

Pharmacological Inhibition of Histone Deacetylases by Suberoylanilide Hydroxamic Acid Specifically Alters Gene Expression and Reduces Ischemic Injury in the Mouse Brain

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Received June 14, 2006; accepted August 30, 2006

ABSTRACT

Pharmacological manipulation of gene expression is considered a promising avenue to reduce postischemic brain damage. Histone deacetylases (HDACs) play a central role in epigenetic regulation of transcription, and inhibitors of HDACs are emerging as neuroprotective agents. In this study, we investigated the effect of the HDAC inhibitor suberoylanilide hydroxamic acid (SAHA) on histone acetylation in control and ischemic mouse brain. We report that brain histone H3 acetylation was constitutively present at specific lysine residues in neurons and astrocytes. It is noteworthy that in the ischemic brain tissue subjected to 6 h of middle cerebral artery occlusion, histone H3 acetylation levels drastically decreased, without evidence for a concomitant change of histone acetyl-transferase or deacetylase activities. Treatment with SAHA (50 mg/kg i.p.) increased histone H3 acetylation within the normal brain (of approximately

8-fold after 6 h) and prevented histone deacetylation in the ischemic brain. These effects were accompanied by increased expression of the neuroprotective proteins Hsp70 and Bcl-2 in both control and ischemic brain tissue 24 h after the insult. It is noteworthy that at the same time point, mice injected with SAHA at 25 and 50 mg/kg had smaller infarct volumes compared with vehicle-receiving animals (28.5% and 29.8% reduction, $p < 0.05$ versus vehicle, Student's t test). At higher doses, SAHA was less efficient in increasing Bcl-2 and Hsp70 expression and did not afford significant ischemic neuroprotection (13.9% infarct reduction). Data demonstrate that pharmacological inhibition of HDACs promotes expression of neuroprotective proteins within the ischemic brain and underscores the therapeutic potential of molecules inhibiting HDACs for stroke therapy.

Epigenetic rearrangement of chromatin architecture is a key event in maintenance of nuclear homeostasis and gene expression. Acetylation, phosphorylation, methylation, and ubiquitination at specific amino acid residues of histone tails regulate high-order chromatin folding and establish the so-called “histone code” (Jenuwein and Allis, 2001). A complex and dynamic interplay between histone acetyl-transferases (HATs) and histone deacetylases (HDACs) participates in the interconversion between permissive and repressive chromatin structures, modulating transcription regulating factor binding to the DNA template. Recruitment of HDACs at

specific transcription initiation elements leads to decreased histone acetylation, chromatin compaction, and gene silencing. Paradoxically, HDAC-dependent silencing of gene repressor can also result in increased transcription (Dokmanovic and Marks, 2005).

Given the role of histone acetylation in transcriptional activation, as well as the significance of altered gene expression in disease pathogenesis, a great deal of effort has been directed to the development of chemical inhibitors of HDACs (Johnstone, 2002; Dokmanovic and Marks, 2005). Numerous studies demonstrate that HDAC inhibitors are potent inducers of growth arrest, differentiation, and apoptosis of transformed cells *in vitro* and *in vivo*. Therefore, these drugs are currently evaluated for treatment of patients affected by different types of cancer, such as lymphoma, melanoma, and breast and brain tumors (Butler et al., 2000; Marks et al.,

This study was supported by grants from the University of Florence, the Ministero dell'Università e della Ricerca Scientifica e Tecnologica (Programma Cofinanziato 2002), and Ente Cassa di Risparmio di Firenze.

Article, publication date, and citation information can be found at <http://molpharm.aspetjournals.org>.
doi:10.1124/mol.106.027912.

ABBREVIATIONS: HAT, histone acetyl transferase; HDAC, histone deacetylase; SAHA, suberoylanilide hydroxamic acid; PaO₂, arterial oxygen pressure; PaCO₂, partial pressure of carbon dioxide; MCAO, middle cerebral artery occlusion; PBS, phosphate-buffered saline; TBST, phosphate-buffered saline containing 0.1% Tween 20; iNOS, inducible nitric-oxide synthase; COX-2, cyclooxygenase-2; 3D, three-dimensional; Hsp70, 70-kDa heat shock protein; ANOVA, analysis of variance; GFAP, glial fibrillary acidic protein.

2000; Richon et al., 2001; Minucci and Pelicci, 2006). In apparent contrast with this, cytoprotection can occur when nontransformed cells are exposed to HDAC inhibitors, and trials investigating the potential therapeutic effect of these drugs in infective, neurological, and respiratory diseases are currently ongoing (see <http://www.clinicaltrials.gov>). However, reasons underlying selective toxicity of HDAC inhibitors toward transformed cells remain to be clearly established (Dokmanovic and Marks, 2005; Ungerstedt et al., 2005).

Perturbation in acetylation homeostasis is emerging as a central event in the pathogenesis of neurodegeneration (for a comprehensive review, see Saha and Pahan, 2006). Hence, recent studies have indicated that HDAC inhibitors might prove useful in treatment of such neurodegenerative disorders as Huntington's disease (Ferrante et al., 2003; Hockly et al., 2003; Gardian et al., 2005), spinal muscular atrophy (Chang et al., 2001), amyotrophic lateral sclerosis (Corcoran et al., 2004; Ryu et al., 2005; Petri et al., 2006), and experimental autoimmune encephalomyelitis (Camelo et al., 2005). Alteration of gene expression occurs after ischemic brain injury (Papadopoulos et al., 2000), and manipulation of transcription is thought to be of therapeutic relevance to stroke therapy (Read et al., 2001). In particular, experimental evidence indicates that pharmacological modulation of transcriptional programs activated in injured neurons and reactive glial cells may prove useful to reduce neurodegeneration within the ischemic penumbra (Dirnagl et al., 1999; Lo et al., 2003).

In the present study, we sought to determine whether the potent and selective HDAC inhibitor suberoylanilide hydroxamic acid (SAHA) (Gottlicher et al., 2001; Phiel et al., 2001) affects histone acetylation levels and gene expression profile within the ischemic brain, as well as the sensitivity to post-ischemic brain damage.

