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BRIEF COMMUNICATION

Viral genome silencing by neuronal sirtuin 1

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Abstract Neurotropic viruses remain dormant in sensory neurons for years, but upon reactivation, they can produce multiple disease states including pain symptoms. Latent viral DNA is extrachromosomal, maintained as a circular episome bound to histones. Here, we show the regulation of an adenoviral genome by the nicotinamide adenine dinucleotide (NAD^+)-dependent histone deacetylator Sirt1 in dorsal root ganglion neurons. Pharmacological modulation of Sirt1 and Sirt1 overexpression both affected viral transgene expression. We propose that age or stress-related neuronal NAD^+ depletion may be a trigger for viral reactivation.

Keywords Histone deacetylase · Viral reactivation · Shingles · Nicotinamide adenine dinucleotide · Aging · Pain

Introduction

Recent case reports suggest that histone deacetylase (HDAC) inhibitor therapy in cancer patients can result in the reactivation of DNA viruses (Ritchie et al. 2009). HDACs catalyze the deacetylation of lysine residues on histones leading to strengthening of the histone–DNA contacts and transcriptional silencing (Borrelli et al. 2008). There are three classes of HDACs—class I, class II, and class III. Sirtuins belong to class III and are nicotinamide adenine dinucleotide (NAD^+)-dependent. Sirt1 is a nuclear protein involved in regulating many processes, including transcription, senescence, metabolism, and apoptosis. Sirt1 is a highly conserved NAD^+-dependent deacetylase important for determining longevity and is expressed in many species from bacteria to mammals. Deletion of *Sir2* in yeast, Sir2 caused a decreased life span and extra copies of Sir2 produced a prolonged life span (Kaeberlein et al. 1999).

With aging, the biosynthesis of NAD^+ declines and the resultant loss of genome silencing causes aberrations in gene expression (Imai 2009). Specific organs like the brain and pancreas are highly susceptible to changes in biosynthesis of NAD^+ since they have very low levels of NAD^+ biosynthetic enzymes (Revollo et al. 2007). Coincidently, shingles, which is caused by varicella zoster virus (VZV) reactivation in latently infected neurons, correlates with aging. Shingles, with an annual US incidence of 1 million cases per year, occurs most often after the age of 50. Unfortunately, the molecular triggers responsible for VZV reactivation are still unknown. In this study, the potential for Sirt1 to regulate viral transgene expression was investigated. We show that Sirt1 is expressed in dorsal root ganglia (DRG) neurons, which are known to harbor latent VZV infections. Inhibition, activation, and overexpression of Sirt 1 were employed to determine the effects on viral transgene expression.

Sirt1 expression in DRG neurons

We examined the expression of Sirt1 in both rat embryonic DRG neuronal cultures and intact adult slices. In mice,

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Sirt1 was shown to be embryonically expressed in DRG but expression levels declined during development (Sakamoto et al. 2004a). Under our culture conditions, E15 DRG neurons form a homogenous population of cells expressing neuroinflammatory peptides and Neurofilament. Sirt1 is universally expressed in these neurons and was found mainly in the nucleus with some diffuse cytoplasmic staining (Fig. 1a). Residual non-neuronal cells after araC treatment did not show nuclear Sirt1 staining, indicating that expression is limited to neurons. We also found abundant Sirt1 expression in neuronal nuclei of intact adult rat DRG neurons. Specific punctuate staining is visible in the nuclei of large-, medium-, and small-sized DRG neurons (Fig. 1b). Diffuse cytoplasmic staining was also observed. The faint 4′-6-diamidino-2-phenylindole (DAPI) staining of neuronal nuclei is likely the result of the large nuclear size and sectioning of the DRG. In addition, the image acquisition was optimized to minimize stray light from other focal planes by using an apotome. We therefore feel that the image is an underrepresentation of the actual amount of Sirt1 present in adult DRG neurons. In contrast, there is no visible nuclear Sirt1 staining within the satellite cells of the DRG although a few cells exhibited cytoplasmic staining. In the central nervous system, neuronal Sirt1 expression and distribution were shown to be equivalent in adult rats and humans (Zakhary et al. 2010). Our results provide the first evidence of Sirt1 expression in adult DRG neurons and contrast with previous reports of expression only occurring during embryogenesis (Sakamoto et al. 2004b).

