Extension of Human Cell Lifespan by Nicotinamide Phosphoribosyltransferase

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†Extending the productive lifespan of human cells could have major implications for diseases of aging, such as atherosclerosis. We identified a relationship between aging of human vascular smooth muscle cells (SMCs) and nicotinamide phosphoribosyltransferase (Nampt/PBEF/Visfatin), the rate-limiting enzyme for NAD⁺ salvage from nicotinamide. Replicative senescence of SMCs was preceded by a marked decline in the expression and activity of Nampt. Furthermore, reducing Nampt activity with the antagonist FK866 induced premature senescence in SMCs, assessed by serial quantification of the proportion of cells with senescence-associated β-galactosidase activity. In contrast, introducing the Nampt gene into aging human SMCs delayed senescence and substantially lengthened cell lifespan, together with enhanced resistance to oxidative stress. Nampt-mediated SMC lifespan extension was associated with increased activity of the NAD⁺-dependent longevity enzyme SIRT1 and was abrogated in Nampt-overexpressing cells transduced with a dominant-negative form of SIRT1 (H363Y). Nampt overexpression also reduced the fraction of p53 that was acetylated on lysine 382, a target of SIRT1, suppressed an age-related increase in p53 expression, and increased the rate of p53 degradation. Moreover, add-back of p53 with recombinant adenovirus blocked the anti-aging effects of Nampt. These data indicate that Nampt is a longevity protein that can add stress-resistant life to human SMCs by optimizing SIRT1-mediated p53 degradation.

Age is the greatest risk factor for myocardial infarctions and strokes (1). This risk is partly attributable to an age-related decline in the ability of vascular cells to resist stress and efficiently remodel the arterial wall. Vascular smooth muscle cells (SMCs) are especially important in this regard; the efficiency with which SMCs stabilize a developing atherosclerotic lesion determines whether the lesion will rupture, a potentially fatal event. Strategies to prevent the premature senescence of SMCs could be a promising approach for reducing vascular disease if molecular targets can be identified.

Nicotinamide phosphoribosyltransferase (Nampt, also known as P–B cell colony-enhancing factor and Visfatin) is the rate-limiting enzyme for NAD⁺ biosynthesis from nicotinamide. The intracellular levels of NAD⁺ and nicotinamide have recently been identified as important for certain cell survival reactions, including those linked to the sirtuin family of protein deacetylases (3, 4). Sirtuins, such as Sir2 and its mammalian homolog SIRT1, consume NAD⁺ and generate nicotinamide as they hydrolytically remove a targeted acetyl group (3). Nicotinamide is a known inhibitor of NAD⁺-dependent deacetylation reactions. Therefore, pathways that both replenish NAD⁺ and clear nicotinamide could be vital to SIRT1 activity.

Recently, we discovered that Nampt was substantially up-regulated when a uniquely long-lived human vascular SMC line was subjected to the stress of complete serum withdrawal (5). Here, we report that Nampt is a longevity protein that extends the lifespan of human SMCs by activating SIRT1 and restraining the accumulation of p53.

Experimental Procedures

Cell Culture—Experiments were performed using primary human vascular SMCs derived by outgrowth from fragments of internal thoracic artery and the HITC6 SMC clonal line, also originally generated from the human internal thoracic artery (6). Dermal fibroblasts from an individual with Hutchinson-Gilford progeria syndrome were obtained from the Coriell Cell Repository.

To quantify replication, cells were plated at 4,500 cells/cm², and growth medium with 10% FBS was changed every 2 days until cells reached 90–95% confluence. Harvested cells were counted from triplicate plates, and the number of population doublings was calculated based on: log₁₀ (number of cells harvested) − log₁₀ (number of cells seeded))/log₁₀ (2). Population growth curves were compared using nonlinear regression.