Materials and Methods

Animals. Male C57 mice (25–27 g; Harlan, San Pietro al Natisone, Italy) were housed five per cage and kept at constant temperature ($21 \pm 1^\circ\text{C}$) and relative humidity (60%) with regular light/dark schedule (7 AM–7 PM). Food and water were available ad libitum. Procedures involving animals and their care were conducted in compliance with the Italian guidelines for animal care (DL 116/92) in application of the European Communities Council Directive (86/609/EEC) and was formally approved by the Animal Care Committee of the Department of Pharmacology of the University of Florence.

Surgical Procedure and Measurement of Infarct Volume. Permanent distal middle cerebral artery occlusion (MCAO) was induced in age-matched male C57 mice ($n = 8$ per group). Animals (25–30 g) were anesthetized with 4% isoflurane and maintained on 1.5% isoflurane in air. Rectal temperature was monitored and maintained between 36.5 and 37.5°C with a homeothermic blanket. A 1-cm vertical scalp incision was made between the right eye and ear. The temporalis muscle was bisected, and a 2-mm burr hole was made at the junction of the zygomatic arch and squamous bone. The distal middle cerebral artery was exposed and permanently occluded by cauterization above the rhinal fissure. In randomly selected animals, the left femoral artery was cannulated with a PE-10 polyethylene tube for arterial blood pressure measurement and blood gas determination. Arterial blood samples (50 μl) were analyzed for pH, arterial oxygen pressure (PaO_2), and partial pressure of carbon dioxide (PaCO_2) using a Ciba-Corning 248 PH/blood gas analyzer (Bayer Diagnostics, Tarrytown, NY). Physiological parameters such

as rectal temperature, mean arterial blood pressure, pH, PaO_2 , and PaCO_2 did not differ between groups before, during, and 1 h after ischemia. In addition, as revealed by a flexible skull probe connected to a Laser Doppler imaging system (PF2B; Perimed, Stockholm, Sweden), drug treatment did not affect regional cerebral blood flow in control brain tissue or regional cerebral blood flow drop upon artery cauterization. After surgery, mice were kept at 37°C for 1 h in an incubator and then placed in their cages until sacrifice. Mice were sacrificed at different times (Western blotting and immunohistochemistry) or 24 h (infarct determination) after MCAO, and their brains were snap-frozen in nitrogen vapor for cryostat sectioning. For infarct determination, toluidine blue-stained coronal sections (20 μm) were imaged using the Image 3.0 ProPlus analysis software. Twelve sections per animal were analyzed, and infarct areas were calculated by subtracting the area of intact tissue in the ipsilateral hemisphere from the area of the contralateral hemisphere to minimize the error that is introduced by edema, which distorts and enlarges the infarcted tissue and surrounding white matter. Infarct volumes were calculated by multiplying the infarct area by the distance among sections as described previously (Swanson and Sharp, 1994).

Drug Administration Protocol. SAHA was provided by Italfarmaco (Cinisello Balsamo, Milan, Italy). The compound was >99% pure as assessed by high-performance liquid chromatography. SAHA was dissolved in 100 μl of a solution containing 25% dimethyl sulfoxide and 75% phosphate-buffered saline (PBS) and then injected i.p. Control animals received an equal amount of vehicle. Two i.p. injections of SAHA were administered immediately and 6 h after ischemia. Pilot experiments showed that 25% dimethyl sulfoxide per se did not affect ischemic brain injury in this experimental model.

Immunohistochemistry. For immunohistochemistry, mice were anesthetized and transcardially perfused with ice-cold PBS followed by 4% paraformaldehyde in phosphate-buffered saline. Brains were removed, stored overnight in the same fixative at 4°C, and then submerged in 30% sucrose solution for at least 2 days. Coronal sections (20 μm) were incubated (free floating) in PBS with 0.3% Triton X-100 (Sigma, St. Louis, MO) and 20% of bovine albumin. One hour later, sections were incubated with the anti-acetylated histone H3 lysines Lys18, Lys23, and Lys9 (rabbit polyclonal, 1:200; Cell Signaling Technology, Danvers, MA) at 4°C overnight. After three 10-min washes, brain slices were incubated with a Cy3-conjugated secondary antibody (donkey anti-rabbit 1:500; Jackson ImmunoResearch Laboratories, West Grove, PA). Sections were double-stained with anti-NeuN monoclonal antibody (mouse monoclonal; 1:1000; Chemicon International, Temecula, CA) or anti-GFAP (monoclonal, clone G-A-5, 1:200; Sigma). Brain sections were then mounted on slides and immunostaining visualized by means of a Nikon microscope equipped with piezoelectric motorization, charge-coupled device camera and Metamorph/Metafluor software. For 3D imaging, stacks of images were acquired through the depth of the section and deconvolved using Image Autodeblur software as described previously (Cipriani et al., 2005). Quantification of fluorescence (intensity of optical density, IOD) was performed using the Metamorph/Metafluor software. Values correspond to the mean of at least six different microscopic fields of three different mouse brain sections containing the same number of cells.

Western Blotting. For Western blotting, mice were transcardially perfused with ice-cold PBS. Coronal sections of 20- μm thickness were cut using a cryostat. Samples from the ischemic cortex corresponding to the right and left middle cerebral artery territory were excised from the coronal section of SAHA- and vehicle-treated mice and homogenized in lysis buffer (50 mM Tris, pH 7.4, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 4 $\mu\text{g}/\text{ml}$ aprotinin and leupeptin, and 1% SDS). After 4 to 20% SDS-polyacrylamide gel electrophoresis (50 μg of protein loaded per lane) and blotting, membranes (Hybond ECL; GE Healthcare, UK, Ltd., Little Chalfont, Buckinghamshire, UK) were blocked with Tris-buffered saline containing 0.1% Tween 20 and 5% skimmed milk (TBST/5% milk) and then

probed overnight with primary antibodies (1:2000 in TBST/5% milk). The anti-acetyl-histone H3 (Lys18) antibody was the same of that used for immunohistochemistry (see above). The anti-inducible NO synthase (iNOS) and anti-interleukin-1 β antibodies were polyclonal from Santa Cruz Biotechnology (Santa Cruz, CA); the anti-cyclooxygenase-2 (COX-2) polyclonal antibody was from Cayman Chemical (Ann Arbor, MI); the 70-kDa anti-heat shock protein polyclonal antibody was from Nventa Biopharmaceuticals (Victoria, BC, Canada); the anti-Bcl-2 (N-19) rabbit polyclonal was from Santa Cruz Biotechnology, Inc.; the anti- β -actin antibody was monoclonal from Sigma. Membranes were then washed with TBST and incubated for 1 h in TBST/5% milk containing the corresponding peroxidase-conjugated secondary antibody (1:2000). After washing in TPBS, ECL (GE Healthcare) was used to visualize the peroxidase-coated bands.

