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Interplay between histone acetylation/deacetylation and poly(ADP-ribosyl)ation in the development of ischemic tolerance *in vitro*

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ABSTRACT

Ischemic tolerance is an endogenous defense program in which exposure to a subtoxic preconditioning insult results in resistance to a subsequent, otherwise lethal, episode of ischemia. Herein, we evaluated the role of histone acetylation/deacetylation in an *in vitro* model of preconditioning, using rat organotypic hippocampal slices exposed to 30 min oxygen-glucose deprivation (OGD), which leads to CA1 injury 24 h later: tolerance was induced by exposing the slices to preconditioning bouts of NMDA (3 μM for 60 min) 24 h prior to the toxic OGD challenge. Under these conditions, CA1 damage induced by OGD was reduced. The induction of tolerance was prevented by incubating the slices with HDAC inhibitors. NMDA preconditioning was associated with a mild increase in poly(ADP-ribose) polymerase (PARP) activity that was apparently followed, 3 h later, by a mild increase in histone acetylation. Use of PARP and HDAC inhibitors suggests a possible interaction between PARP and HDAC activities in the development of ischemic tolerance. Finally, both PARP and HDAC inhibitors were able to prevent the increase in pERK1/2 induced by NMDA preconditioning. We propose a model in which mild histone acetylation and PARP activity cooperate in producing a neuroprotective response in the development of ischemic tolerance.

1. Introduction

Post-translational (epigenetic) modifications of chromatin architecture, such as acetylation, methylation, phosphorylation, and ubiquitination at specific amino acid residues of histone proteins, have profound effects on the maintenance of nuclear homeostasis and on gene expression. Histone acetyl-transferases (HATs) and histone deacetylases (HDACs) represent two enzyme families that catalyze the interconversion between permissive and repressive chromatin structures: acetylation at lysine-rich amino-terminal tails of core histone proteins results in charge neutralization and a more relaxed and transcriptionally active chromatin structure, deacetylation shifts the balance towards chromatin condensation, thereby silencing gene expression. During normal conditions, the enzymatic activities of HATs and HDACs remain in a harmonized state of balance that is a prerequisite of neuronal survival. Under conditions of neurodegeneration, it has been repeatedly demonstrated that histone acetylation generally decreases, reflecting a derangement of the acetylation/deacetylation homeostasis (see for reviews: Saha and Pahan, 2006; Abel and Zukin, 2008). HDAC inhibitors have initially been proposed as effective therapeutic agents for cancer, due to their remarkable induction of growth arrest, differentiation and, in some cases, apoptosis. More recently, despite their efficacy as proapoptotic agents, HDAC inhibitors have been widely recognized as neuroprotective agents, in that they prevent and delay neuronal deterioration and death *in vitro* and *in vivo*, and hence have been proposed for the treatment of a number of neurological (Hahnen et al., 2008; Chuang et al., 2009; D'Mello, 2009), psychiatric (Deutsch et al., 2008; Sananbenesi

Abbreviations: HAT, histone acetyl transferase; HDAC, histone deacetylase; OGD, oxygen-glucose deprivation; SAHA, suberoylanilide hydroxamic acid; PARP, poly(-ADP-ribose) polymerase; TIQ-A, 4H-thieno[2,3-c]isoquinolin-5-one; ERK, extracellular-signal-regulated kinases; SAPK/JNK, stress-activated protein kinases (SAPK)/Jun amino-terminal kinases (JNK).

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and Fischer, 2009); age-related (Calvanese et al., 2009; Gravina and Vijg, 2010) and neurodevelopmental (Abel and Zukin, 2008; Kazantsev and Thompson, 2013) disorders.

A number of studies (reviewed in: Langley et al., 2009; Gibson and Murphy, 2010; Schweizer et al., 2013) have shown that histone acetylation is reduced following cerebral ischemia and that class I, II and IV HDAC inhibitors are able to ameliorate neuronal death and cognitive deficits following transient (Endres et al., 2000; Ren et al., 2004; Qi et al., 2004; Faraco et al., 2006; Yildrim et al., 2008; Wang et al., 2012) and permanent(Kim et al., 2007; Langley et al., 2008; Kim et al., 2009) focal ischemia and in a model of transient global ischemia (Xuan et al., 2012). These effects appear to be due to the maintenance of histone acetylation levels, the expression of cell survival (Bcl-2, Bcl-XL, Hsp70) and regenerative (BDNF) pathways, and the down-regulation of pro-inflammatory genes (COX-2, iNOS, TNF-α, IL-β) (Faraco et al., 2006; Kim et al., 2007, 2009). In a more recent study, the combined pretreatment with a class I HDAC inhibitor and an activator of SIRT1 (a class III HDAC belonging to the sirtuin family) restored acetylation of RelA and histones and limited post-ischemic injury in *in vivo* an *in vitro* models of stroke (Lanzillotta et al., 2013).

