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Age-related NAD+ decline

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Abstract

Nicotinamide adenine dinucleotide (NAD^+) is an essential metabolite that is reported to decline in concentration in tissues of aged animals. Strategies to increase NAD^+ availability have shown promise in treating many conditions in rodents, including age-related degeneration, which has in turn driven intense interest in the effects of supplements on human health. However, many aspects of NAD^+ metabolism remain poorly understood, and human data are limited. Here, we discuss the state of the evidence for an age-related decline in NAD^+, along with potential mechanistic explanations, including increased consumption or decreased synthesis of NAD^+ and changes in the composition of cells or tissues with age. Key challenges for the field involve the development of better tools to resolve information on the NAD^+ content of specific cells and subcellular compartments as well as determining the threshold levels at which NAD^+ depletion triggers physiological consequences in different tissues. Understanding how NAD^+ metabolism changes with age in humans may ultimately allow the design of more targeted strategies to maintain its availability, such as inhibition of key consumers in specific tissues or direct delivery of precursors to sites of deficiency. In the meantime, human clinical trials with oral supplements are poised to provide some of the first direct evidence as to whether increasing NAD^+ availability can impact human physiology. Thus, it is an exciting time for NAD^+ research, with much remaining to be learned in terms of both basic biology and potential therapeutic applications.

NAD^+ is critical to life:

Nicotinamide adenine dinucleotide (NAD^+) is an electron (hydride) acceptor that is fundamental to life. It is the product of vitamin B3 metabolism and severe deficiency leads to a condition termed pellagra that is characterized by the 4 Ds: dermatitis, dementia, diarrhea, and death. Accepting and donating electrons interconverts NAD^+ with its reduced form, NADH, in a process that is essential for central carbon metabolism, including glycolysis, the Krebs cycle, and oxidative phosphorylation, as well as hundreds of other

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metabolic reactions within the cell. In addition, NAD^+ serves as a co-substrate for several classes of enzymes, including the sirtuins, poly ADP-ribose polymerases (PARPs), cyclic ADP-ribose synthases such as CD38 and CD157, mono ADP-ribosyltransferases, and the "executioner" enzyme Sterile alpha and Toll/interleukin-1 receptor motif containing 1 (SARM1). All of these enzymes consume NAD^+ and release the nicotinamide (NAM) moiety, necessitating a means to constantly replenish cellular NAD^+ pools. In mammals, NAD^+ is primarily synthesized via the salvage pathway from nicotinamide (NAM), *de novo* from tryptophan, or through the Priess-Handler pathway from nicotinic acid (NA). An alternate pathway for NAD synthesis that was more recently discovered is phosphorylation of nicotinamide (or nicotinic acid) riboside by nicotinamide riboside kinases[1]. Under conditions of stress or disease, these mechanisms may not be sufficient to fully maintain the NAD^+ pool, leading to a decline in its concentration. Given its role at the nexus of many biochemical pathways related to redox balance, energy production, and intracellular signaling, the loss of NAD^+ homeostasis may have dire consequences. Consistently, supplementation with NAD precursors yields therapeutic benefits in many rodent models of acute or chronic stress, metabolic and age-related diseases[2].

Evidence that NAD^+ declines with age:

There is substantial evidence that the concentration of NAD^+ in rodent tissues decreases with age[3–13] (reviewed in Yoshino et al.[2]). Reductions in NAD^+ have been observed during aging in worms[7], rodent tissues and human samples. However, the degree of decline and which tissues are affected vary considerably across studies. For instance, the NAD^+ decline in skeletal muscle for aged rodents has been reported to be anywhere from ~15-65%. In aged liver, most reports suggest from ~10-50% decline, with the notable exception of one study in which NAD^+ concentration increased with age in the livers of female C57BL/6JBomTac mice[6]. The same authors previously reported that this strain maintains hepatic NAD^+ concentration when challenged with high fat diet[14], and speculate that it might be particularly resistant to NAD^+ decline. The increase in NAD^+ with age likely reflects *de novo* synthesis, since it still occurs when *Nampt* is deleted in hepatocytes. For other tissues, there are fewer studies available, but some degree of decline is generally reported. Intriguingly, the NAD^+ concentration in the brains of mice was found to decrease between weaning and young adulthood, and then to decrease further by middle age (12 months old), effects that precede most of the observations in other tissues[13].