Measurement of HAT and HDAC Activities. HAT and HDAC activities were measured by means of kit assays (Upstate Technol-

ogy, Lake Placid, NY) according to the manufacturer's instructions. In brief, contralateral and ipsilateral cortices were homogenized [1:10 (w/v)] in radioimmunoprecipitation assay buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 0.25% sodium deoxycholate, and protease inhibitor cocktail [1:100 (v/v); Sigma]. Aliquots of the homogenates were added to the assay buffer. For HAT activity determination 100 μ M acetyl-CoA was added to the assay buffer. Reaction was started by adding the substrate (biotinylated histone H3 peptide for HAT and *para*-nitroanilide-conjugated acetylated histone H3). The mixtures were incubated 10 min at 30°C. After addition of the developer absorbance was detected by means of a spectrophotometer (Victor₃; PerkinElmer Life and Analytical Sciences) at wavelengths of 450 nm (HAT) and 405 nm (HDAC). Specificity of the signal was obtained by adding 4 μ M trichostatin A to the HDAC assay mixture and by omitting addition of acetyl-CoA to the HAT reaction mixture.

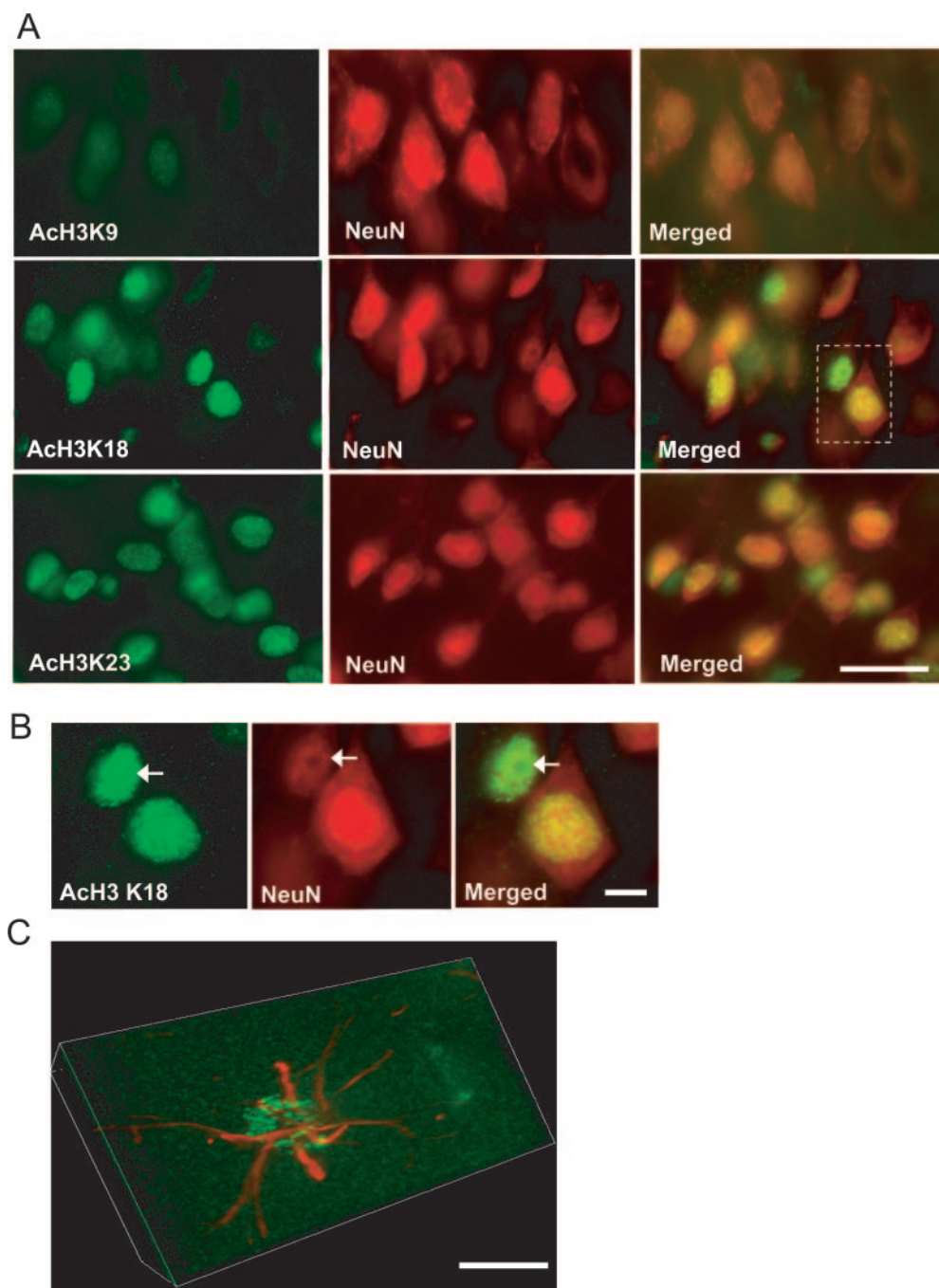


Fig. 1. Levels of H3 acetylation on different lysine residues in the mouse cortex. A, acetylation of histone H3 on Lys9 (AcH3K9) was hardly detectable. The acetylation status of Lys23 on histone H3 (AcH3K23) was higher than that of Lys9 but lower than that of Lys18 (AcH3K18). Immunoreactivity of acetylated histone H3 colocalized with Neu-N positive neurons. B, high magnification of the inset shown in A shows that in neurons, histone acetylation was confined to specific regions scattered in the nucleoplasm with the exception of the nucleolus (arrow). C, 3D reconstruction of a GFAP-positive astrocyte (red) immunopositive for acetylated histone H3 Lys18 (green). Scale bar, 30 μ m (A) and 5 μ m (B and C).

Results

Brain Levels of H3 Acetylation and Effects of HDAC Inhibition.

