Nitric oxide and histone deacytases
A new relationship between old molecules

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Histone deacytases (HDACs) are enzymes that catalyze the removal of acetyl groups from a range of nuclear and cytoplasmic proteins. Recently, we described a novel route to neurotrophin-dependent gene activation in neurons, which requires the S-nitrosylation of nuclear HDAC2 by the gaseous molecule nitric oxide (NO). We have further investigated the NO-dependent regulation of HDACs in neurons. Using a fluorogenic deacytation assay, we show that NO decreases the enzymatic activity of a sub-group of neuronal HDACs in vitro and that this inhibition is not due to damaging modifications such as oxidation or tyrosine nitration. The neuronal HDACs whose catalytic activity is inhibited by NO are entirely those that are localized in the cytoplasm. These observations support and extend the concept that nitric oxide is a key regulator of HDAC function in mammalian neurons.

Amongst the genes residing within modern eukaryotic genomes, some are truly ancient, with their origins stretching back to the earliest days of cellular evolution. These genes play fundamental roles in cell biology across the eukaryotic phylogenetic tree. Histone deacytases are one such family of archaic enzymatic proteins. HDACs are found in all modern eukaryotic organisms and are essential for cellular proliferation, differentiation and homeostasis. The HDACs are also implicated in the onset and progression of many human diseases which affect an increasingly aging population, such as neurodegenerative disease, cancer, cardiovascular disease and chronic obstructive pulmonary disease. The development of effective treatments based on HDAC inhibition is however hampered by a lack of specificity in currently available molecules which often block many, if not all, HDAC isoforms.

We recently described that HDAC2, a nuclear histone deacytase that is highly expressed in neurons, is regulated by S-nitrosylation. NO generated in response to neurotrophin stimulation diffuses throughout the neuronal soma into the cell nucleus and nitrosylates HDAC2 at cysteine residues 262 and 274, releasing it from chromatin to facilitate gene expression. S-nitrosylation of HDAC2 stimulates its release from DNA but does not alter the enzyme’s catalytic activity.

Histone deacytases would be more aptly referred to as “lysine deacytases” as they deacytlate these residues in a wide range of non-histone proteins, including cytoskeletal proteins and transcription factors. Mammalian HDACs fall into one of four different classes based on their amino acid sequence and structure. Class I HDACs are trichostatin A (TSA)-sensitive enzymes, which are typically expressed ubiquitously in mammalian cells and are predominantly nuclear in localization. Class II and IV HDACs are tissue-specific, TSA-sensitive enzymes that may be found in both the nucleus and the cytoplasm where they undergo stimulus-dependent nucleo-cytoplasmic shuttling. Class III HDACs are the sirtuins family. Sirtuins have no homology to Class I/II/IV HDACs and are NAD+-, rather than zinc, dependent deacytases that can be found in the nucleus, cytoplasm or mitochondria. The sirtuins are insensitive to inhibitors of class I/II/IV HDACs, such as TSA. HDACs may therefore act in the cytoplasm or nucleus on a range of histone or non-histone proteins to exert their effect on numerous intracellular events.

The diversity in HDAC localization and function, in addition to the nature of NO as an ancient and important neuronal signaling molecule, prompted us to study whether NO-dependent regulation of HDACs might occur at several levels in neurons. Neuronal whole cell lysates were incubated with the donor S-nitrosoglutathione (GSNO), which yields NO in aqueous solution. GSNO inhibited whole-cell histone deacytase enzymatic activity in a dose-dependent manner (Fig. 1A), although glutathione had no effect. Interestingly, GSNO caused only a partial inhibition of whole cell HDAC activity that reached a plateau between 100 and 500 μM concentration. In contrast, the pan-HDAC inhibitor TSA caused a complete (>99%) inhibition of neuronal HDAC activity (Fig. 1B). This observation has two important implications: first, the total cellular HDAC activity measured by this assay is dependent upon the TSA-sensitive class I, II and IV HDACs, with no contribution from the TSA-insensitive sirtuins. Second, the partial inhibition of HDAC activity observed upon treatment with GSNO is due to a sub-group of HDACs that account for approximately 40% of total measurable...
Nitric oxide regulates histone deacetylase functions.

The inhibition is specific to NO, as the effect of GSNO on HDAC activity is completely abolished by the NO-scavenger PTIO (Fig. 1B). Oxidative damage and tyrosine nitration has been shown to reduce the enzymatic activity of HDACs. The decrease in HDAC activity here is unlikely to be due to these damaging post-translational modifications, as the potent oxidizing agent hydrogen peroxide did not cause a significant decrease in HDAC activity (Fig. 1B) and specific anti-nitrotyrosine antibodies did not detect any modification when neuronal cell lysates were exposed to GSNO (Fig. 1C). These results suggest that the change in HDAC activity may occur via a physiological NO-based effect, such as S-nitrosylation.