Ischemic tolerance is an endogenous protective mechanism in which exposure to a sublethal preconditioning stimulus results in resistance to subsequent otherwise lethal ischemic insult (Gidday, 2006; Stenzel-Poore et al., 2007; Obrenovitch, 2008; Dirnagl et al., 2009). Because the preconditioning stimulus is known to generate an epigenetic reprogramming of the brain leading to inhibition of programmed cell death or augmentation of cell survival, the acetylation/deacetylation system appears to be an ideal candidate as a possible neuroprotective mediator in the development of ischemic tolerance (Thompson et al., 2013). So far, this hypothesis has only been explored for SIRT1, which appears to be required for preconditioning-induced ischemic tolerance *in vitro* (Raval et al., 2006; Yan et al., 2013) and *in vivo* (Della-Morte et al., 2009), but not for class I, II and IV HDACs.

We have previously reported that pharmacological preconditioning with glutamate receptor agonists promotes neuroprotective mechanisms that implicate the mild and sublethal activation of poly(ADP-ribose polymerase (PARP) in rat organotypic hippocampal slices exposed to oxygen-glucose deprivation (OGD) (Gerace et al., 2012). In the present study, we examined the histone H3 acetylation levels and the effects of HDAC inhibition in our *in vitro* model of ischemic tolerance and investigated the possible relationship between acetylation/deacetylation and the protective activation of PARP and ERK1/2 that occurs under our experimental conditions.

2. Materials and methods

Experiments and animal use procedures were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23, revised 1996). The experimental protocols were approved by the Animal Care Committee of the Department of Health Sciences, University of Florence, in compliance with the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (ETS no. 123) and the European Communities Council Directive of 24 November 1986 (86/609/EEC). The authors further attest that all efforts were made to minimize the number of animals used and their suffering.

2.1. Materials

NMDA, propidium iodide (PI), suberoylanilide hydroxamic acid (SAHA), and sodium butyrate were purchased from Sigma–Aldrich (St Louis, MO, USA). The MEK inhibitor U0126 was from Promega (Milano, Italy). Thieno(2,3-c)isoquinolin-5-one (TIQ-A) was synthesized as described in Pellicciari et al. (2003). Tissue culture reagents were obtained from Gibco-BRL (San Giuliano Milanese, MI, Italy) and Sigma–Aldrich.

2.2. Preparation of rat organotypic hippocampal slice cultures

Organotypic hippocampal slice cultures were prepared as previously reported (Pellegrini-Giampietro et al., 1999a, 1999b). Briefly, hippocampi were removed from the brains of Wistar rat pups (Harlan, MI, Italy), and transverse slices (420 μm) were prepared using a McIlwain tissue chopper and then transferred onto 30 mm diameter semiporous membranes inserts (Millicell-CM PICM03050; Millipore, Italy), which were placed in sixwell tissue culture plates containing 1.2 ml medium per well. The slices culture medium consisted of 50% Eagle's minimal essential medium, 25% heat-inactivated horse serum, 25% Hanks' balanced salt solution, 5 mg/ml glucose, 2 mM L-glutamine, and 3.75 mg/ml amphotericin B. Slices were maintained at 37 °C in an incubator in atmosphere of humidified air and 5% CO2 for two weeks. Before experiments all slices were screened for viability by incubating them for 30 min with PI (5 mg/ml); slices displaying signs of neurodegeneration were discarded from the study.

