2$ and at 6h, there was a significant reduction (30%) in cell survival (Figure 1D). Time-dependent effect of HDAC inhibitors on $CoCl_2$-treated NT2-N cells indicated dramatic maintenance in cell survival after 6h of treatment, which was 80 - 90% in SAHA treated group and 85 - 95% in MGCD0103-treated group compared to hypoxia group (80 - 85%). When compared to 24h of $CoCl_2$-treated hypoxia condition, both HDAC inhibitors improved cell survival at an average of 20%. Furthermore, increased oxidative stress by ROS production in hypoxia condition (2.5 fold compared to control) was found to be significantly decreased by 40% in both the inhibitor treatment groups (Figure 1E).

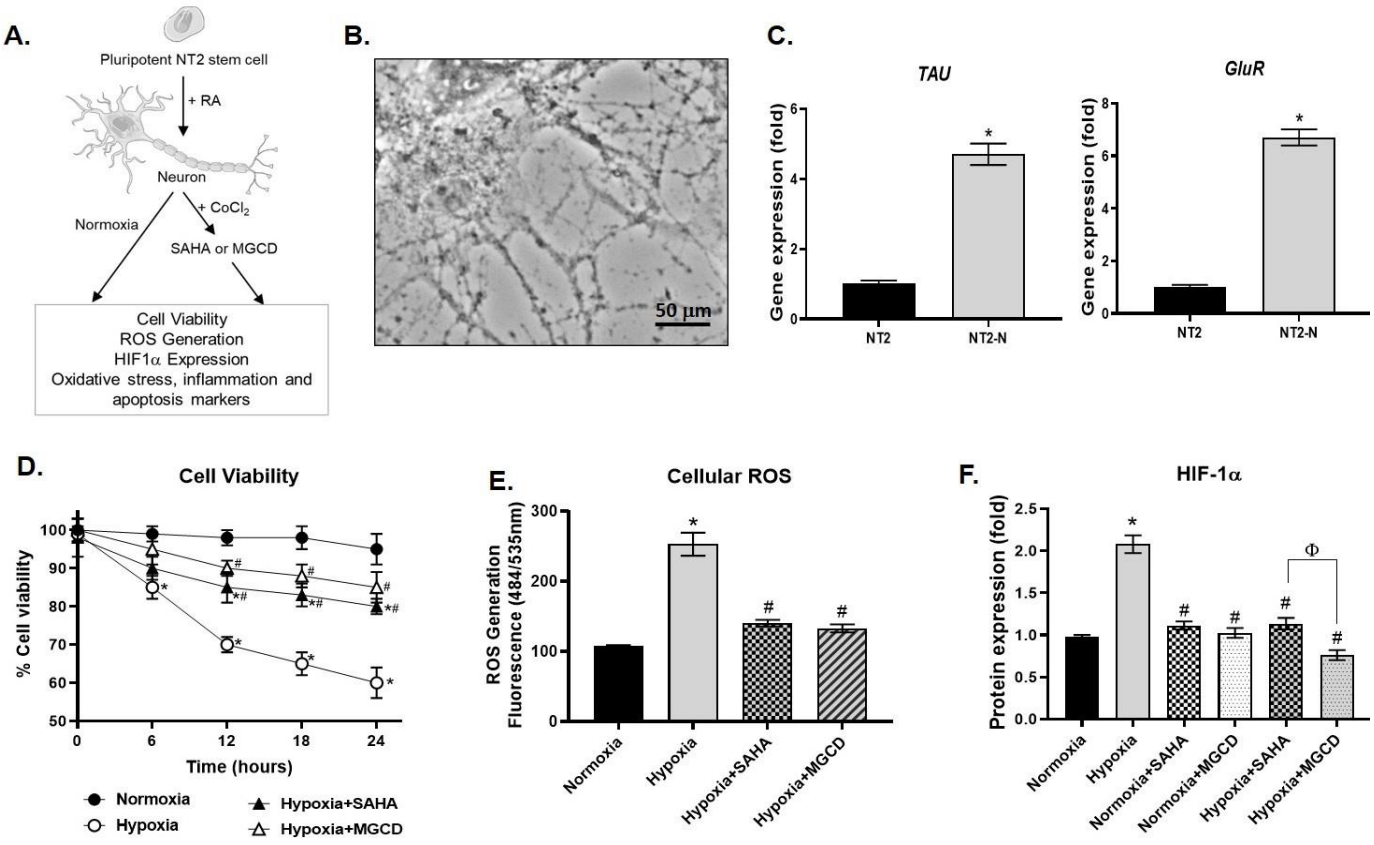

Figure 1. Effect of HDAC inhibitors on hypoxia-induced cell death, ROS generation, and HIF-1α expression. (**A**) Schematic for experimental design of chemically induced hypoxia (+$CoCl_2$) and cell treatments. (**B**) Bright-field image of NT2 differentiated neurons, scale bar= 50µm. (**C**) NT2-N differentiation markers, *TAU* and *GluR*, as assessed by qRT-PCR. *p<0.05 vs. NT2, n=3, mean±SEM. (**D**) Cell viability, assessed via trypan blue exclusion assay, at various time points. *p<0.05 vs. normoxia, $^{\#}p$<0.05 vs. hypoxia at respective time points, n=6, mean±SEM. (**E**) ROS generation quantification at endpoint (24h). *p<0.05 vs. normoxia, $^{\#}p$<0.05 vs. hypoxia, n=3, mean±SEM; (**F**) HIF-1α protein expression assessed via ELISA at endpoint. *p<0.05 vs. normoxia, $^{\#}p$<0.05 vs. hypoxia, $^{\Phi}p$<0.05 as indicated, n=4, mean±SEM.

