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Acetylation: A lysine modification with neuroprotective effects in ischemic retinal degeneration

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ABSTRACT

Neuroretinal ischemic injury contributes to several degenerative diseases in the eye and the resulting pathogenic processes involving a series of necrotic and apoptotic events. This study investigates the time and extent of changes in acetylation, and whether this influences function and survival of neuroretinal cells following injury. Studies evaluated the time course of changes in histone deacetylase (HDAC) activity, histone-H3 acetylation and caspase-3 activation levels as well as retinal morphology and function (electroretinography) following ischemia. In addition, the effect of two HDAC inhibitors, trichostatin-A and valproic acid were also investigated. In normal eyes, retinal ischemia produced a significant increase in HDAC activity within 2 h that was followed by a corresponding significant decrease in protein acetylation by 4 h. Activated caspase-3 levels were significantly elevated by 24 h. Treatment with HDAC inhibitors blocked the early decrease in protein acetylation and activation of caspase-3. Retinal immunohistochemistry demonstrated that systemic administration of trichostatin-A or valproic acid, resulted in hyperacetylation of all retinal layers after systemic treatment. In addition, HDAC inhibitors provided a significant functional and structural neuroprotection at seven days following injury relative to vehicle-treated eyes. These results provide evidence that increases in HDAC activity is an early event following retinal ischemia, and are accompanied by corresponding decreases in acetylation in advance of caspase-3 activation. In addition to preserving acetylation status, the administration of HDAC inhibitors suppressed caspase activation and provided structural and functional neuroprotection in model of ischemic retinal injury. Taken together these data provide evidence that decrease in retinal acetylation status is a central event in ischemic retinal injury, and the hyperacetylation induced by HDAC inhibition can provide acute neuroprotection.

1. Introduction

Retinal ischemia is associated with a number of vision impairing degenerative diseases including glaucoma, diabetic retinopathy, and retinopathy of prematurity. Knowledge of the cellular events occurring in retinal neurons after ischemic injury is important in understanding the pathophysiological response to ischemia and searching for new treatments for blinding diseases. Protein acetylation, like phosphorylation, plays a significant role in regulation of cellular activity and is controlled by the competing actions of two enzyme families, histone acetyltransferases and histone deacetylases (HDACs) (Crosson et al., 2010). The balance between the actions of these enzymes serves as a key regulatory mechanism for gene expression by modulating chromatin condensation, as well as several signaling events within cells (Haberland et al., 2009).

Dysregulation of acetylation contributes to the pathogenesis of a myriad of diseases, including oncogenic, cardiovascular, and inflammatory disorders (Zhang et al., 2002a; Huang, 2006). In the central nervous system, *in vitro* studies have shown that the inhibition of HDACs can protect neurons from oxidative and nitrosative stress, and glutamate-induced excitotoxicity, as well as promote neuronal growth and prolong neuronal lifespan (Zhong and Kowluru, 2010; Hao et al., 2004; Kanai et al., 2004). *In vivo* studies have provided evidence that HDAC inhibition protected neurons exposed to intracerebral hemorrhage, ischemic injury and stroke (Kim et al., 2007; Sinn et al., 2007). These effects involve regulation of gene expression at the molecular level through

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epigenetic mechanisms, particularly in chromatin remodeling, via direct inhibition of HDACs preventing histone hypoacetylation of specific regions of the chromatin (Phiel et al., 2001).

This article focuses on how protein acetylation is an early event in the post-ischemic environment. Specifically, a rodent model of retinal ischemia was utilized to address changes in the acetylation state of histone-H3 at different time intervals following ischemic injury and to provide a direct comparison to changes in HDAC enzymatic activity levels as well as changes in an apoptotic marker, retinal cleaved caspase-3. This study expanded on previous studies from this laboratory, and evaluated how pharmacologically inhibiting these changes, using two structurally distinct HDAC inhibitors, trichostatin-A (TSA) and valproic acid (VPA), may provide similar structural and functional neuroprotection.

2. Material and methods

2.1. Animals

Adult male or female brown Norway rats (3–5 months of age, 150–200 g; Charles River Laboratories, Inc., Wilmington, MA) were used in this study. Rats were maintained in an environmental cycle of 12-h light and 12-h dark. Animal handling was performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research; and the study protocol was approved by the Animal Care and Use Committee at the Medical University of South Carolina. Previous studies from this laboratory have demonstrated effectiveness of TSA at a dose of 2.5 mg/kg i.p. (Crosson et al., 2010). Studies on VPA have shown neuroprotective effects at 200 mg/kg/day (Dou et al., 2003); however in the current study animals developed motor defects immediately after treatment with 200 mg/kg VPA. Subsequent preliminary dosing studies found that control animal treated twice daily with 100 mg/kg, did not exhibit any motor defects, and hyperacetylation was noted in the retina. Therefore, for neuroprotection studies, trichostatin-A (TSA) (2.5 mg/kg), valproic acid (VPA) (100 mg/kg), or vehicle (0.9% sodium chloride) was administered by intraperitoneal (i.p.) injection 1 h prior and 3 h following ischemic injury on the day studies were initiated. On post-ischemic days 1, 2, and 3, TSA, VPA or vehicle was administered twice daily. In animals receiving any i.p. treatment, functional and morphological results from contralateral eyes were used as control comparisons. For timing experiments, ischemic injury was induced in identical fashion, and retinal lysates were obtained at several early time points within the initial 24 h after ischemia induction to analyze levels of acetylated histone-H3 and cleaved caspase-3 using Western blotting, and histone deacetylase (HDAC) activity using a fluorometric enzymatic assay.