In humans, NAD^+ concentration has been reported to decrease with age in skin samples. Although the precise degree of decline was not calculated, average concentration appeared to decrease at least 50% over the course of adult aging, and to be several fold-lower in adults as compared to newborns[15]. NAD(H) was also reported to decline by approximately 14% in the cerebrospinal fluid of subjects > 45 years of age as compared to those ≤ 45 years[5]. Two MRI-based studies have also provided evidence for NAD^+ decline in human brains with age, ranging from ~10% to ~25% between young adulthood and old age[10,16]. In contrast, a third study that has been made available prior to peer review suggests that there is not a consistent difference in NAD^+ content between the brains of young and old humans[17]. Therefore larger studies may still be required to provide a final definitive answer as to how the NAD^+ content of human brain changes with age. Human liver samples from patients > 60 years of age

were found to exhibit an approximately 30% decline in NAD^+ concentration compared to samples from patients < 45 years of age[11]. In a small cohort of older individuals NAD^+ content was also shown to decrease in monocyte-derived macrophages[18]. Finally, plasma NAD^+ levels (which are at nanomolar concentrations, 3-4 orders of magnitude below tissue concentrations, and are of uncertain significance) were recently shown to decline sharply with age in humans[19] in a study that used improved methodology as compared to a prior report that showed only a modest trend[5]. Therefore, a decline in NAD^+ concentration in at least some tissues appears to be a conserved feature of aging across species, including humans.

Why does NAD^+ fall?

Many different mechanisms have the potential to contribute to an age-related decline in NAD^+. The most straightforward is increased activity of NAD^+ consuming enzymes, which may be related to inflammation (CD38) or DNA damage (PARPs). However, it remains unclear whether NAD^+ synthesis is fully maintained with age, and more nuanced changes, such as alterations in the cellular composition or redox state of aging tissues may also play a role. Finally, it should not be overlooked that organelles such as mitochondria concentrate NAD^+, and a change in the mitochondrial content of a cell could therefore result in a net NAD^+ change without necessarily affecting the concentration in any specific compartment. Here, we consider the available evidence for each of these potential contributors.

CD38/CD157

CD38 and CD157 are members of a family of bi-functional enzymes that use NAD^+ to generate ADP ribose and cyclic ADP-ribose, which serves as an intracellular second messenger for calcium signaling. CD38 can also generate nicotinic acid adenine dinucleotide phosphate (NAADP), another calcium mobilizing metabolite, *in vitro* but it remains unclear whether this activity is relevant *in vivo*[20,21]. CD38 is the only NAD^+ consumer that has been documented to increase in mRNA expression across multiple tissues of aging mice, and is thus a strong candidate to explain an age-related increase in consumption of NAD^+ [4]. CD38 null mice have elevated NAD^+ concentrations in multiple tissues, and exhibit a blunted or absent decline with age. Intriguingly, a CD38 inhibitor given late in life also elevates NAD^+ levels in multiple tissues and ameliorates age-related phenotypes in mice[22]. Despite these straightforward conclusions, there are a number of puzzling aspects to the CD38 story. First, the enzyme is restricted largely to immune cells[23–26], with very low expression in hepatocytes[27] or myotubes[28], making it hard to understand why total NAD^+ concentrations in tissues such as liver or muscle are so dramatically affected. On this point, it is worth noting that immune cells have been documented to infiltrate aging tissues, which may account for the observed increases in CD38 mRNA and enzymatic activity. In addition, it has recently been discovered that factors secreted from senescent cells can induce CD38 expression in macrophages and endothelial cells, amplifying the increase in tissue CD38[29]. A second mystery centers around the configuration of the enzyme. While there is a small amount of intracellular CD38 on vesicles, more than 90% of CD38 functions as an ecto-enzyme, facing the extracellular space and lacking access to intracellular NAD^+ stores[30]. Interestingly, CD38

also has a second substrate; in addition to cleaving NAD^+, it can cleave the precursor nicotinamide mononucleotide (NMN)[4]. NMN is present in the plasma, although the apparent concentration can vary by two orders of magnitude depending on the technique used to detect it[31–34]. Thus, CD38 could potentially decrease NAD^+ by direct consumption and by destruction of extracellular NMN that would otherwise serve as a precursor. Given that the loss of CD38 activity causes a large increase in the NAD^+ content of aged tissues, it is also worth considering whether the more subtle decline in NAD^+ observed with natural aging is truly prevented, or merely "swamped" by the larger effect in CD38 null animals. Clearly, this is an intriguingly story with details yet to be elucidated.