A comparison of the acetylation status of histone residues in neural cells has not been reported. Hence, we first evaluated the acetylation levels of Lys9, Lys18, and Lys23 of histone H3 in the mouse brain parietal cortex. As shown in Fig. 1, histone H3 acetylation in the mouse cortex mainly occurred on Lys18. Indeed, as revealed by fluorescence quantitation (see *Materials and Methods*), acetylation of Lys23 and Lys9, respectively, was 63 ± 14 ($p < 0.05$) and $6 \pm 2\%$ ($p < 0.001$) of that occurring at Lys18. It is noteworthy that the majority of cells immunopositive for acetylated histone H3 also expressed the neuronal marker NeuN (Fig. 1, A and B). High magnification of neuronal nuclei revealed that histone acetylation was not homogeneous but confined to specific regions evenly scattered in the nucleoplasm. The negative image of the nucleolus was also evident (Fig. 1B). The histone acetylation status of GFAP-positive astrocytes was not easily detectable. 3D reconstruction was necessary to surely assign acetylated H3 immunoreactivity to a GFAP-positive cells (Fig. 1C). At variance with neurons, differences among the acetylation status of lysine residues were not detected in astrocytes (data not shown). These features were not restricted to the cortex but were also found in other regions of the mouse brain such as the striatum, brainstem, and hippocampus. Cells of the ependyma and pia mater had the highest levels of acetylation among the other brain structures (data not shown).

Next, we investigated the effect of HDAC inhibition on H3 acetylation levels in the mouse brain. SAHA at 50 mg/kg (see *Materials and Methods* for treatment protocol), a dose previ-

ously adopted in an animal model of Huntington's disease (Hockly et al., 2003), was well tolerated by mice. As revealed by Western blotting, increase of brain histone H3 acetylation levels occurred only in some animals at 1.5 to 3 h after the first injection, whereas at later time points (6–7.5 h) histone acetylation significantly increased in all the injected mice (Fig. 2). Such an increase was maintained up to 12 h and still present in some mice after 24 h, whereas at 48 h, levels of H3 acetylation returned to basal values (data not shown).

Effect of SAHA on Histone H3 Deacetylation and Measurement of HAT and HDAC Activities in the Ischemic Brain Cortex.

Despite the relevance of histone acetylation to gene expression, as well as that of gene expression to postischemic brain damage, it is still unknown whether histone acetylation levels are altered within the ischemic brain. To address this issue, we evaluated H3 acetylation in different brain areas of mice subjected to permanent occlusion of the middle cerebral artery. We found that acetylation of histone H3 was reduced 3 h after the insult (data not shown) and almost disappeared at 6 h in the ischemic tissue (Fig. 3A). The contralateral cortex did not undergo alteration of histone acetylation (data not shown). We next investigated the effect of SAHA on ischemia-induced histone deacetylation. As shown in Fig. 3B, injection of SAHA efficiently prevented reduction of histone acetylation in the ischemic tissue. It is noteworthy that the drug's effects were maintained up to 24 h (Fig. 3C). The reduced acetylation levels found in the brain tissue subjected to 6 h ischemia could be explained with a decreased HAT activity and/or increased deacetylating efficiency of HDACs. However, both enzymatic activities

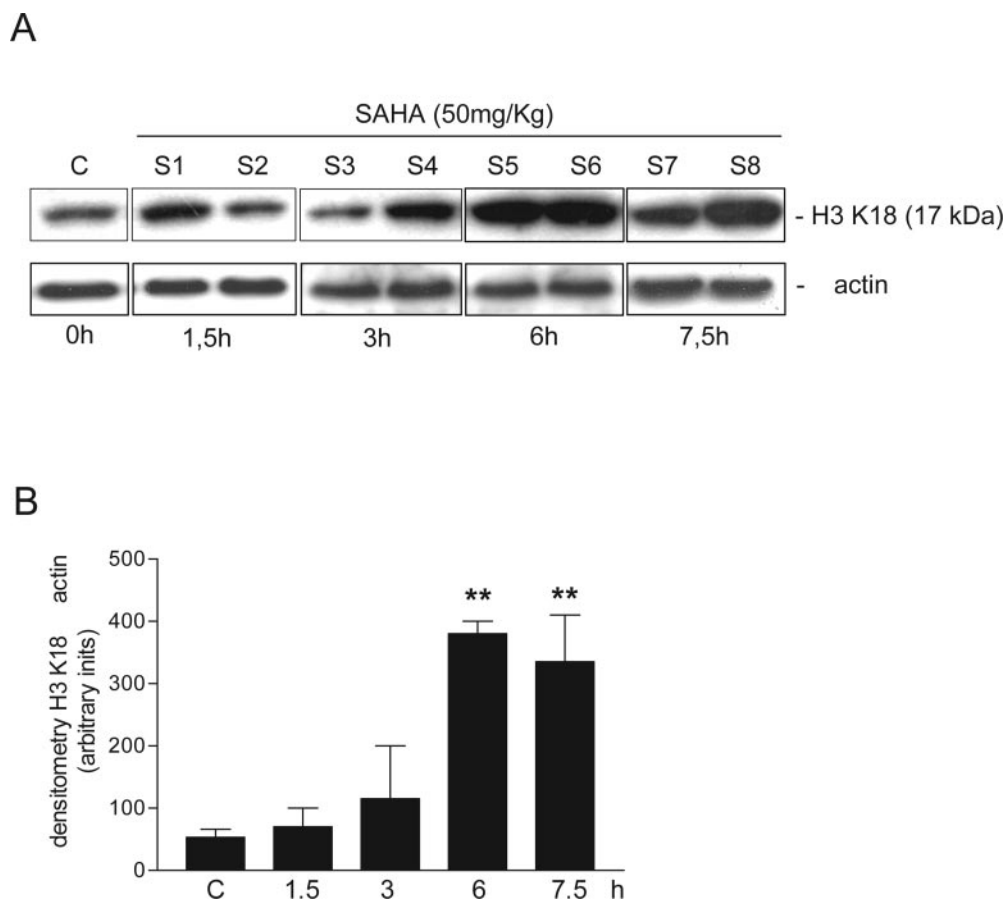


Fig. 2. Effect of SAHA on H3 acetylation levels in the mouse cortex. A, 1.5 to 3 h after the injection of SAHA (50 mg/kg, i.p.) histone H3 acetylation was inconsistently increased in the mouse cortex. At later time points (6–7.5 h) histone acetylation increased in the brain of all the injected mice (two animals per group are shown). Note that the group of mice sacrificed at 7.5 h received a second injection at 6 h (see *Materials and Methods*). β -Actin is shown as loading control. One experiment representative of 3 is shown. B, densitometric evaluation of SAHA-induced histone H3 hyperacetylation. Columns represent the mean \pm S.E.M. of three experiments. **, $p < 0.001$ versus control (ANOVA followed by Tukey W test).

did not change up to 6 h after ischemia in the ischemic cortex compared with the contralateral one (Fig. 3D).

