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ANTI-INFLAMMATORY PROPERTIES OF HISTONE DEACETYLASE INHIBITORS: A MECHANISTIC STUDY

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Abstract

Background—We have demonstrated that post-shock administration of suberoylanilide hydroxamic acid (SAHA), a histone deacetylase inhibitor (HDACI), can significantly improve early survival in a highly lethal model of hemorrhagic shock. As the primary insult in hemorrhagic shock is cellular hypoxia, and transcription factor hypoxia-inducible factor-1 (HIF-1) controls pro-inflammatory gene expression in macrophages, we hypothesized that SAHA would attenuate the HIF-1 associated pro-inflammatory pathway in a hypoxic macrophage model.

Methods—Mouse macrophages were exposed to hypoxic conditions (0.5% O₂, 10% CO₂, and 89.5% N₂) at 37°C in the presence or absence of SAHA (10 µmol/L). The cells and culture medium were harvested at 1 hour, 4 hours and 8 hours. Sham (no hypoxia, no SAHA) served as a control. Western blots were performed to assess protein levels of prolyl hydroxylase 2 (PHD2), HIF-1 and inducible nitric oxide synthase (iNOS) in the cells. Colorimetric biochemical assay and ELISA were utilized to analyze the release of nitric oxide (NO) and secretion of tumor necrosis factor (TNF-) respectively in the cell culture medium.

Results—Hypoxia significantly increased cellular level of HIF-1 (1 and 4 hours), gene transcription of iNOS (4 and 8 hours), iNOS protein (8 hours), NO production (8 hours), and TNF- secretion (4 & 8 hours). SAHA treatment attenuated all of above hypoxia-induced alterations in the macrophages. In addition, SAHA treatment significantly increased cellular level of PHD2, one of the upstream negative regulators of HIF-1, at 1 hour.

Conclusions—Treatment with SAHA attenuates hypoxia- HIF-1 -inflammatory pathway in macrophages, and suppresses hypoxia-induced release of proinflammatory NO and TNF-. SAHA also causes an early increase in cellular PHD2, which provides, at least in part, a new explanation for the decrease in the HIF-1 protein levels.

Keywords

Hypoxia; macrophages; SAHA; HIF-1; PHD2

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INTRODUCTION

Hemorrhagic shock (HS) initiates a systemic inflammatory response that contributes to organ injury and early multi-organ failure,¹ and accounts for up to 40% of trauma-related deaths.² The tissue hypoxia that occurs following hemorrhagic shock has been thought to play a major role in the alteration of cell metabolism and organ function.³

It is well known that hypoxic adaptation is primarily regulated by hypoxia-inducible factors (HIFs) in mammalian cells. Under normal O₂ levels, prolyl hydroxylases (PHDs) modify the subunits of HIFs at two conserved proline residues, causing HIFs to be ubiquitinated via a specific von Hippel-Lindau (pVHL)-E3 ligase complex and subsequently degraded by proteasomes.⁴⁻⁷ Hypoxia reduces PHD hydroxylase activities and stabilizes subunits of HIFs. Stabilized subunits of HIFs translocate to the nucleus, form dimers with constitutive HIF-1 and bind to co-activators, permitting transcriptional activation of many hypoxia-response element (HRE)-bearing genes encoding metabolic, angiogenic and metastatic factors.⁸

HIFs are recognized to be important transcription factors that regulate the expression of many hypoxia-related pro-inflammatory cytokine/chemokine genes in macrophages.⁹ Of the subunits of HIFs, HIF-1 appears to be expressed ubiquitously.⁸ Studies have shown that HIF-1 mediates macrophage inflammatory responses, and plays pivotal roles in the development of myeloid cell-mediated inflammation and regulation of bactericidal capacity.¹⁰

We have demonstrated that post-shock administration of suberoylanilide hydroxamic acid (SAHA), a histone deacetylase inhibitor (HDACI), can significantly improve early survival in a highly lethal model of hemorrhagic shock, even in the absence of conventional fluid resuscitation.¹¹ The objective of the present study was to investigate the underlying protective mechanisms. More specifically, we wanted to determine whether SAHA treatment attenuates the hypoxia- HIF-1 -inflammatory pathway.