Sirt1 modulation alters viral transgene expression

We subsequently used the cultured DRG neurons to assess viral genome silencing by Sirt1. Specific activators and inhibitors of Sirt1 were used to examine the modulation of the ZsGreen transgene in a replication-defective A5 adenovirus. Culture media were supplemented with either nicotinamide (4 and 10 mM), resveratrol (50 μM), trichostatin A (tsA; 500 pM), or SRT1720 (500 nM) during viral infection. Induction of ZsGreen was quantified by Western analysis and visualized by fluorescence microscopy. A representation of untreated neurons is shown for each drug treatment because experiments were performed using different inoculations. Nicotinamide, a potent inhibitor of Sirt1, significantly increased expression of ZsGreen (Fig. 2a, b). Addition of 4 mM nicotinamide and 10 mM increased ZsGreen 1.47×±0.15 (n=4; p=0.0477) and 2.28×±0.39 (n=4; p=0.0457), respectively (Fig. 2c, k). With 10 mM nicotinamide, ZsGreen expression was so robust that it appeared in the cell body and in neuronal projections. Resveratrol is a polyphenol found in wines, a strong antioxidant, and a specific activator of Sirt1. Resveratrol significantly decreased expression of ZsGreen (Fig. 2d, e) to 0.39±0.18 of control levels (n=4; p=0.0459; Fig. 2f, k). SRT1720, which is structurally unrelated to resveratrol, but a potent Sirt1 activator, also decreased ZsGreen expression (n=2; data not shown). Addition of tsA, which inhibits class I and class II HDACs, produced a dramatic increase in ZsGreen expression. (Fig. 2g, h). tsA (500 pM) increased ZsGreen 5.93×±1.14 (n=3; p=0.0491; Fig. 2i, k). These findings show that all three classes of HDACs are involved in silencing viral genomes.

Overexpression of Sirt1 reduces transgene expression

To further illustrate the ability of Sirt1 to regulate transgene expression, we overexpressed Sirt1 in neurons. We similarly inoculated with 500 multiplicity of infection (MOI) adenovirus containing ZsGreen as a transduction marker; however, we supplemented our inoculations with either an adenovirus encoding mouse Sirt1 (250 MOI; Alcendor et al.

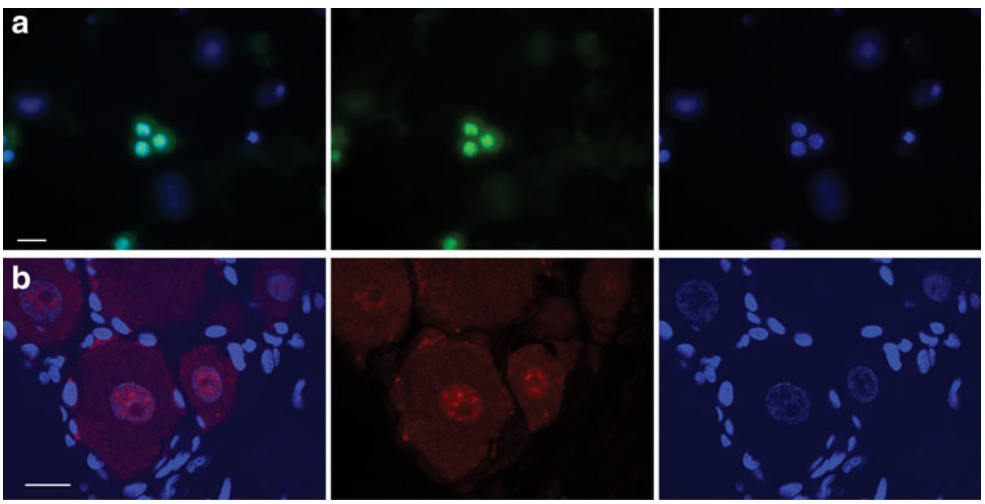

Fig. 1 Sirt1 is expressed in cultured embryonic and adult DRG neurons. **a** Sirt1 (*green*) expression localized to the nucleus (*blue*) of cultured embryonic DRG neurons. **b** Sirt1 (*red*) expression localized to the nucleus (*blue*) of intact adult DRG neurons. Incubation with secondary antibody alone did not produce any specific staining (data not shown). (*left*, merged *middle* Sirt1, *right* DAPI) *Scale bar*=20 μm