SAHA Specifically Affects Protein Expression within the Ischemic Brain. Given the relevance of histone acetylation to gene expression, we next sought to determine whether SAHA changed the protein expression profile in normal and ischemic brain tissue by means of Western blotting and densitometric analysis. Evidence that pharmacological inhibition of HDAC prevents transcription of proinflammatory mediators (Leoni et al., 2002), along with the pathogenetic role of neuroinflammation in ischemic brain

injury (Iadecola and Alexander, 2001), prompted us to evaluate the expression levels of iNOS, COX-2, and IL1 β in the contralateral and ipsilateral cortex of vehicle- and SAHA (50 mg/kg)-treated mice. As shown in Fig. 4A, 24 h after the ischemic challenge expression of COX-2 was similarly reduced in the ischemic cortex of both animal groups. At this time point, both iNOS and IL1 β expression levels were below detection limits in the healthy or injured tissue of vehicle- or SAHA-treated mice. Although Akt (also known as PKB) is a kinase involved in ischemic neuroprotection (Yano et al., 2001), levels of Akt phosphorylation (an index of kinase ac-

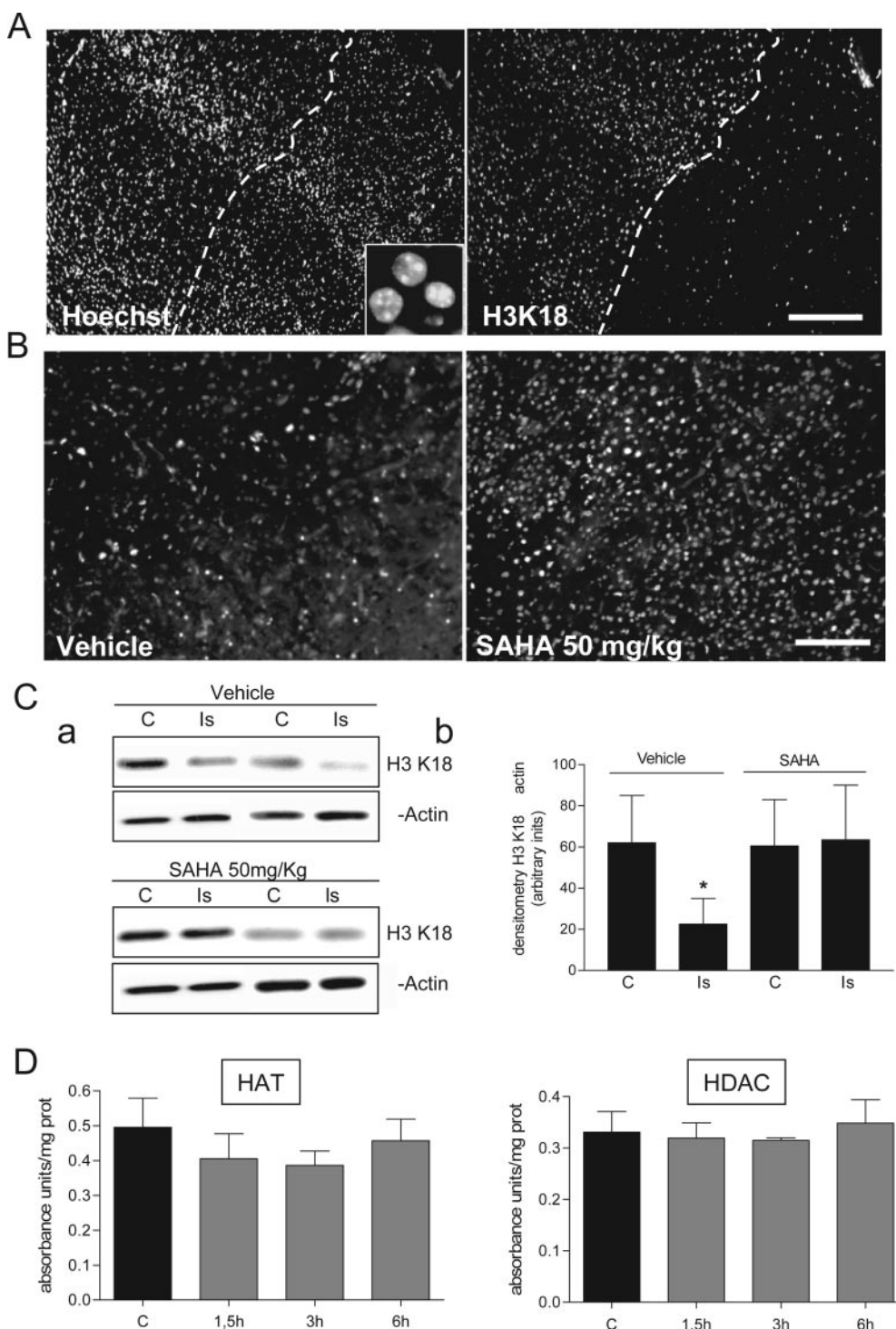


Fig. 3. SAHA prevents ischemia-induced histone H3 hypoacetylation. A, acetylation of histone H3-Lys18 is drastically reduced in the ischemic cortex of mice subjected to 6-h MCAO despite the fact that Hoechst staining indicates maintenance of nuclear integrity (inset) and cortex layers. The dotted line demarcates the edge of the ischemic tissue. B, SAHA (50 mg/kg, i.p. at time 0) prevents histone H3-Lys18 hypoacetylation in the ischemic core 6 h after MCAO. C, a, Western blotting evaluation of H3 acetylation levels in the ipsilateral and contralateral cortex of vehicle- or SAHA-injected mice subjected to 24 h MCAO. b, densitometric analysis of H3 acetylation in control (C) and ischemic (Is) brain tissue 24 h after the insult, *, $p < 0.05$ versus control (ANOVA followed by Tukey W test). Note that SAHA prevents ischemia-induced hypoacetylation of histone H3. Two animals per group ($n = 5$) are shown in a. Note the variability of basal brain H3 acetylation among animals. β -Actin is reported as loading control. D, HAT and HDAC activities do not change in the ischemic cortex compared with the contralateral cortex up to 6 h after ischemia. In A–C, one experiment/blot representative of three is shown. Scale bar, 500 μ m (A) and 80 μ m (B). In D, bars represent the mean of three experiments conducted in duplicate.