MATERIALS AND METHODS

Cell culture and treatment

Murine macrophages RAW 264.7 cells (American Type Culture Collection, Manassas, VA) were maintained in advanced D-MEM (Invitrogen, Grand Island, NY) supplemented with 10% FBS (Invitrogen, Grand Island, NY). These cells were grown at 37°C in a humidified incubator in 5% CO₂ and 95% air. The cells were subcultured before 70–80% confluence and seeded at a density of 0.25×10⁶ cells/mL, allowed to attach to the bottom of the 75 cm² flasks for 24 hours and then serum starved (0.5% FBS) overnight before exposure to hypoxic conditions (0.5% O₂, 10% CO₂, and 89.5% N₂) at 37°C with humidification in the presence or absence of SAHA (10 μmol/L). Sham (no hypoxia, no SAHA) macrophages served as the control. In addition, macrophages treated with SAHA under normoxic conditions were used for comparing protein expressions. The starting time of treatment served as the reference point (time zero). The cells were harvested in pellets and the cell culture supernatants were collected at 1 hour, 4 hours and 8 hours. Whole cell lysates were made with Whole Cell Extraction Kit (Millipore Corporation, Temecula, CA).

Western blot analysis

Equal amounts of whole cell lysate samples were separated by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis on polyacrylamide gels and transferred onto nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA). The membranes were blocked in 0.05% PBS-tween (PBST) containing 5% milk (Bio-Rad Laboratories) and then

incubated with primary antibodies against following proteins; Actin, HIF-1 (Sigma-Aldrich, Inc., St. Louis, MO), iNOS and PHD2 (Cell Signaling Technology Inc, Danvers, MA), at 4°C overnight. The primary antibodies were detected by incubation with horseradish peroxidase-coupled second antibodies against rabbit or mouse IgG (Amersham Biosciences, Piscataway, NJ) (1:3,000 in PBST with 5% milk) at room temperature for 2 hours. The chemiluminescence detections were performed by using Western Lighting Chemiluminescence Reagent Plus (Perkin-Elmer LAS, Inc., Boston, MA). Films were developed using standard photographic techniques and quantitative analysis of detected bands was carried out by densitometer scanning using the VersaDoc Imaging System (Bio-Rad Laboratories).

RNA Isolation and Real-Time Polymerase Chain Reaction (real-time PCR)

Total RNA was prepared with the RNeasy Mini Kit (Qiagen, Valencia, CA), and mRNA was reverse transcribed into cDNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) according to the manufacture's instructions. Equal amounts of cDNA were submitted for PCR in the presence of SYBR Green Master Mix and forward and reverse primers using the ABI PRISM 7300 Real-Time PCR detection machine (Applied Biosystems). The primers for TNF- (forward: 5'-CCC ACT CTG ACC CCT TTA CT-3'; reverse: 5'-TTT GAG TCC TTG ATG GTG GT-3') and iNOS (forward: 5'-CTT TGT GCG AAG TGT CAG TG-3'; reverse: 5'-CAC CTG GAA CAG CAC TCT CT-3') were synthesized by Real Time Primers (Elkins Park, PA). GAPDH (forward: 5'-CCTGGAGAAACCTGCCAAGTAT-3'; reverse: 5'-CTCGGCCGCCTGCTT-3') was used as an internal control and was synthesized by Invitrogen (Carlsbad, CA). PCR was performed with 50 cycles of 10 seconds at 95°C, and 45 seconds at 58°C. Each sample was run in triplicate. Relative quantification of mRNA expression was performed using the $2^{-\Delta\Delta C_t}$ method where ΔC_t is the calculated difference in the C_t (threshold cycle) values between TNF-, iNOS and GAPDH, and $\Delta\Delta C_t$ is the calculated difference between the ΔC_t for a given sample and the ΔC_t for sham, such that the relative quantity of mRNA, normalized to GAPDH and sham, is calculated as $2^{-\Delta\Delta C_t}$.