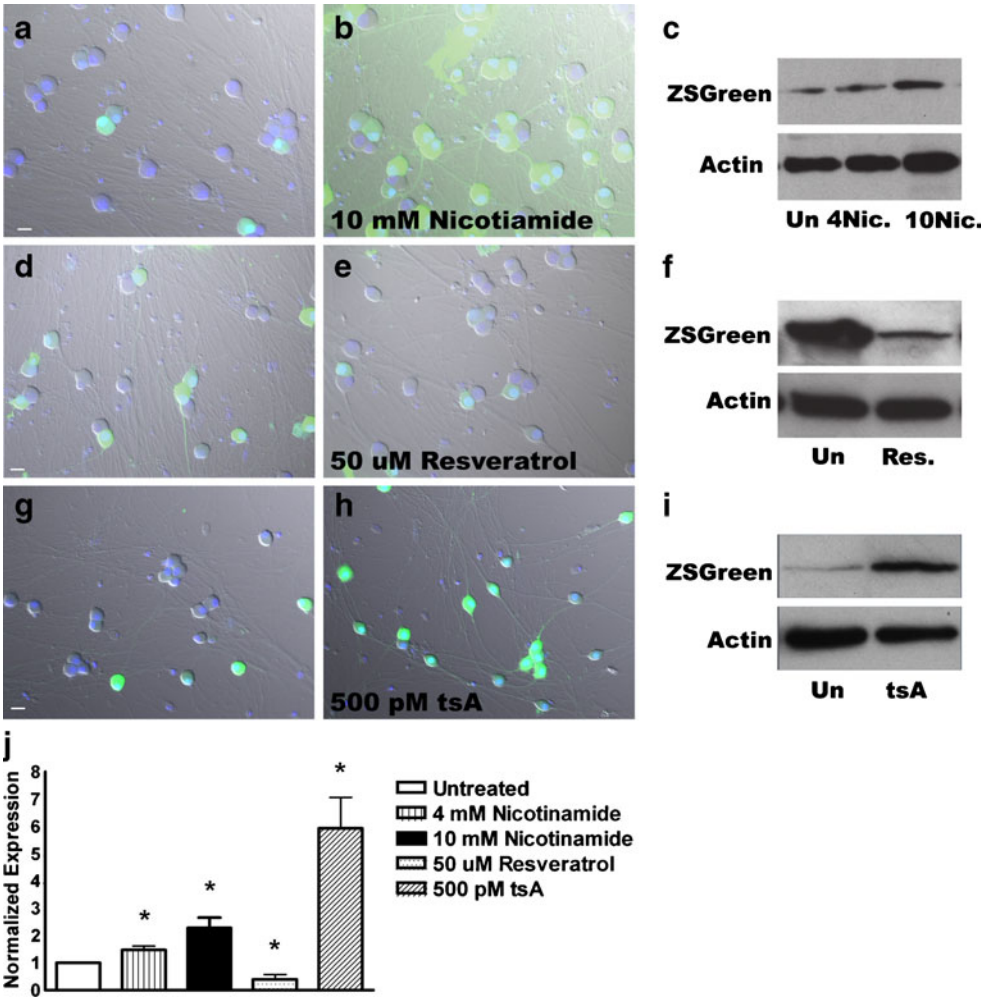

Fig. 2 Modulation of Sirt1 affects adenoviral transgene expression. **a** Representative expression of ZsGreen in untreated neurons or **b** neurons treated with 10 mM nicotinamide. **c** Representative western blot. **d** Representative expression of ZsGreen in untreated neurons or **e** neurons treated with 50 μM resveratrol. **f** Representative western blot. **g** Representative expression of ZsGreen in untreated neurons or **h** neurons treated with 500 pM tsA. **i** Representative western blot. **j** ZsGreen expression normalized to actin (4 mM nicotinamide $n=4$, $^*p<0.05$; 10 mM nicotinamide $n=4$, $^*p<0.05$; Resveratrol $n=4$, $^*p<0.05$; tsA $n=3$, $p<0.05$). DIC images are overlaid with DAPI (*blue*) and ZsGreen. *Scale bar*=20 μm, *error bars* indicate SEM

2004) or a control non-coding sequence (250 MOI). Again, we examined ZsGreen expression 48 h after adenovirus inoculation. Increased Sirt1 caused a significant reduction in the expression of ZsGreen when compared to the non-coding control or adenovirus-ZsGreen alone (Fig. 3a, b). Sirt1 overexpression reduced ZsGreen expression to 0.5913± 0.078 ($n=3$, $p=0.0363$) when compared to neurons only inoculated with the adenovirus-ZsGreen. Similarly, supplementation with a non-coding adenovirus did not alter ZsGreen expression 0.9874±0.1819 ($n=3$, $p=0.9509$) when compared to neurons only inoculated with adenovirus-ZsGreen. Lysates were harvested at 48 h for consistency with the experiments containing pharmacological activators as described above. It is possible that additional reduction of ZsGreen could have been observed at later time points or at higher MOIs. Unfortunately, because of the fragility of cultured neurons, these experiments were not performed.