tivity) slightly but similarly increased in the ischemic tissues of both animal group (Fig. 4A). Other proteins involved in protection from postischemic brain damage are the chaperone protein HSP-70 (Sharp et al., 1999; Weinstein et al., 2004) and the powerful antiapoptotic effector Bcl-2 (Chen et al., 1995; Chen et al., 1997). It is noteworthy that Western blotting and densitometric analysis (Fig. 4) revealed that both proteins slightly increased in the ischemic tissues in line with previous results in rats (Chen et al., 1995, 1996, 1997). SAHA further increased Hsp70 and Bcl-2 levels in both ischemic and contralateral cortices (Fig. 4, A and B). It is noteworthy that the effects of SAHA on expression of both proteins

showed a bell-shaped curve; the increase obtained by 50 mg/kg was higher than that induced by 25 or 100 mg/kg (Fig. 4C).

SAHA Reduces the Vulnerability to Ischemic Brain Injury of Mice Subjected to MCAO. We then asked whether prevention of ischemia-induced histone deacetylation and alteration of protein expression levels by SAHA could modulate the sensitivity of the brain tissue to ischemic injury. We found that rectal temperature, mean arterial blood pressure, pH, PaO₂, and PaCO₂ did not differ between vehicle- and SAHA-treated animals before, during, and 1 h after ischemia (data not shown). Measurement of brain infarct areas and volumes 24 h after MCAO revealed that

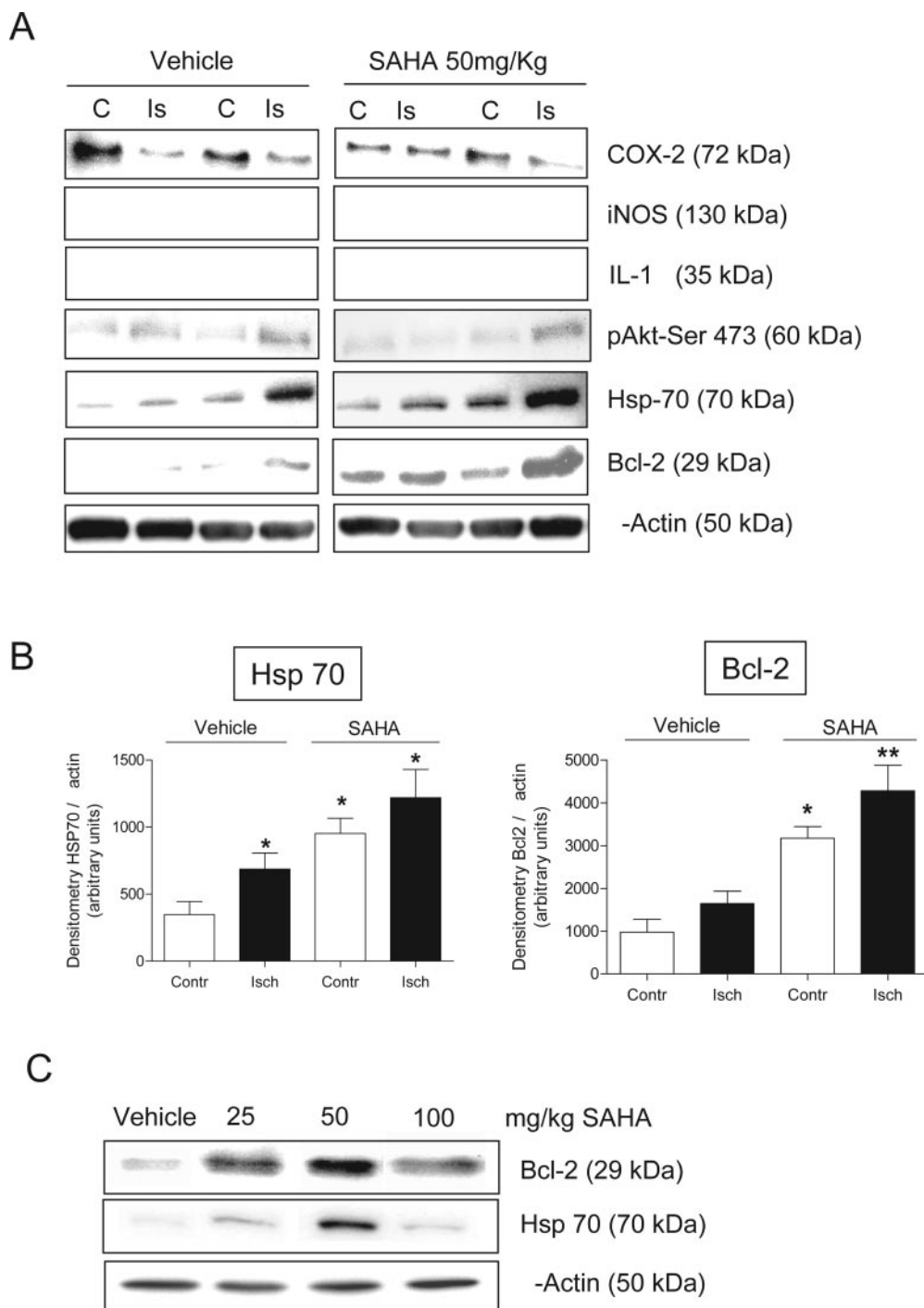


Fig. 4. SAHA specifically alters protein expression profile within the ischemic brain. **A**, COX-2 expression is similarly reduced in the ischemic cortex of vehicle- and SAHA (50 mg/kg)-treated mice 24 h after the induction of ischemia. At this time point, neither iNOS nor interleukin-1 β is expressed in either animal group. In the same animals, treatment with SAHA does not significantly affect ischemia-induced Akt phosphorylation on Ser473. Conversely, SAHA potentiated the ischemia-induced increase of Hsp70 and Bcl-2 expression. Note that expression of both proteins is also increased in the contralateral cortex of SAHA-injected compared with vehicle-receiving mice. **B**, densitometric analysis of Hsp70 and Bcl-2 expression in the cortex of animals treated with vehicle or SAHA (50 mg/kg). *, $p < 0.05$; **, $p < 0.01$ versus contralateral cortex of vehicle treated animals (ANOVA followed by Tukey W test). **C**, dose effect of SAHA on induction of Bcl-2 and Hsp70 in the ischemic cortex of mice. Note that protein induction decreases at doses of 100 mg/kg. In A, two mice per group are shown. A and B are representative of three independent experiments. In C, one blot representative of two is shown.