Evaluation of hypoxia-induced NO release and TNF- α secretion from RAW264.7 cells

Macrophage NO production was determined by measuring the concentration of the stable NO degradation products nitrate and nitrite in the cell culture medium using Parameter colorimetric biochemical assay kit for NO (R&D Systems, Minneapolis, MN). Briefly, cell culture medium was thawed, and nitrate in the samples was converted to nitrite by the addition of nitrate reductase. Nitrite concentration was then detected by the addition of Griess reagent and quantitatively measured by a SpectramaxPlus 384 microplate reader (Molecular Devices, Sunnyvale, CA) at 540 nm/690 nm. Quantitative determination of TNF- in the cell culture medium was made using the Quantikine ELISA Kit (R&D Systems, Minneapolis, MN) according to manufacturer's instruction. The concentrations of TNF- were measured by optical densitometry at 450 nm/540 nm in the same microplate reader mentioned above. All of the analyses were performed in triplicate.

Statistical analysis

All quantitative data are described as the mean $\pm$ SD (SD). Data were analyzed using Microsoft Office Excel 2003. Statistical differences were determined by Student t tests. A p 0.05 was considered as statistically significant.

RESULTS

Effects of SAHA treatment on cellular protein expression of PHD2 and HIF-1 . Immunoblotting with anti- HIF-1 antibody showed that hypoxia significantly increased

HIF-1 protein levels at 1 and 4 hours, while treatment with SAHA reversed these changes (Fig. 1, $p < 0.01$). To explore whether the effect of SAHA on HIF-1 correlates with its upstream negative regulator PHD2, we immunoblotted the whole cell lysate harvested at 1 and 4 hours with anti-PHD2 antibody. Significant up-regulation of PHD2 protein expression caused by SAHA treatment in hypoxia-stimulated macrophages appeared at 1 hour (Fig. 2, $p < 0.01$), and disappeared afterward. Under normal (non-hypoxic) condition, SAHA had no effect on HIF-1 and PHD2 proteins (see Fig 1b and Fig 2b).

SAHA suppresses hypoxia-induced iNOS gene transcription, iNOS protein expression and iNOS activity (NO production). It is well known that HIF-1 can activate transcription of iNOS gene. To find out whether inhibition of HIF-1 by SAHA could affect iNOS gene transcription, we used real-time PCR. It showed that hypoxia up-regulated iNOS gene expression within 4 hours ($p < 0.05$), and SAHA treatment significantly down-regulated it compared with the hypoxia group (Fig. 3a, $p < 0.001$).

To determine whether the inhibition of gene transcription of iNOS by SAHA can decrease protein levels of iNOS, immunoblottings were performed with anti-iNOS antibody. Hypoxia significantly increased level of iNOS protein (8 hours) whereas SAHA treatment significantly attenuated this change (Fig. 3b, $p < 0.01$).

Nitrite levels were used as a measure of NO released into the culture medium as a result of hypoxia-induced iNOS production. Significant increase in NO release was noted at 8 hours, which was significantly reduced by SAHA (Fig. 4, $p < 0.01$).

SAHA suppresses transcription and secretion of TNF- α protein. HIFs are important transcription factors regulating expression of many pro-inflammatory cytokine/chemokine genes. Real-time PCR was employed to determine whether inhibition of HIF-1 by SAHA could affect transcription of TNF- α gene. TNF- α gene transcription was significantly decreased at 1, 4 and 8 hours compared with the untreated macrophages (Fig. 5a, $p < 0.01$).

To determine the impact of SAHA treatment on levels of the protein production, ELISA was used to measure TNF- α concentration in the cell culture medium. Hypoxia significantly increased TNF- α secretion within 4 hours, which remained even till 8 hours. SAHA significantly attenuated the alteration (Fig. 5b, $p < 0.001$).