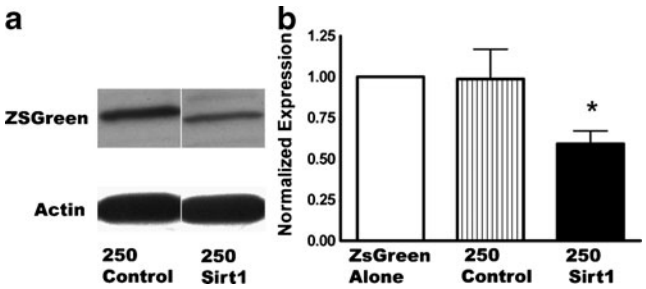

Fig. 3 Overexpression of Sirt1 attenuates ZsGreen expression. **a** Representative Western blot comparing ZsGreen expression of adenovirus containing a non-coding control sequence (*lane 1*) and adenovirus with Sirt1 (*lane 2*). **b** Densiometric analysis of ZsGreen expression with and without Sirt1 overexpression. Data were normalized to actin (250 MOI Sirt1 $n=3$, $^*p<0.05$)

Our data show high levels of expression of Sirt1 in both embryonic and adult DRG neurons. Using cultured DRG neurons, we were able to study the effects of various Sirt1 modulators on adenoviral transgene expression. Inhibition of Sirt1 by nicotinamide significantly increased expression of adenoviral transgenes while activation of Sirt1 by resveratrol significantly reduced expression. Sirt1 likely acts in concert with other HDACs because tsA had an equivalent effect on adenoviral transgene expression. Previous studies have shown that tsA treatment caused the derepression of HSV-1 genome in persistently infected trigeminal neurons (Terry-Allison et al. 2007). We provided

further evidence for the role of Sirt1 in viral genome repression in overexpression experiments. Sirt1 activation by resveratrol and Sirt1 overexpression had the same effect: a reduction in adenoviral transgene expression.

Reactivation of neurotropic viruses has been considered the consequence of a compromised immunity. Clearly, the development of latency and the mechanisms of reactivation differ among neurotropic viruses. Compared to HSV, VZV does not express a latency-associated transcript (LAT). LAT is thought to be important for both the development of HSV latency and reactivation (Hay and Ruyechan 1994). However, HSV reactivation occurs with a much higher frequency than VZV reactivation. Nonetheless, in VZV reactivation, resultant shingles is still thought to result from an age-related decline in cellular immunity. However, DRG neurons do not express major histocompatibility complex proteins (Turnley et al. 2002), and unlike HSV, immune cells are absent in VZV-infected ganglia (Theil et al. 2003; Verjans et al. 2007). Moreover, so-called "latency-associated" proteins are rarely expressed, if at all, in infected ganglia (Zerboni et al. 2010a; Zerboni et al. 2010b). The VZV genome is episomal and condensed with histones, and the viral burden is low, with only an estimated six to 31 copies of VZV DNA per 10,000 ganglionic neurons (Mahalingam et al. 1993). Viral latency and reactivation should be in large part dependent upon the activity of HDACs. Indeed, many groups have shown the importance of Class I HDACs in the development of latency of HSV and VZV. Inhibition of the repressive activity of HDACs can lead to viral replication (Walters et al. 2010), while in neurons, it has been proposed that viruses can use HDACs to develop a latent infection (Du et al. 2010; Roizman et al. 2005). As opposed to compromised immunity, we propose that a reduction in neuronal Sirt1 activity caused by declining NAD^+ levels during aging and/or stress is a molecular trigger for viral reactivation. Therefore, Sirt1 activators such as resveratrol could be used to prevent viral reactivation. In fact, resveratrol has been shown to decrease replication of VZV in non-neuronal cells (Docherty et al. 2006).