SAHA reduced ischemic neurodegeneration with a bell-shaped dose-response curve. In particular, in vehicle-treated mice, infarct volume was $26.8 \pm 1.5 \text{ mm}^3$. The infarct volume was unaffected by 12.5 mg/kg SAHA ($26.6 \pm 3.5 \text{ mm}^3$), whereas it was reduced when the dose rose to 25 mg/kg (28.5% reduction, $19 \pm 4.2 \text{ mm}^3$, $p = 0.03$ versus vehicle; Student's t test). Reduction by 29.8% was observed in mice receiving SAHA at 50 mg/kg ($18.8 \pm 1.5 \text{ mm}^3$, $p = 0.0034$ versus vehicle; Student's t test) (Fig. 5). When the dose of SAHA was raised to 100 mg/kg, ischemic neuroprotection was lost ($23.1 \pm 2.4 \text{ mm}^3$) (Fig. 5).

Discussion

In this study, we show that histone acetylation dramatically decreases in the ischemic brain tissues. We also demonstrate that pharmacological inhibition of HDACs with SAHA maintains histone acetylation within the ischemic

brain, increases expression of neuroprotective proteins, and affords protection from ischemic brain injury.

Our results are in line with evidence that valproic acid, a mood-stabilizing and antiepileptic drug recently found to be able to inhibit HDACs, reduces postischemic brain damage (Ren et al., 2004). The present data are also in agreement with studies indicating that HDAC inhibitors reduce neurodegeneration and ameliorate neurological deficit in experimental models of Huntington disease (Ferrante et al., 2003; Hockly et al., 2003; Gardian et al., 2005), amyotrophic lateral sclerosis (Corcoran et al., 2004; Ryu et al., 2005; Petri et al., 2006), spinal muscular atrophy (Chang et al., 2001), and experimental autoimmune encephalomyelitis (Camelo et al., 2005). Evidence that several HDAC inhibitors including SAHA prevent oxidative neuronal death (Ryu et al., 2003) strengthens the neuroprotective potential of pharmacological strategies aimed at inhibiting histone deacetylation.

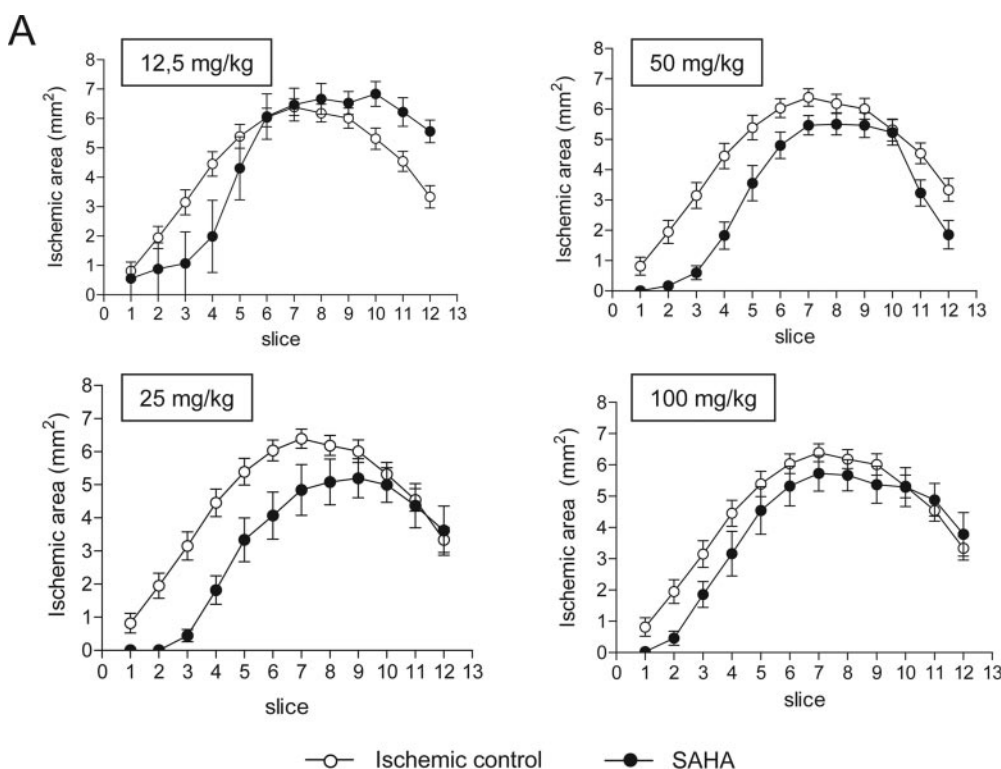


Fig. 5. Effect of SAHA on ischemic brain injury. SAHA reduces ischemic areas (A) and volumes (B) of mice subjected to 24 h MCAO ($n = 8$ per group) when administered at 25 and 50 mg/kg. Note that SAHA does not significantly reduce postischemic brain damage at lower (12.5 mg/kg) and higher (100 mg/kg) doses. *, $p < 0.05$; **, $p < 0.001$ versus vehicle (ANOVA and Dunnett's post hoc test).