DISCUSSION

Hemorrhagic shock (HS) results in cellular hypoxia and a systemic inflammatory response, and modulation of these changes can potentially have a beneficial impact. We have previously shown that treatment with HDACIs improves survival in highly lethal HS and poly-trauma models in rat and swine.^{11–15} In these studies, survival advantage was noted even in the absence of conventional fluid resuscitation, and it was equally impressive regardless of whether the treatment was given pre- or post-insult. In the present study, we examined the effects of SAHA, one of the HDACIs, on the hypoxia-HIF-inflammatory pathway in mouse macrophages. Our results showed that treatment with SAHA: 1) increases cellular levels of PHD2 and decreases the levels of HIF-1 proteins, respectively; 2) represses hypoxia-induced gene transcription, cellular protein level and activity (NO production) of iNOS; and 3) suppresses hypoxia-induced TNF- α secretion.

It has been reported that stabilization of HIF subunits is dependent on a decrease in PHD protein levels as well as enzyme activity. PHD2 serves as a decisive oxygen sensor in the HIF degradation pathway. These proteins are intimately involved in regulating each others expression. Hypoxia decreases overall PHD activity, whereas an up-regulation of HIF-1

induces PHD2, which can cause a negative feedback to decrease the HIF-1 levels (“autoregulatory loop”).¹⁶

SAHA is a pan-HDAC inhibitor which inhibits class I/II/IV HDAC activity.¹⁷

Accumulating evidences suggest that inhibition of class I/II HDAC activity represses HIF, but detailed molecular and biochemical mechanisms of HDACs’ effects on HIF remain unclear. There are several possibilities: 1) HDACs mediate destabilization of HIF-1 ; 2) HDACs mediate repression of the transactivation potential of the carboxyl-terminal transactivation domain of HIF-1 ; 3) HDACs repress DNA binding ability; 4) HDACs inhibit nuclear translocation of HIF-1.¹⁸ Among three isoforms of PHD in mammalian cells, PHD2 plays a dominant role as a negative regulator of HIF-1. It is a decisive oxygen sensor in the HIF degradation pathway and the rate-limiting enzyme that sets the low steady-state level of HIF-1 in normoxia.^{19–21} In oxygenated cells, prolyl hydroxylation targets the proteins for degradation by promoting their interaction with VHL, a component of an E3 multiprotein ubiquitin-ligase complex in the cytoplasm. As a result, α -subunits of HIF are not translocated into the nucleus and HIF remains largely non-functional. As the current study was designed to determine whether SAHA had any effect on the α -subunit of HIF1, we analyzed the whole cell lysate. We now plan to investigate whether SAHA could decrease translocation of HIF into the nucleus.

In the present study, we found that an increase in cellular PHD2 was concurrent with a drop in HIF-1 levels (1 hour after SAHA treatment). It has been reported that apicidin, an HDAC2 and HDAC3 selective inhibitor,²² up-regulates PHD2 prolyl hydroxylase gene expression in cervical cancer cells.²³ However, in the present study, SAHA affected cellular PHD2 level too rapidly (1 hour) to implicate transcriptional regulation as the dominant mechanism. It is most likely to be an effect of SAHA on the function of the PHD2 protein itself. This result has never been reported before. We also made an attempt to determine whether the effect of SAHA is through acetylation of PHD2 but failed because of technical difficulties with immunoprecipitation.

Clinical dose-escalating studies with daily administration of SAHA (vorinostat) have demonstrated that it is a safe drug in patients with hematological and solid tumors. Intravenous administration of SAHA (in doses of 75, 150, 300, 600, and 900 mg/m²/d) results in plasma concentrations $\approx$ 2.5 μ mol/L, which inhibits cell proliferation in vitro.²⁴ In the current study, we incubated macrophages with 10 μ mol/L of SAHA which is 4 fold larger, compared with the dose used in patients. It should be pointed out that doses that alter gene transcription in transformed cells are not the same as those that are required to influence acute inflammatory response. We selected this large dose based on our previous data²⁵ as well as findings by other researchers.²⁶ In this proof-of-concept study, we wanted to ensure that SAHA concentration was large enough to clearly elicit an anti-inflammatory response.