To study whether NO inhibits cytoplasmic and/or nuclear HDACs, NO donors were incubated with either cytoplasmic or nuclear extracts, and the effect on HDAC activity was measured. The results showed that NO donors inhibited HDAC activity in both cytoplasmic and nuclear fractions, but the inhibition was more pronounced in the cytoplasmic fraction.

**Figure 1.**

(A) Whole cell lysates prepared from E18 embryonic cortical neurons were used in a fluorogenic HDAC assay as previously shown. Lysates were incubated with the NO donor GSNO, or control glutathione (GSH) for 1 hour at room temperature before HDAC activity was assayed. HDAC activity was normalized to untreated control (100% activity). Data were analyzed using paired t-tests comparing GSNO vs. GSH-treated lysate at each concentration point (n = 5). *p = 0.0161; **p = 0.0085; ***p = 0.0006. (B) Whole cell neuronal lysates were incubated with GSNO, GSH, hydrogen peroxide (all 500 μM), TSA (1 μM) and GSNO (500 μM) in the presence of the NO scavenger PTIO (200 μM) for one hour at room temperature. Data are normalized to untreated (100%) and presented as mean ± SEM (n = 3): *p < 0.05 and ***p < 0.001 (versus control), #p < 0.05 (GSNO vs. GSNO + PTIO) by one-way ANOVA with Tukey’s post hoc test. (C) Anti-nitrotyrosine western blot analysis of neuronal lysates following 1 hour incubation with NO donors. Tyrosine-nitrated myosin, BSA and superoxide dismutase were used as positive control. Tyrosine nitration was not observed with concentrations of GSNO up to 500 μM (n = 2). Some tyrosine nitration appeared following treatment with S-nitrosocysteine (SNOC). (D) Neuronal cytoplasmic and nuclear extracts were incubated with GSNO (500 μM), GSH (500 μM) or TSA (1 μM) for 1 hour at room temperature and subjected to fluorogenic HDAC assay. Data are presented as mean ± SEM (n = 3) after normalization to untreated (100%) HDAC activity. Statistical differences were calculated using one-way ANOVA with Tukey’s post hoc test: *p < 0.05 and ***p < 0.001 (versus control), #p < 0.05 (500 μM GSNO vs. 500 μM GSH).
nuclear neuronal extracts and subjected to HDAC activity assay. We observed that NO-dependent inhibition of HDAC activity was entirely confined to the cytoplasmic fraction (Fig. 1D). In contrast, both cytoplasmic and nuclear fractions were fully inhibited by TSA. One interesting candidate whose catalytic activity may be specifically inhibited by NO is HDAC6, a cytoplasmic class II HDAC that plays a key role in regulating tubulin deacetylation and protein turnover.20-22 However, we were unable to find any NO-induced inhibition of tubulin acetylation (unpublished observations).

Our observations indicate that NO regulates both nuclear and cytoplasmic neuronal HDACs. This may entail HDAC dissociation from promoter regions in the nucleus and inhibition of HDAC catalytic activity in the cytoplasm. A plausible mechanism is that NO regulates the assembly of HDAC-containing macromolecular complexes in neurons through S-nitrosylation of HDACs or HDAC binding partners. Full HDAC enzymatic activity is critically dependent upon their association with other proteins within large macromolecular complexes.23,24 NO may regulate such protein-protein interactions by promoting assembly or disassembly of complexes incorporating S-nitrosylated proteins.25 In the nucleus for example, NO induces the disassembly of complexes containing S-nitrosylated HDAC2 from the chromatin, resulting in decreased histone deacetylation levels and gene transcription. In the cytoplasm, NO may promote the disassembly of HDAC-containing complexes resulting in an inhibition of HDAC catalytic activity. Our observations provide evidence that NO is a key regulator of multiple HDAC functions in mammalian neurons. As NO may act on HDACs in the nucleus or the cytoplasm, it has the potential to influence multiple intracellular events in neurons. The discovery of a sub-group of NO-sensitive cytoplasmic HDACs implies that events in the cytoplasm, such as cytoskeleton rearrangement or regulation of protein stability and turnover, could potentially be modulated through NO-dependent changes in HDAC activity. Finally, the identification of NO as a modulator of specific HDACs in neurons may also prove useful for targeting those isoforms that are involved in the progression of neurodegenerative disease.

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