PARPs

Poly ADP-ribose polymerase activity is a major driver of NAD^+ catabolism and can be further stimulated in response to DNA damage and genotoxic stress. PARPs have been proposed to mediate the age-related decline in NAD^+ primarily based on increases in PARP1 activity and PARylation of proteins in aged tissues, which is suggestive of increased NAD^+ consumption[15,22,35]. This model is attractive because DNA damage is a well-established stimulus for PARPs and is known to accumulate with age[36]. Moreover, animal models with increased DNA damage exhibit NAD^+ depletion that can be restored by PARP inhibition[37,38], and mutant mice lacking PARP1 have been reported to have increased tissue NAD^+ levels[39]. However, several key points of this model have not yet been tested. First, the activity of PARG, the enzyme that removes PARylation[40], is not well-documented in aged tissues. Thus, an alternative explanation for increased PARylation with age is that the rate of removal decreases, rather than increase in synthesis. Second, it has not been established how much PARylation actually contributes to total NAD^+ flux *in vivo*. The contribution of PARPs to NAD^+ flux is substantial (~1/3 of total flux) in cultured T47D breast cancer cells[31], but whether this holds up across tissue in animals remains to be seen. Third, it remains possible that the PARylation observed in tissue homogenates represents only a small minority of cells that are stressed or apoptotic, in which case it would not explain the loss of a large fraction of total tissue NAD^+. In an alternative to the view that DNA damage precedes NAD^+ depletion, it was recently shown that low NAD^+ levels initiate an interaction between DBC1 and PARP1, leading to decreased PARP activity and subsequent accumulation of DNA damage[41]. In support of this model, NAD^+ precursor supplementation inhibits the interaction between PARP1 and DBC1, restoring PARP1 activity and reducing DNA damage in the livers of aged mice. Thus, further studies are required to fully determine the tissue-specific cause and effect relationships between NAD^+ concentration, DNA damage, and PARP activity during aging.

SARM1

SARM1 is protein involved in axonal (Wallerian) degeneration that was recently shown to possess an intrinsic NAD^+ cleavage activity, generating NAM, ADP ribose and cyclic ADP ribose[42,43]. Activation of SARM1 rapidly depletes the NAD^+ pool[43,44], whereas inhibition of SARM1's cleavage activity or *Sarm1* deletion protects against axonal degeneration, improves survival in mouse model of axonopathy, and attenuates diabetic peripheral neuropathy[42–47]. Accordingly, SARM1 is now being investigated as a potential therapeutic target in neurodegenerative diseases, although early results from mouse models

of Amyotrophic lateral sclerosis have been mixed, with no clear improvement in behavioral deficits[48,49]. *SARM1* is also expressed in peripheral tissues including the liver and kidneys, where its role has not been characterized in detail[50]. Almost no data are currently available on the expression or activity of SARM1 in neurons or peripheral tissues with age. Thus, its role in age-related NAD^+ decline, if any, remains to be determined.

NAD^+ Synthesis

Whether NAD^+ synthesis changes with age in mammalian tissues is largely unknown. Most tissues synthesize NAD^+ primarily from NAM via the salvage pathway, whereas the liver also synthesizes a substantial portion of NAD^+ via *de novo* synthesis from tryptophan, and limited synthesis from tryptophan and nicotinic acid occurs in other tissues, including kidney and macrophages[18,31]. NAD^+ can also be synthesized via nicotinamide riboside kinase (NRK) enzymes[51], but the significance of this pathway to endogenous NAD^+ turnover is not yet clear, since NR concentrations are low in the absence of supplementation, and tissue NAD^+ concentrations are maintained in young mice lacking NRKs[52,53].

Decreased expression of the key salvage pathway enzyme nicotinamide phosphoribosyltransferase (*Nampt*) in with age has been reported in some tissues, including adipose tissues, skeletal muscle, retinal pigment epithelial cells, and certain brain regions[4,12,13,54–58]. Moreover, circulating levels of extracellular NAMPT packaged in extracellular vesicles decline with age both in rodents and humans[58]. Adipose-tissue specific overexpression of *Nampt* and treatment of aged mice with extracellular vesicles from young mice led to improvements in physical activity, sleep quality, glucose homeostasis, and lifespan. The expression of the *de novo* pathway enzyme quinolate phosphoribosyltransferase (*QPRT*) is decreased in macrophages isolated from aged humans and mice[18], suggesting a potential decrease in NAD^+ synthesis from tryptophan. NRK1 and NRK2 protein levels are unchanged in the adipose tissue and skeletal muscle, respectively of aged individuals[55]. All pathways to NAD^+ synthesis required the activity of nicotinamide mononucleotide adenylyltransferases (NMNATs). A significant decline in mRNA for *Nmnat1* and trends for *Nmnat2* and *Nmnat3* were reported in the livers of aged mice[4], and expression levels of *Nmnat* isoforms are also reduced in the kidneys, oocytes and colons[59–61]. Collectively, these studies support a potential decrease in NAD^+ synthetic capacity with age. Ultimately, flux studies with NAD^+ precursors will be required to determine whether net synthesis of NAD^+ decreases with age, or if tissue concentration is reduced primarily due to increased consumption.