2.3. OGD and exposure to drugs in rat organotypic hippocampal slices

Cultures were exposed to OGD as previously reported in detail (Pellegrini-Giampietro et al., 1999a, 1999b). Briefly, the slices were subjected to OGD by exposing them to a serum- and glucose-free medium saturated with 95% N_2 and 5% CO_2. Following 30 min of incubation at 37 °C in an airtight anoxic chamber equipped with an oxygen gas controller (BioSpherix, New York, USA), the cultures were transferred to oxygenated serum-free medium (75% Eagle's minimal essential medium; 25% Hank's balanced salt solution; 2 mM L-glutamine; and 3.75 μg/ml amphotericin B) containing 5 mg/ml glucose and returned to the incubator under normoxic conditions until neuronal injury was evaluated 24 h later.

Exposure to drugs (NMDA, SAHA, sodium butyrate, TIQ-A and U0126) was carried out in the incubator using serum-free medium as previously described (Pellegrini-Giampietro et al., 1999a, 1999b; Gerace et al., 2012). After OGD, hippocampal slices were cultured for an additional 24 h in a fresh serum-free medium (with or without drugs) and then evaluated for CA1 pyramidal cell injury. To achieve maximal neuronal injury, hippocampal slices were exposed for 24 h to 10 mM glutamate in the incubator using serum-free medium.

2.4. Assessment of CA1 pyramidal cell injury

Cell injury was assessed using the fluorescent dye PI (5 μg/ml). PI was added to the medium at the end of the 24 h period following OGD. Thirty minutes later, fluorescence was viewed using an inverted fluorescence microscope (Olympus IX-50; Solent Scientific, Segensworth, UK) equipped with a xenon-arc lamp, a low-power objective (4×) and a rhodamine filter. Images were digitized using a video image obtained by a CCD camera (Diagnostic Instruments Inc., Sterling Heights, MI, USA) controlled by software (InCyt Im1™; Intracellular Imaging Inc., Cincinnati, OH, USA) and subsequently analyzed using the Image-Pro Plus morphometric analysis software (Media Cybernetics, Silver Spring, MD, USA). In order to quantify cell death, the CA1 hippocampal subfield was identified and encompassed in a frame using the drawing function in the image software (ImageJ; NIH, Bethesda, USA) and the optical density of PI fluorescence was detected. There was a linear correlation between CA1 PI fluorescence and the number of injured CA1 pyramidal cells as detected by morphological criteria (Pellegrini-Giampietro et al., 1999a).

2.5. Western blot analysis

Cultured slices were washed with cold 0.01 M phosphate-buffered saline, pH 7.4 and four slices/sample were gently transferred and dissolved in a tube containing 1% sodium dodecyl sulfate (SDS). Total protein levels were quantified using the Pierce (Rockford, IL, USA) BCA (bicinchoninic acid) Protein Assay. Lysates (20 μg per lane of protein) were resolved by electrophoresis on a SDS-polyacrylamide gel and transferred onto nitrocellulose membranes. Blots were probed with a polyclonal rabbit anti-acetyl-histone H3 (Lys 18) (Cell Signaling Technology, Beverly, MA, USA), a monoclonal mouse anti-PAR (10H) antibody (Alexis Biochemicals, Vinci, Italy) or polyclonal rabbit anti-PARP-1, anti-Bcl-2, anti-BAD, anti-phospho ERK1/2 (Thr202/Thr204) and anti-phospho SAPK/JNK (Thr183/Thr185) (Cell Signaling Technology, Beverly, MA, USA), all diluted 1:1000 (Gerace et al., 2012). Immunodetection was performed with secondary antibodies (1:2000 anti-mouse or anti-rabbit IgG from donkey, Amersham Biosciences, UK) conjugated to horseradish peroxidase. The reactive bands were detected using chemiluminescence (ECLplus; Euroclone, Padova, Italy). Quantitative analysis was performed using the QuantityOne analysis software (Bio-Rad, Hercules, CA, USA).

2.6. PARP activity assay

^{3}H-PAR was synthesized *in vitro* as previously described in (Chiarugi et al., 2003). Briefly, 0.05 U of purified bovine PARP-1 was incubated for 60 min at 37 °C in a final volume of 100 ml containing 50 mM Tris–HCl buffer (pH 7.4), 5 mM $MgCl_2$, 2 mM DL-dithiothreitol, 10 mg sonicated DNA, 0.5 mg/ml bovine serum albumin, 10 μM NAD^+ and 0.2 μCi of [adenine-2,8-^{3}H]NAD, with or without 10 μM TIQ-A or 10 μM SAHA. The reaction was stopped by adding 1 ml 10% (w/v) of trichloroacetic