It has been known that iNOS is up-regulated in lung, liver, and gut during hemorrhagic shock and resuscitation.^{27,28} On a cellular level, hypoxia has been shown to increase iNOS expression and activity in macrophages.²⁹ HIF-1 can mediate iNOS expression under hypoxic conditions, and the promoter region of iNOS contains a HIF-1 binding element.³⁰ NO derived from iNOS has been implicated in many pathologic events,^{31–33} including the development of tissue injury,³⁴ and selective inhibition of iNOS can attenuate trauma-hemorrhage/resuscitation induced hepatic injury.³⁵ In the present study, SAHA showed an ability to repress many components in the hypoxia-HIF1- α -iNOS-NO pathway, including cellular level of HIF-1 α , iNOS gene transcription, iNOS protein expression and NO production.

It is already known that levels of cytokines (TNF- α , IL-6) and chemokines (KC and MCP-1) increase following trauma-hemorrhage in plasma, hepatic tissue, hepatocytes, and Kupffer cells.³⁶ Even hemorrhagic shock without significant tissue trauma can increase pro-inflammatory mediators.³⁷ In fact, hypoxemia alone (without hemorrhage) causes a release of inflammatory cytokines.³⁸ In our study, macrophages secreted TNF- α promptly in response to hypoxia before there were any measurable changes in the iNOS expression and NO production. Moreover, although SAHA treatment decreased TNF- α gene expression at all time points compared with hypoxia groups; there was no significant increase in the hypoxia group at 1 hour compared with the sham macrophages. This could be a sampling time issue as the peak of TNF- α gene expression could have occurred before the 1 hour time point. It is also highly likely that there are additional pathways through which hypoxia stimulates TNF- α secretion in macrophages that are independent of HIF-1 α . For example, it is well known that hypoxia can activate NF- κ B by inhibiting prolyl hydroxylases that negatively modulate IKK catalytic activity, resulting in the transcription of downstream target genes, including inflammatory cytokines.³⁹ Similarly, SAHA modulates many diverse pathways. For example, in addition to gene transcription and protein synthesis, it may also modulate the mechanisms that control secretion of TNF- α by the cells.⁴⁰ Similarly, in a rat model of intestinal ischemia/reperfusion, HDACI treatment improved survival and attenuated distant organ damage (acute lung injury) through its systemic anti-inflammatory and anti-oxidant effects.⁴¹ A number of mechanisms are currently being investigated to further improve our understanding of this complex process.

In summary, we have demonstrated that SAHA attenuates hypoxia-HIF-1 α inflammatory pathway in macrophages, and suppresses release of pro-inflammatory NO and TNF- α . SAHA also causes an early increase in the PHD2 protein, which could also explain the decrease in cellular HIF-1 α protein levels (Fig. 6).

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Fig. 1. Effects of SAHA on level of HIF-1 protein in hypoxia-stimulated macrophages (1a) and normal macrophages (1b)

Mouse macrophages RAW264.7 were harvested at 1 and 4 hours after hypoxia in the presence or absence of SAHA. Sham (no SAHA, no hypoxia) macrophages served as a control. Whole cell lysates were subjected to western blotting with anti-HIF-1 and anti-actin antibodies. Specific bands were quantified by densitometry and expressed as mean values $\pm$ SD ($n = 3$). # denotes a significant difference compared with the sham group ($p < 0.01$). * denotes a significant difference compared with the hypoxia groups ($p < 0.01$). All time points (hours) in this study used start of treatment as the reference point (time zero).

Fig. 2. Effects of SAHA on level of PHD2 protein in hypoxia-stimulated macrophages (2a) and normal macrophages (2b)

Mouse macrophages RAW264.7 were harvested at 1 and 4 hours after hypoxia in the presence or absence of SAHA. Sham (no SAHA, no hypoxia) macrophages served as a control. Whole cell lysates were subjected to western blotting with anti-PHD2 and anti-actin antibodies. Specific bands were quantified by densitometry and expressed as mean values $\pm$ SD ($n = 3$). * denotes a significant difference compared with the hypoxia group ($p < 0.01$). All time points (hours) in this study used start of treatment as the reference point (time zero).