Tissue composition

Changes in the cellular composition of some tissues occur with age. One place where changes in cell type are particularly obvious is in adipose, in which there is a switch from brown/beige to white adipocytes over time[62]. Thus, care must be taken to separate effects of cell composition from effects of aging per se when studying NAD^+ concentration and turnover in aged adipose tissues. Another example of cell-type specific changes that occur with age is fiber type switching in skeletal muscle[63]. Aging is also characterized by persistent chronic immune cell infiltration that contributes to declines in physiological function at the tissue and organismal level[64]. In addition, tissues can accumulate fluid, lipid

or glycogen that can affect the total weight in a way that does not reflect cell content. For this reason, it may be preferable to normalize to protein or another internal standard, rather than to the wet or dry weight of tissue in many cases. This should be especially considered for animals with obvious differences in adiposity, such as comparisons between lean and western diet-fed mice Advanced mass spectrometry-based technologies such as single cell metabolomics and imaging mass spectrometry will allow a better understanding of the contribution of specific cell types to changes in tissue NAD^+ concentration.

Redox status

The majority of studies to date have focused on NAD^+, rather than NADH measurements, largely because NADH requires more difficult extraction conditions, is less stable, and is generally present at lower concentrations, decreasing confidence in the measurements. Nevertheless, NADH concentration is critical to the interpretation of lower NAD^+ with age, since a redox shift to a more reduced state could lower NAD^+ concentration without changing the (NAD^+ + NADH) pool. Indeed, an MRI-based study on NAD^+ content in human brains determined that NAD^+ decreases with age, but NADH increases such that the decline in the total pool size is slower than it would appear from NAD^+ alone[10]. A shift toward a more reduced NAD^+/NADH ratio has also been reported in the plasma of aged individuals[19]. In addition, a study in rats that included both NAD^+ and NADH across multiple tissues with age similarly concluded that there is a redox shift in favor of NADH that exaggerates the NAD^+ decline[3]. Improving the quality of NADH measurements and factoring redox changes into tissue NAD^+ measurements are important goals for the field moving forward.

Mitochondrial/organellar NAD

NAD^+ is highly compartmentalized within the cell. Total and free NAD^+ concentrations are higher in the mitochondria than in the cytosol[65,66] and mitochondrial NAD can be preserved under conditions that deplete total tissue concentrations[67,68]. The same may be true for multiple other organelles such as peroxisomes, which also contain NAD^+ and possess a transporter[69], but these concentrations have not been measured to date. The mitochondrial NAD^+ redox state is also more reduced than that of cytosolic NAD^+ [70]. Thus, changes in the number of mitochondria could lead to changes in total tissue NAD^+ and apparent redox state (based on total extractable NAD^+ and NADH) without actually changing NAD^+ concentration or redox state in any subcellular compartment, i.e., only changing the fraction of the tissue that is composed of mitochondria. A crude assessment of the mitochondrial NAD^+ pool can be made by extracting the organelles, but this technique cannot provide reliable information on redox state, and it remains unknown whether NAD^+ in the mitochondrial matrix is recovered quantitatively. Fluorescent NAD^+ sensors can provide a more reliable indication of mitochondrial free NAD^+ content and redox state in live cells[66,70], but have not yet been applied to animal models.

Whether nuclear NAD^+ content varies from that in the cytosol is less clear. The nuclear pore is large enough to allow diffusion of NAD^+, cells can tolerate loss of the nuclear or cytosolic NMNAT isoforms, and genetically encoded NAD^+ biosensors indicate similar free NAD^+ concentrations in the nucleus and cytosol of 293T cells at rest and throughout the

time course of NAD^+ depletion after NAMPT inhibition[66,71]. However, recent observations suggest that nuclear and cytoplasmic NMNATs can compete for NMN to drive local NAD^+ synthesis[65], and the recruitment of NMNAT1 to specific promoter regions to support gene transcription[72] highlights the potential importance of microdomains within organelles. Biosensor studies have also suggested the possibility of slight differences in steady state concentration between nuclear and cytoplasmic NAD^+ pools in U2OS cells[70]. Understanding organellar NAD^+ pools *in vivo*, including the use of organelle-specific NAD^+ biosensors, will be a high priority in future studies on the roles of NAD^+ in health and aging.