2.2. Retinal ischemia

Prior to the induction of retinal ischemia, rats were anesthetized by i.p. injection of ketamine (75 mg/kg) and xylazine (8 mg/kg) (Ben Venue Laboratories, Bedford, OH), and corneal analgesia created by the application of proparacaine (0.5%; 5 μL; Akorn, Inc., Buffalo Grove, IL). Body temperature was maintained at 37 °C by means of a heating pad (Harvard Apparatus; Holliston, MA). Retinal ischemia was created using methods previously described by Whitlock and colleagues (Whitlock et al., 2005). Briefly, the anterior chamber was cannulated with a 30-G needle that was connected to a container of sterile normal saline via polyethylene tubing (PE-50; Fischer, Atlanta, GA). To induce retina ischemia, the reservoir was elevated to raise the intraocular pressure (IOP) above systolic blood pressure to 160 mmHg for 45 min. The IOP was monitored by an in-line pressure transducer connected to a computer. Each pressure then returned to normal and the eye was examined to ensure that retinal blood flow was reestablished. The contralateral eye was left untreated, serving as control.

2.3. Electroretinograms

To quantitate baseline and post-ischemic neuroretinal function, electroretinograms (ERGs) were performed. Baseline values were obtained one day prior to ischemic injury and seven days post-injury. For these studies, rats were dark-adapted overnight. On the following day, rats were anesthetized with i.p. ketamine and xylazine administration as described above, and pupils dilated with a 10 μL drop of a solution containing phenylephrine HCl (2.5%) and tropicamide (1%) (Akorn, Inc., Buffalo Grove, IL). A needle ground-electrode was placed subcutaneously in the back of the animal and a reference electrode on the tongue. A contact lens gold-ring electrode was held in place on the cornea with a drop of methylcellulose. A stimulus-intensity series of ERGs was recorded in response to single-flash intensities, from 40 dB attenuation (low-intensity flash), to no attenuation (high-intensity flash). Responses were an average of 2 flashes with an inter-stimulus interval of 2 min. Electroretinograms were recorded by means of an UTAS-2000 system (LKC Technologies, Gaithersburg, MD). Amplitudes of ERG a- and b-waves from ischemic eyes of VPA-treated animals were compared to contralateral control responses and corresponding responses from vehicle-treated animals.

2.4. Morphometric analysis

For histological examination, rats were euthanized by an overdose of pentobarbital. Eyes were then enucleated and fixed for 1 h in 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS) at 4 °C. The eyes were opened at the ora serrata and fixation continued for 24 h. Following fixation, the anterior segment was removed and the posterior eyecup dehydrated and embedded in paraffin. Retina cross-sections 5 μm thick were then cut and stained with hematoxylin and eosin (Sigma Chemical Co., St. Louis, MO). Retina sections were photographed and measured 450–550 μm from the edge of the optic nerve head by means of a Zeiss Axioplan-2 fluorescent microscope (Maple Grove, MN).

For immunohistochemical analysis, selected eyes were fixed in 4% paraformaldehyde for 1 h. Globes were washed in PBS and transferred into 15% sucrose solution for 1 h, followed by 30% sucrose solution overnight at 4 °C. Tissues were embedded in optimal cutting temperature (OCT) compound (Tissue Tek; Sakura Finetech, Torrance, CA) and 10 μm slices sectioned at −26 °C. The sections were washed in PBS to remove OCT and blocked with 5% normal donkey serum, 3% bovine serum albumin, and 0.1% Triton X-100 in PBS for 1 h at room temperature. Sections were incubated in primary antibody specific to acetyl histone-H3 (1:500 dilution) (Cell-Signaling Technologies) at 4 °C overnight. Sections were then washed and incubated for two hours at room temperature with FITC-labeled secondary antibody (1:100 dilution) (Invitrogen). For negative controls, the staining with primary antibody was omitted and sections were stained with only FITC-labeled secondary antibody. Retina sections were observed and photographed by means of a Zeiss Axioplan-2 fluorescence microscope (Maple Grove, MN).

2.5. Western blot analysis

Western blot analysis was performed after homogenization of whole retina in lysis buffer (50 mM Tris-base; 10 mM EDTA; 0.5 mM sodium orthovanadate; 0.5% sodium deoxycholic acid; 1% Triton X-100) and protease inhibitors. Equivalent amounts of protein were loaded onto 10% SDS polyacrylamide gels; proteins were separated by PAGE and transferred to nitrocellulose paper. The membranes