Recombinant Retrovirus and Adenovirus Infection—A retroviral gene delivery system was used to generate primary human cells stably overexpressing Nampt, using methods described previously (7). Retrovirus containing pQCXIP-Nampt-IRES-PURO or pQCXIP-IRES-PURO (Clontech Laboratories) was generated by calcium phosphate-mediated transfection of the Phoenix-amphotropic retrovirus packaging cell line (ATCC, Manassas, VA). Stable transductants were selected

The abbreviations used are: SMC, smooth muscle cell; Nampt, nicotinamide phosphoribosyltransferase; TSA, trichostatin A; FBS, fetal bovine serum; SA-β-Gal, senescence-associated β-galactosidase; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; ANOVA, analysis of variance.

The online version of this article (available at http://www.jbc.org) contains two supplemental movies showing time-lapse delineation of the changes discussed in the legend for Fig. 4.

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Nampt expression and Nampt enzyme activity decline in presenescence of human SMCs. A, Hoffman-modulated contrast images of human HITC6 SMCs in a healthy and proliferating state (left) and after eight further subcultures, at which time cell replication ceased and the cells were flat and spread (right). B, HITC6 SMCs incubated with X-gal solution at pH 6.0 for 5A β-Gal activity and photographed in a proliferating state (left) and after having become senescent eight subcultures later (right). The transition to senescence was associated with a decline in Nampt expression, indicated by Western blot analysis of lysates harvested from primary human SMCs (C) and clonal HITC6 SMCs (D), studied at designated subcultures (s) following harvesting from fragments of adult internal thoracic artery. Nampt activity in SMCs depicted in panel C, assessed by incubating lysates with [14C]nicotinamide and phosphoribosylpyrophosphate, is shown. *, p < 0.01 versus s26 and s32, ANOVA with Bonferroni post hoc test.

Senescence-associated β-Galactosidase Activity Assay—SMCs at ∼70% confluence were fixed in 2% formaldehyde/0.2% glutaraldehyde in phosphate-buffered saline for 3 min and incubated with X-gal-containing reaction mixture, as described (10). Cells were stained with Hoescht 33258 (2.5 μg/ml), and the proportion of senescence-associated β-galactosidase (SA β-Gal)-positive cells was quantified (Olympus BX51, ×20 objective, ~1200 cells). Senescence-free survival was determined using Kaplan-Meier analysis of survival versus replicative age.

In Vivo Assessment of SIRT1 Deacetylase Activity—Cell-based assessment of SIRT1 enzymatic activity was performed using the Fluor de Lys-SIRT1 substrate (Biomol, Plymouth Meeting, PA), as described (11). SMCs in phenol red-free M199 with 5% FBS were incubated for 2 h with 5 μM TSA followed by the addition of the fluorogenic substrate for 4 h. Signal was quantified by spectrofluorometry (Wallac, Wellesley, MA) and normalized to total protein content.

Time-lapse Analysis of SMC Response to Oxidative Stress—The morphologic response to oxidative stress was dynamically assessed by digital time-lapse microscopy, using methods previously described (12). Hoffman-modulated contrast images (Axiovert S100; Carl Zeiss, Inc. Thornwood, NY) were digitally acquired every 5 min over 3 h, beginning immediately after the addition of 150 μM H2O2 to SMCs in M199 containing 1% FBS.

RESULTS
Nampt Expression and Activity Decline as Human SMCs Undergo Replicative Aging—To track Nampt expression as SMCs undergo replicative aging, two human SMC preparations with different in vitro lifespans were studied: 1) primary cultures of SMCs, initiated by outgrowth from the internal thoracic artery harvested from adult patients undergoing coronary artery bypass surgery; and 2) a clonal line of human SMCs (HITC6) that also originated from the internal thoracic artery but displayed enhanced longevity in culture (6). SMCs were serially subcultured until they reached senescence, indicated by cessation of proliferation, an enlarged flattened morphology, and cytoplasmic β-galactosidase activity at pH 6.0 (Fig. 1, A and B). In both SMC preparations, Nampt protein expression declined significantly as SMCs approached senescence (Fig. 1, C and D). Nampt enzyme activity, assessed by quantifying the conversion of nicotinamide to nicotinamide mononucleotide (5), fell in presenescence SMCs even more strikingly than Nampt expression, reaching 14 ± 3% (mean ± S.D., n = 3) of basal activity (Fig. 1C). These findings identify the regeneration of NAD+ from nicotinamide as a metabolic pathway that becomes exhausted as SMCs approach senescence.
Nampt Regulates and Extends Human Cellular Lifespan