The classical hypoxia marker, HIF-1α, was elevated by approximately two-fold in $CoCl_2$-treated NT2-N cells and shown to decrease HIF-1α expression upon HDAC inhibitor treatment. This suggests that HDAC inhibitors improve cellular response to hypoxia-induced oxidative stress (Figure 1F). As compared to SAHA treated group, HIF-1α expression in MGCD0103-treated hypoxic NT2-N cells was lower with improved cell survival compared to SAHA, suggesting MGCD0103 may be a potent anti-hypoxic inhibitor. These inhibitors showed no effect in normoxic cells.

3.2 HDAC inhibitors promote anti-oxidant response and mitigate heme oxygenase 1- induced apoptosis and inflammation

Effect of SAHA and MGCD0103 on anti-oxidant *HMOX1* (Figure 2A), as well as pro-inflammatory cytokine IL-1β (Figure 2B), in $CoCl_2$-treated neuronal cells, was assessed by qRT-PCR. There was a substantial increase (~4-fold) in *HMOX1* gene expression in hypoxic NT2-N cells. Administration of HDAC inhibitors significantly inhibited *HMOX1* gene expression by 50%. Similarly, the pro-inflammatory cytokine *IL-1β* gene expression was reduced to about 50% by these two HDAC inhibitors in hypoxic neurons. The ratio of pro-apoptotic BAX and anti-apoptotic BCL-xL genes was determined using qRT-PCR (Figure 2C). As anticipated, the BAX/BCL-xL gene expression ratio was strongly upregulated (1.7-fold) in response to hypoxia. Intriguingly, this increase in BAX/BCL-xL ratio was dramatically reduced to normal levels in the presence of HDAC inhibitors, SAHA or MGCD0103.

