学 位 論 文 の 要 旨

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学 位 論 文 名	Effects of NAD^+ Synthesis Levels on Sirtuin 1 Deacetylase Activity in Mammalian Cells
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論 文 内 容 の 要 旨

INTRODUCTION

Sirtuin 1 (SIRT1) is an evolutionarily conserved NAD^+-dependent protein deacetylase that plays critical roles in various biological processes, such as carbohydrate and fat metabolism, the circadian cycle, and aging. Increasing the deacetylase activity of SIRT1 has been reported to protect against age-related functional decline and diseases, such as metabolic syndrome, neurodegeneration, and cancer. Attention has been focused on physiological and pharmacological interventions that boost cellular NAD^+ levels to promote healthy aging. In mammalian cells, NAD^+ synthesis is accomplished predominantly through the salvage pathway from nicotinamide (Nam) and nicotinic acid (NA).

We have demonstrated previously that NA elevates cellular NAD^+ levels in cells expressing NAPRT, thus protecting cells from stress. Using mass spectrometry and cell volume measurements, we recently determined absolute concentrations of NAD^+ ($[NAD^+]$) in primary cultured cells and various mammalian cell lines, and found that $[NAD^+]$ was maintained within a relatively narrow range of 400–700 μM, irrespective of cell origins. As the reported SIRT1 K_m value for NAD^+ (~90 μM) is much lower than the measured cellular $[NAD^+]$, we hypothesized that increased cellular NAD^+ levels may not stimulate SIRT1 deacetylase activity.

In this study, we employed two human cell lines as model systems, altered cellular $[NAD^+]$ with NA and the Nampt inhibitor FK866, and examined the effects of changes in $[NAD^+]$ on the

acetylation of histone H4 lysine 16 (H4K16) and histone H3 lysine 9 (H3K9), the preferred targets of the class III histone deacetylase (HDAC) SIRT1.

MATERIALS AND METHODS

Human hepatoma HepG2 cells stably expressing NAPRT (HepG2-NAPRT) were cultured in Nam-free MEM supplemented with dialyzed serum containing various concentrations of NA, FK866, EX-527, or trichostatin A (TSA). For knockdown experiment, HepG2-NAPRT cells were transfected with SIRT1 siRNA or control siRNA. Human cervical carcinoma HeLa cells were cultured in Nam-free MEM supplemented with Nam containing the indicated concentrations of NA or FK866. Harvested cells were counted and determined approximate volume using a Scepter instrument. NAD^+ was extracted with perchloric acid, neutralized with ammonium formate, and analyzed by liquid chromatography with tandem mass spectrometry (LC/MS/MS). For western blotting, cell lysates separated using SDS-PAGE were transferred onto membranes. The membranes were reacted with anti-acetyl (Ac)-lysine, anti-H4K16Ac, anti-H3K9Ac, anti-histone H4, anti-histone H3, or anti-SIRT1 antibodies.

RESULTS AND DISCUSSION

Supplying HepG2-NAPRT cells with 20 μM NA increased cellular [NAD^+] to 1400 μM from the basal level of 450 μM. However, this increase in cellular NAD^+ did not decrease the acetylation levels of H4K16 or H3K9. Decrease in [NAD^+] to 250 μM with 100 nM of a NAMPT inhibitor FK866 from the basal level also did not affect acetylation levels of H4K16 or H3K9. SIRT1 expression also was not altered after the treatment with NA or FK866. Thus, an increase in cellular [NAD^+] from 250 to 1400 μM, which is 15-fold higher than the reported SIRT1 K_m value for NAD^+ (~90 μM), does not affect histone acetylation levels.

Unlike NA or FK866, 1 μM TSA, a class I/II HDAC inhibitor, dramatically increased the acetylation of sites on both histone H4 and H3 without accompanying changes in cellular [NAD^+] or SIRT1 expression, confirming that the signals we observed using targeted acetylated lysine antibodies indeed represent protein acetylation.

In HeLa cells, which express endogenous NAPRT, NA elevated cellular [NAD^+] to around 1000 μM. Such large increases in [NAD^+] did not decrease acetylation levels of H4K16 or H3K9. Adding 30 and 100 nM FK866 decreased cellular [NAD^+] from 600 to near 100 μM, close to the K_m value of SIRT1 for NAD^+, but did not increase acetylation levels of H4K16 or H3K9. Moreover, SIRT1 expression was unchanged by FK866.

To test the possibility that SIRT1 does not deacetylate a majority of histones, the effects of SIRT1 deacetylase activity inhibition or reductions in SIRT1 expression on histone acetylation in

HepG2-NAPRT cells were examined. To our surprise, treatment with neither EX-527 (30 μM), a SIRT1 inhibitor, nor SIRT1 knockdown did increase histone acetylation levels, indicating that SIRT1 deacetylates a limited number of histones.

In this study, whether SIRT1 deacetylase activity is regulated strictly by cellular [NAD^+] was investigated. In contrast to most studies that have described cellular NAD^+ content, we provided absolute cellular NAD^+ concentrations by calculating the molar amounts of NAD^+ based on LC/MS/MS and cellular volume measurements. We manipulated [NAD^+] by boosting NAD^+ synthesis with NA in HepG2 cells overexpressing NAPRT (HepG2-NAPRT) and HeLa cells expressing endogenous NAPRT. In HepG2-NAPRT cells, NA-induced increases in [NAD^+] from 450 to 1400 μM did not reduce H4K16 and H3K9 acetylation. In addition, increasing [NAD^+] in HeLa cells from 600 to 1000 μM using NA did not reduce histone acetylation. We also manipulated cellular NAD^+ levels using the Nampt inhibitor FK866. In HepG2-NAPRT and HeLa cells, treatment with FK866 decreased cellular [NAD^+] from 450 to 250 μM and from 600 to 100 μM, respectively, which did not result in increased H4K16 and H3K9 acetylation. Cellular NAD^+ concentrations obtained here could represent the concentrations of nuclear/cytosol fractions. Thus, our observations are consistent with the notion that normal levels of cellular NAD^+ are more than what is required for the nuclear enzyme SIRT1, and thus, [NAD^+] is not a limiting factor for deacetylase activity under normal conditions if SIRT1 is a major deacetylase of acetylated H4K16 and H3K9.

We found that SIRT1 knockdown or inhibition with EX-527 did not influence H4K16 or H3K9 acetylation. In contrast, TSA elevated H4K16 and H3K9 acetylation dramatically. H4K16 and H3K9 may thus be deacetylated mainly by class I/II HDACs but not SIRT1. These observations provide evidence that global acetylation levels of H4K16 and H3K9 may not always be proper indicators of intracellular SIRT1 deacetylase activity.

CONCLUSION

We found that increasing cellular [NAD^+] to 1000–1400 μM from basal levels of 450–600 μM or decreasing the [NAD^+] to 100 μM did not affect histone H4 and H3 acetylation, whereas treatment with TSA increased acetylation levels dramatically. Unexpectedly, neither EX-527, a SIRT1 inhibitor, nor SIRT1 knockdown increased histone acetylation, indicating that global acetylation levels of H4K16 and H3K9 may not be appropriate indicators of intracellular SIRT1 deacetylase activity.