To determine whether human SMC lifespan could be extended by overriding this innate decline in Nampt expression and activity, we augmented Nampt gene dosage by introducing human Nampt cDNA into SMCs using retrovirus. This yielded a 7.1 ± 3.1-fold increase in Nampt activity in stably transduced SMCs. Primary adult SMCs overexpressing Nampt surpassed the maximal lifespan of vector-infected SMCs by an additional 2.1 ± 0.3 population doublings, which, given their otherwise short in vitro lifespan, constituted a 34 ± 4% prolongation of lifespan (Fig. 2A). Equally notably, this extension proceeded in cells that were already well advanced along their path to replicative senescence. In the longer lived HITC6 SMCs, lifespan extension by Nampt was even more striking, with an additional 6.3 ± 0.3 population doublings or a 71 ± 7% extension of lifespan (Fig. 2A). Cell lifespan extension by Nampt was not limited to SMCs and was also seen with human fibroblasts derived from a subject with Hutchinson-Gilford progeria syndrome, a condition associated with markedly premature atherosclerosis (13) (Fig. 2A).

To determine the role of endogenous Nampt in SMC lifespan and senescence, SMCs were incubated with the specific Nampt antagonist FK866 (14). FK866 is a long, almost linear molecule that binds Nampt from within a narrow tunnel at the Nampt dimer interface, an unusual structural relationship that accounts for its specificity (15). We quantified the proportion of senescent SMCs in successive subcultures incubated with 10 nM FK866, a concentration we determined reduced Nampt activity in SMCs to 22 ± 2% of baseline. Kaplan-Meier survival analysis revealed a substantially shortened senescence-free survival of Nampt-inhibited cells SMCs (p < 0.0001) (Fig. 2B). In contrast, there was markedly extended senescence-free survival in Nampt-overexpressing SMCs versus vector-infected cells (p < 0.0001) (Fig. 2C). Therefore, a direct relationship exists between Nampt activity and the number of replication cycles a SMC can undergo before becoming senescent. Together with the age-related decline in Nampt activity, these data firmly establish Nampt as a longevity enzyme for human SMCs.

Nampt Postpones Senescence by Activating SIRT1—To explore the mechanism by which Nampt regulates SMC lifespan, we considered that Nampt both stimulates NAD+ production and consumes nicotinamide, positioning this enzyme as a potential regulator of the NAD+-dependent deacetylase, SIRT1 (16). SIRT1 consumes NAD+, is inhibited by nicotinamide, and mediates lifespan extension of caloric restricted animals (17). To test whether Nampt could stimulate SIRT1 activity in human SMCs, TSA-independent deacetylation of a fluorogenic SIRT1 substrate (Biomol) was quantified. As shown in Fig. 3A, SMCs overexpressing Nampt had a 86 ± 4% increase in SIRT1 activity, at the indicated cumulated population doublings (cpd), are shown on the right.