The discrete nuclear distribution of histone H3 acetylation we report here in neurons is in line with the notion that acetylation mainly occurs at foci of euchromatic and highly transcribed genome regions. We also show that histone H3 lysine residues are differently acetylated in mouse neurons. This finding suggests that HATs display a preference toward certain lysine residues and/or that acetylated lysine residues are differentially targeted by HDACs in neurons. Although the functional meaning of this is currently unknown, a thorough understanding of the mechanisms involved in HATs/HDAC regulation could certainly help to decipher the neuronal histone code. Regardless, given that SAHA is a powerful inhibitor of HDAC class I and II (Marks et al., 2004), our data indicate that these two classes of deacetylases are of pathogenetic relevance to ischemic brain injury. At present, we cannot rule out whether inhibition of targets other than HDACs contributed to SAHA-induced ischemic neuroprotection. However, evidence that HDAC inhibitors with molecular moieties different from SAHA also prevent neuronal death in disparate models of neurodegeneration (Chang et al., 2001; Ferrante et al., 2003; Hockly et al., 2003; Ryu et al., 2003; Corcoran et al., 2004; Ren et al., 2004; Camelo et al., 2005; Gardian et al., 2005; Ryu et al., 2005; Petri et al., 2006) corroborates the hypothesis that SAHA-dependent ischemic neuroprotection was causally related to HDAC inhibition. The present findings that SAHA increases histone acetylation in the brain of control animals (Fig. 2) and prevents histone deacetylation within the ischemic tissue confirm that the drug enters the brain and inhibits HDACs (Hockly et al., 2003; Hahnen et al., 2006).

In principle, decrease of histone acetylation in the ischemic cortex might be due to reduced activity of HATs and/or activation of HDAC. In this regard, it is worth mentioning that although homeostatic mechanisms responsible for activation or inhibition of HATs and HDACs are still in large part elusive (Saha and Pahan, 2006), enzyme activities are strictly dependent on recruitment on chromatin active regions by specific transcription-regulating proteins (Kouzarides, 2000; Marks et al., 2003; Dokmanovic and Marks, 2005). We were surprised to find that when histone acetylation levels were highly reduced in the ischemic brain 6 h after ischemia, no changes in HAT and HDAC activities were detected. We reconcile these apparently contrasting findings by considering that during ischemia brain acetyl-CoA contents decrease (Calvani and Arrigoni-Martelli, 1999), thereby limiting HAT activity. The recent evidence that pyruvate dehydrogenase, the enzyme leading to the synthesis of acetyl-CoA, undergoes oxidative denaturation during brain ischemia strengthens the hypothesis that reduced availability of acetyl-CoA underlies the decreased levels of histone acetylation within the ischemic brain. The fact that we detected no change in HAT activity after ischemia may be due merely to the fact that measurement of this enzymatic activity *in vitro* requires addition of acetyl-CoA (see *Materials and Methods*). It is remarkable that our data for the first time hint at an unexpected link between derangement of energetic metabolism and gene expression during cerebral ischemia. The notion that ischemia drastically reduces brain ATP contents, together with evidence that HATs are activated upon phosphorylation (Saha and Pahan, 2006), may also corroborate the hypothesis that HATs are impaired within the ischemic brain tissue.

On this basis, we reason that pharmacological inhibition of HDACs during brain ischemia prevents reduction of histone acetylation due to decreased HAT activity and is of therapeutic relevance to treatment of postischemic brain damage. It is plausible that the neuroprotective effects of SAHA are due to modulation of gene expression within the ischemic tissue. It is well known, indeed, that complex (and in large part still obscure) changes in the protein expression profile occur in the brain upon ischemic challenge (Read et al., 2001; Weinstein et al., 2004) and that pharmacological manipulation of these changes affords neuroprotection (Lo et al., 2003; Stenzel-Poore et al., 2004). In good agreement with this, we report that brain ischemia increases the expression levels of Hsp70 and Bcl-2, which protects from ischemic neuronal death (Martinou et al., 1994; Chen et al., 1995, 1996; Antonawich et al., 1999; Weinstein et al., 2004). The ability of SAHA to further augment such an increase is therefore of pathophysiological relevance and, together with prior work (Chang et al., 2001; Ferrante et al., 2003; Hockly et al., 2003; Ryu et al., 2003; Corcoran et al., 2004; Ren et al., 2004; Camelo et al., 2005; Gardian et al., 2005; Ryu et al., 2005), suggests that the neuroprotective properties of SAHA and other HDAC inhibitors may be due in part to enhanced expression of specific genes. Accordingly, the nonspecific HDAC inhibitor valproic acid increases brain levels of Hsp70 (Ren et al., 2004) in the rat, whereas phenylbutyrate, a more specific HDAC inhibitor, increased Bcl-2 transcripts in the spinal cord of mice with experimental amyotrophic lateral sclerosis (Petri et al., 2006). In addition, the fact that loss of ischemic neuroprotection with high doses of SAHA (100 mg/kg) correlates with decreased induction of the two proteins (compare Figs. 4C and 5) underscores the causative role of Bcl-2 and Hsp70 in SAHA-dependent reduction of postischemic brain damage. Gene array experiments would identify additional proteins whose increased or decreased expression participates to SAHA-dependent ischemic neuroprotection.

At present, we do not know the molecular mechanisms underlying the bell-shaped dose-response curve of SAHA on protection from postischemic brain damage. In theory, high doses of SAHA may impair transcription by excessive histone acetylation and/or activate neurotoxic genes. The possibility that high doses of SAHA lead to hyperacetylation of nonhistone proteins with active roles in cell death, such as p53 (Juan et al., 2000; Luo et al., 2000), may also underlie loss of neuroprotection when SAHA dosage exceeds a certain threshold. The impact of HDAC inhibitors on substrates different from histones, however, could also in part mediate ischemic neuroprotection. Indeed, hyperacetylation-dependent Sp1 transcription factor activation underpins the protective effects of HDAC inhibitors on oxidative stress-dependent neuronal death (Ryu et al., 2003). It is therefore possible that the overall effect of HDAC inhibitors on the brain's stress response is the result of a complex series of events related to both histone modification and hyperacetylation-dependent activation/inhibition of nonhistone proteins. Evidence that SAHA increases Hsp70 and Bcl-2 also in the contralateral cortex of ischemic brain is of pathophysiological significance. Indeed, inhibition of HDACs might be a new pharmacological strategy to increase the CNS stress resistance in an insult-independent manner and may be of relevance to primary or secondary prevention of neurological disorders. The ability of SAHA to increase brain expression of

Bcl-2 is also of significance given that pharmacological tools able to specifically increase tissue levels of Bcl-2 remain to be identified (Letai, 2005; Oltersdorf et al., 2005).

All together, the present study furthers our understanding of the role of histone acetylation during brain ischemia and points to pharmacological inhibition of HDACs as a promising strategy to reduce ischemic neurodegeneration. The fact that SAHA and other HDAC inhibitors are currently being evaluated in clinical trials strengthens the relevance of this class of drugs to stroke treatment.

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