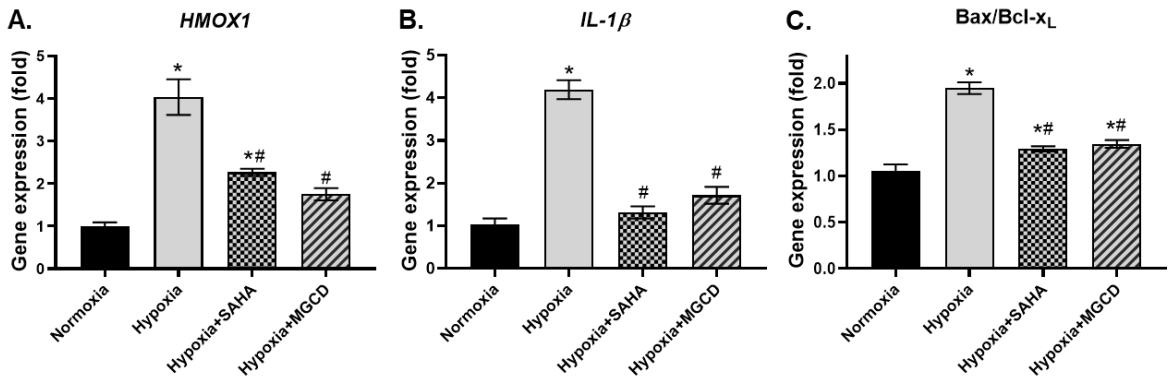

Figure 2. Effect of HDAC inhibitors on hypoxia-induced expression of oxidative stress, inflammatory and apoptotic gene expression. mRNA expression of (**A**) HMOX1; (**B**) IL-1β; and (**C**) Bax/Bcl-x_L at endpoint. *p<0.05 vs normoxia, #p<0.05 vs hypoxia, n=3-4, mean±SEM.

4.0 DISCUSSION & CONCLUSION

The regulation and modification of non-histone proteins are crucial to controlling of a multitude of cellular functions. Histone deacetylases act on these non-histone proteins and alter cellular signaling through post-translational modification [26]. Modification of transcriptional gene regulation by epigenetic modifiers may alter OS and reduce cell death [13,15]. Hypoxia-induced OS-mediated stabilization of HIF-1α is well established [11]. We have shown that inhibitors of epigenetic modifiers such as mocetinostat can greatly influence HIF-1α expression in chemically induced hypoxia in neuronal cells via repression in ROS production. Inhibition of HIF-1α was significantly higher in MGCD0103 treated cells than SAHA, suggesting MGCD0103 modulates HIF-1α induced hypoxia through ROS inhibition stronger than SAHA. Role of HIF-1α in HO-1 gene regulation was previously established [27], which is regulated through E2-related factor 2 (Nrf2)/AKT pathway [12]. HMOX1 gene activity in

oxidative stress condition induced by $CoCl_2$ has been shown. Inhibition of HMOX1 by HDAC inhibitors is essential to inhibit HIF-1α, which is evident through our study. Increased HMOX1 in $CoCl_2$-induced hypoxic NT2-N cells was mitigated by HDAC inhibitors, suggesting that HDAC inhibition destabilizes hypoxic factor HIF-1α and ROS production. Furthermore, HMOX1 regulates many inflammatory responses. HO-1 is induced by HIF-1α to modulate the activation and function of different inflammatory cells [28]. We analyzed inflammatory behavior in these hypoxic neuronal cells in responses to HDAC inhibitors. In $CoCl_2$-induced hypoxia, increased HMOX1 gene activity post-transcriptionally enhanced inflammatory gene IL-1β expression. Furthermore, increased IL-1β in hypoxic neuronal cells was reduced by HDAC inhibitors suggesting that HDAC inhibitors may have anti-inflammatory property. Inflammasome mediated caspase-1 drives maturation of IL-1β. While we did not measure caspase 1 activity, nevertheless it is known that caspase-1 activation leads to caspase-3 activation and apoptotic cell death [29]. On the other hand, Caspase-2,-3 and -9 are transiently activated, during the RA-induced neuronal differentiation of NT2 cells [30]. Also, cleavage of caspase-3 induces apoptosis via mitochondrial pro-apoptotic protein Bax. Increased ratio of Bax over anti-apoptotic protein Bcl-xL was mitigated by HDAC inhibitors. Overall, this study suggests that both Vorinostat and Mocetinostat are potent regulators of ROS-mediated hypoxia in neuronal cells and modulate, resulting apoptosis and inflammation. Further studies directed at studying the effect of these epigenetic modulators using in vivo models of neuroinflammation and neurodegenerative diseases will provide a rationale for the repurposing of these drugs and others in clinical trials to treat patients with neuropathological conditions.

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