**FIGURE 2. Nampt regulates and extends human SMC lifespan.** A, cumulative population doubling curves of primary SMCs (left), HITC6 SMCs (middle), and Hutchinson-Gilford progeria syndrome (HGPS) fibroblasts (right) infected with retrovirus containing control cDNA (pQCXIP, Vector) or cDNA encoding Nampt. Stable transductants were selected with puromycin, and each graph depicts the averaged results from three longevity assessments. Curves were fit and compared based on nonlinear regression analysis. B and C, Kaplan-Meier analysis of senescence-free survival for HITC6 SMCs incubated with or without the Nampt antagonist FK866 (10 nM) (B) and HITC6 SMCs stably transduced either with empty vector or with cDNA encoding Nampt (C). The number of SA-β-Gal-positive SMCs and the total number of SMCs (based on Hoechst 33258 staining) were determined for serial subcultures in 10 randomly chosen fields (p < 0.0001 for both survival plot pairs, log-rank test). Corresponding micrographs of SMCs stained for SA-β-Gal activity, at the indicated cumulated population doublings (cpd), are shown on the right.
that was greater than for vector-infected SMCs expressing SIRT1 H363Y (55 ± 12 versus 15 ± 10%, p = 0.01, Fig. 3C).

Nampt Enhances p53 Degradation and Exogenous p53 Abrogates Nampt-mediated Inhibition of Senescence—p53 is stabilized by acetylation of Lys-382, and this p53 site is a known target of SIRT1-mediated deacetylation (19). p53 promotes aging (20), and in keeping with this, we found that p53 abundance increased as human SMCs approached replicative senescence (Fig. 3D). However, this age-related increase in p53 was blunted in parallel cultures of SMCs overexpressing Nampt. Furthermore, the fraction of p53 that was acetylated on Lys-382 was substantially lower in Nampt-overexpressing SMCs than in control cells (Fig. 3E). This p53 modification was associated with a significantly increased rate of p53 degradation in Nampt-overexpressing SMCs, as assessed in SMCs incubated with cycloheximide (Fig. 3F). To determine whether altered p53 levels underlay the changes in senescence induced by Nampt, p53 was introduced to SMCs using recombinant adenovirus (9). As shown in Fig. 3G, add-back of p53 to Nampt-overexpressing SMCs abrogated the reduction in senescent SMCs afforded by augmented Nampt activity. Collectively, these findings indicate that Nampt postpones cellular senescence by ensuring ongoing and efficient degradation of p53.

Nampt Protects against Oxidative Cell Damage—Finally, using time-lapse microscopy (12), we established that the additional replicative life conferred by Nampt was associated with a healthy, stress-resistant phenotype. Although vector-infected SMCs responded to 150 μM H2O2 by global retraction of plasma membrane, Nampt-overexpressing SMCs, matched for cumulative population doubling, largely maintained their morphology and ability to migrate (Fig. 4A and supplemental video file). Concurrently, H2O2 induced a rapid increase in cytoplasmic p53 in control SMCs, whereas the response was blunted in Nampt-overexpressing SMCs (Fig. 4B).

DISCUSSION

Our study establishes that a programmed diminution in the capacity to regenerate NAD+ from nicotinamide is a critical precursor of human SMC senescence. Moreover, by enhancing Nampt activity, cellular lifespan can be lengthened, a phenomenon that we observed in human primary SMCs, human clonal SMCs, and fibroblasts derived from a patient with Hutchinson-Gilford progeria syndrome. We further established that this anti-aging phenomenon is mediated by enhanced SIRT1