Fig. 3. (a) Effects of SAHA on gene expression of iNOS in hypoxia-stimulated macrophages Mouse macrophages were harvested at 1, 4 and 8 hours after hypoxia with or without SAHA treatment. iNOS mRNA levels were determined by real-time PCR and expressed as mean values $\pm$ SD ($n = 3$). # denotes a significant difference compared with the sham group ($p < 0.05$). *denotes a significant difference compared with the hypoxia group ($p < 0.001$). **(b) Effects of SAHA on cellular protein level of iNOS in hypoxia-stimulated macrophages.** Mouse macrophages RAW264.7 were harvested 1, 4 and 8 hours after hypoxia in the presence or absence of SAHA. Whole cell lysates were subjected to western blotting with anti- iNOS and anti-actin antibodies. Specific bands were quantified by densitometry and expressed as mean values $\pm$ SD ($n = 3$). # denotes a significant difference compared with the sham group ($p < 0.01$). * denotes a significant difference compared with the hypoxia group ($p < 0.01$). Sham (no SAHA, no hypoxia) macrophages served as a control. All time points (hours) in this study used start of treatment as the reference point (time zero).

Fig. 4. Effects of SAHA on production of NO in hypoxia-stimulated macrophages

Cell culture medium of RAW264.7 macrophages was harvested 4 and 8 hours after hypoxia with or without SAHA treatment. Sham (no SAHA, no hypoxia) macrophages served as a control. Nitrite level was used as a measure of NO released into the cell culture medium. Concentrations of Nitrite in the cell culture mediums were determined by colorimetric biochemical assay. The NO concentration was expressed as mean values $\pm$ SD ($n = 3$). # denotes a significant difference compared with the sham group ($p < 0.01$). * denotes a significant difference compared with the hypoxia group ($p < 0.01$). All time points (hours) in this study used start of treatment as the reference point (time zero).

a

b

Fig. 5. (a) Effects of SAHA on expression of TNF- α gene in hypoxia-stimulated macrophages Mouse macrophages were harvested 1, 4 and 8 hours after hypoxia with or without SAHA treatment. TNF- α mRNA levels were determined by real-time PCR and expressed as mean values $\pm$ SD ($n = 3$). *denotes a significant difference compared with the hypoxia groups ($p < 0.01$). **(b) Effects of SAHA on secretion of TNF- α protein by hypoxia-stimulated macrophages.** Cell culture mediums of RAW264.7 macrophages were harvested at 4 and 8 hours after hypoxia stimulation with or without SAHA treatment. Concentrations of TNF- α in the cell culture mediums were determined by ELISA. TNF- α concentration was expressed as mean values $\pm$ SD ($n = 3$). # denotes a significant difference compared with the sham group ($p < 0.001$). * denotes a significant difference compared with the hypoxia groups ($p < 0.001$). Sham (no SAHA, no hypoxia) macrophages served as a control. All time points (hours) in this study used start of treatment as the reference point (time zero).

Fig. 6. Schematic depiction of mechanism by which SAHA could attenuate hypoxia- HIF-1 inflammatory pathway in macrophages

SAHA treatment increases cytoplasmic levels of PHD2 protein, decreases HIF-1 protein, inhibits transcription of iNOS and TNF- α genes, decreases iNOS expression, attenuates NO production and TNF- α secretion. SAHA, suberoylanilide hydroxamic acid; PHD2, prolyl hydroxylase 2; HIF-1, hypoxia-inducible factor-1; iNOS, inducible nitric oxide synthase; TNF- α , tumor necrosis factor; NO, nitric oxide. Figure numbers in parentheses refer to figures that present more detailed data related to these steps in the pathway.