Materials and methods

Neuronal culture DRG were isolated and cultured using standard techniques. Briefly, DRG were dissected from E15 embryos of Sprague–Dawley rats. The ganglia were dissociated in 2.5 mg/ml trypsin (Invitrogen, Carlsbad, CA) for 40 min. Cells were plated on poly-D-lysine (Sigma, St. Louis, MO) and laminin-coated coverslips (Invitrogen, Carlsbad, CA), and maintained in serum-free medium containing 100 ng/ml nerve growth factor (Harlan Bio-products, Indianapolis, IN). One day after dissection, cells were treated with 1 μM cytosine-D-arabinofuranoside (Sigma, St. Louis, MO), an inhibitor of DNA synthesis to inhibit growth of non-neuronal cells, for 2 days. Cells were allowed to recover for 2 days prior to manipulation.

Immunocytochemistry Cultured neurons were fixed in 4% paraformaldehyde (PFA) over 30 min then washed with phosphate-buffered saline (PBS) over 30 min. Neurons were permeabilized with 0.1% Triton X-100 for 5 min then washed again with PBS. Neurons were blocked with PBS containing 5% bovine serum albumin (BSA) for 2 h at room temperature (RT) and then incubated with primary antibody anti-SIRT1 (Santa Cruz, Santa Cruz, CA) in PBS containing 5% BSA overnight at 4°C. After several rinses in PBS, secondary antibody Alexa Fluor 488 goat anti-mouse was added (Molecular Probes, Eugene, OR) for 1 h. Coverslips were then mounted on slides using Prolong Gold antifade reagent with DAPI (Molecular Probes, Eugene, OR). Neurons were imaged using a Zeiss Axioimager.

Immunohistochemistry Lumbar and thoracic DRG were isolated from adult male Sprague–Dawley rats. Animals were anesthetized with sodium pentobarbital (60 mg/kg) and perfused transcardially with 60 ml PBS followed by 60 ml of cold 4% PFA. DRG were removed, cleaned of surrounding tissue, post-fixed in 4% PFA, and transferred to 20% sucrose. After embedding in freezing media, frozen sections of DRG were sectioned at 20 μm. Sections were permeabilized with a PBS solution containing 0.4% Triton X-100. Sections were then blocked for 2 h at RT with PBS containing 5% BSA and then incubated with the primary antibody anti-SIRT1 (Santa Cruz, Santa Cruz, CA) in PBS containing 5% BSA overnight at 4°C. After several rinses in PBS, secondary antibody Alexa Flour 546 goat anti-mouse was added (Molecular Probes, Eugene, OR) for 2 h. Coverslips were then mounted on slides using Prolong Gold antifade reagent with DAPI. Sections were imaged using a Zeiss Axioimager.

Western analysis DRG cultures were collected in lysis buffer containing protease inhibitors. Lysates were then sonicated for 10 min and centrifuged (14,000×*g* for 10 min at 4°C). Reducing buffer was added to the supernatant and lysates were boiled for 5 min then loaded on to a 4–15% Tris–HCl gel (BioRad, Hercules, CA). After electrophoresis, the gel was transferred to a PVDF membrane and then blocked with 5% non-fat dry milk. Primary antibodies to ZsGreen (Clonetech, Mountain View, CA) were incubated overnight at 4°C. Then, membranes were stripped and reprobed for actin (Santa Cruz, Santa Cruz, CA). Horseradish peroxidase-conjugated secondary antibodies were used (Santa Cruz, Santa Cruz, CA). The signal was detected using a chemiluminescent substrate kit (KPL, Gaithersburg, Maryland)

and collected on X-ray film. Densitometry analyses obtained from Western blots using Quantity One software.

Adenovirus transduction Adeno-X containing ZsGreen (Clonetech, Mountain View, CA) was transfected using lipofectamine (Invitrogen, Carlsbad, CA) into HEK 293A cells and then amplified. After titers were calculated in HEK 293A cells, neurons were transduced with 500 MOI adenovirus. Neurons were treated with nicotinamide, tsA, resveratrol, or SRT1720 at time of transduction. Forty-eight hours post-transduction, fluorescence microscopy and Western analysis were performed. For Sirt1 overexpression experiments, neurons were co-transduced with 500 MOI adenovirus-ZsGreen and either 250 MOI of a control non-coding sequence or 250 MOI of adenovirus encoding mouse Sirt1. Forty-eight hours post-transduction, Western analysis was performed.

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