FIGURE 3. Nampt extends SMC lifespan and postpones senescence via enhanced SIRT1 activity and p53 degradation. A, SIRT1 activity, measured by quantifying NAD+ dependent deacetylation of an acetylated p53 peptide substrate (Fluor de LyS-SIRT1) in the presence of TSA (p = 0.03, two-tailed t test). HITC6-Vector, control SMCs; HITC6-Nampt, Nampt-overexpressing SMCs. B, Western blot showing modestly increased expression of SIRT1 in Nampt-overexpressing SMCs. C, proportion of senescent SMCs in late passage (37th subculture) HITC6 SMCs double-transduced with amphotropic retrovirus containing cDNA for Nampt or pQCXIP empty vector and SIRT1H363Y mutant or pBABE empty vector (ANOVA with Bonferroni post hoc test). D, Western blot showing up-regulation of p53 as SMCs approach the end of their replicative lifespan but little increase in Nampt-overexpressing SMCs at the equivalent subcultures. E, Western blots of total p53 and p53 acetylated on lysine 382. A 25% overload of Nampt-overexpressing SMC lysates was used, yielding similar total p53 content, to facilitate comparison between deacetylated p53 signals. F, p53 degradation profile in HITC6-Vector and HITC6-Nampt SMCs, assessed by treating cultures with cycloheximide (10 μg/ml) and TSA (5 μM). Degradation kinetics were fit with mono-exponential decay curves, averaged from three experiments, and statistical comparison between slopes (t test, two-tailed) was made after natural logarithm transformation (inset) (p = 0.03). G, proportion of senescent SMCs in HITC6 SMCs double-transduced with amphotropic retrovirus containing cDNA for Nampt or pQCXIP empty vector and adenovirus containing p53 or enhanced green fluorescent protein (ANOVA with Bonferroni post hoc test).
Nampt activity that we observed in presenescent cells might depend on cellular age. That is, the endogenous decline in protective/longevity response to mild stress would itself be human cells. Our data also suggest, however, that such a pro-
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ability of Nampt to enhance resistance to stress and extend restriction into Sir2-mediated extended longevity (23). The protective action that, in yeast, translates the stress of caloric restriction (22).

FIGURE 4. Nampt protects against NAD<sup>+</sup>-dependent oxidative stress-induced cell damage and p53 accumulation. A, Hoffman-modulated contrast images of control (HITC6-Vector) and Nampt-overexpressing (HITC6-Nampt) SMCs immediately after and 2.5 h after the addition of 150 μM H<sub>2</sub>O<sub>2</sub> to medium containing 1% FBS and 5 μM TSA. Side-by-side images depict identical fields of view, demonstrating the pronounced degradation of morphology of individual cells in control but not Nampt-overexpressing SMCs. Time-lapse delineation of these changes can be seen in the supplemental movies. B, accumulation of cytoplasmic p53 is seen within 1 h after addition of H<sub>2</sub>O<sub>2</sub> in control SMCs but minimally in Nampt-overexpressing SMCs.

deacetylase activity that, in turn, holds p53 levels below those which induce senescence. These findings implicate a Nampt-SIRT1-p53 axis as a fundamental determinant of human cell aging.

The role of Nampt as a driver of cellular stress resistance and longevity is noteworthy in the context of its expression profile. Nampt is up-regulated by stressful stimuli including infection and proinflammatory cytokines (21). In human SMCs, Nampt expression increased substantially in response to the stress of complete serum removal (5). This latter response is reminiscent of the up-regulation of <i>PNC1</i> in yeast subjected to caloric restriction (22). <i>PNC1</i> encodes a nicotinamide deaminase, and the resulting clearance of nicotinamide is considered to be a protective action that, in yeast, translates the stress of caloric restriction into Sir2-mediated extended longevity (23). The ability of Nampt to enhance resistance to stress and extend cellular longevity in a SIRT1-dependent manner suggests that Nampt may orchestrate an analogous longevity paradigm in human cells. Our data also suggest, however, that such a protective/longevity response to mild stress would itself be dependent on cellular age. That is, the endogenous decline in Nampt activity that we observed in presenescent cells might underlie a switch in the response to low level stress from lifespan extension to accelerated aging.

Cell senescence is strongly implicated in age-related pathologies as well as the recognized decline in tissue regenerative potential with age (24). Vascular SMC senescence, a hallmark of atherosclerotic lesions (25), can be particularly dangerous because the resulting proinflammatory and nonreparative state can incite lesion disruption and acute vascular occlusion. The current findings identify Nampt as underlying an aging suppression pathway in SMCs, with potential relevance to controlling atherosclerosis and possibly other diseases of aging.

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