How much NAD^+ is enough?

Knowing that measured NAD^+ concentrations in tissues fall with age or disease, it becomes important to understand how much NAD^+ is actually required for normal tissue function. Given the essential nature of NAD^+ in hundreds of biochemical reactions, including glycolytic and mitochondrial energy production, it seems intuitive that any change in concentration could have major consequences. However, mice with a drastic (~85%) reduction in skeletal muscle NAD^+ content have surprisingly mild phenotypes into early adulthood, and increasing skeletal muscle NAD^+ content is almost without effect in young adult animals, requiring a prolonged exercise training regimen to tease out differences in performance[8,73,74]. Similarly, the respiratory capacity of mitochondria isolated from cultured myotubes is maintained until NAD^+ is depleted by ~80% or more. These observations raise a number of questions: Is NAD^+ concentration in excess under normal circumstances? Is it more important or limiting in some tissues than in others? If large changes in NAD^+ concentration can be tolerated, then do the milder changes in aging and disease states actually matter? We will consider each of these questions in turn.

Is “normal” NAD^+ in excess of actual need?

This question has proven surprisingly difficult to answer, in part due to the compartmentalization and protein binding properties of NAD^+. Because the concentration of the free nucleotide varies throughout the cell, and the activities of many NAD^+ consuming enzymes may be modulated by other factors including local NAD^+/NADH redox state, feedback inhibition from nicotinamide, binding partners, or post-translational modifications, simply measuring the *in vitro* K_m of specific consumers for NAD^+ and comparing to the concentration measured in tissue homogenates is not sufficient to understand whether their activities will be affected by small concentration changes in intact cells or tissues. Recent advances using targeted fluorescent biosensors have allowed approximate concentrations to be assigned for free NAD^+ in the nucleus, cytosol, and mitochondria (e.g., 109, 106, and 230 μM, respectively in HeLa cells)[66]. These values suggest that enzymes such as GAPDH (K_m for NAD: 11 μM)[75] and dehydrogenases of the Krebs cycle (K_m for NAD dependent reactions catalyzed by isocitrate dehydrogenase, malate dehydrogenase and alpha-ketoglutarate dehydrogenase are 40, 114 and 25 μM, respectively)[76–78] should be able to function unless a substantial degree of depletion occurs, whereas some NAD^+ consumers, such as sirtuins (K_m for NAD^+ ranges from 26 μM for Sirt6, to 880 μM for Sirt3 and 980 μM for Sirt5, reviewed in[79]) and PARP1 (K_m ~100 μM)[80], may be more responsive to small changes in NAD^+. Accordingly, a study in T47D breast cancer cells showed that

NAD^+ depletion occurs with approximately first-order kinetics, indicating that the major consumers are directly responsive to NAD^+ concentration[31]. In the same cells, inhibition of either SIRT1/2 or PARPs was able to reduce NAD^+ turnover by about one third. Although it has been suggested that the age-dependent decline in NAD^+ limits Sirt3 and PARP1 activity in tissues[4,22], analogous experiments looking at NAD^+ turnover rates during aging have not yet been performed *in vivo*. In this regard, it is worth noting that the half turnover time of NAD^+ in some tissues is as little as 20 minutes, as compared to 6-8 hours in cells (and other tissues), suggesting that there are important NAD^+-intensive processes that are not replicated in cultured cells[31].

Are specific tissues more sensitive to NAD^+ levels?

Addressing the effects of incremental NAD^+ depletion in specific tissues is challenging. Eliminating NAD^+ salvage by deleting *Nampt* throughout the body causes embryonic lethality, and heterozygous mice have normal hepatic NAD^+ levels and a mild phenotype; glucose-stimulated insulin secretion is decreased only in females[34]. Inducible knockout of *Nampt* in the whole body of adult mice results in reduced NAD^+ levels in the liver and intestine, atrophy of intestinal villi, depletion of visceral adipose depots, a decrease in nutrient absorption, weight loss and death within 7-10 days after induction[81]. Pellagra, the disease triggered by nutritional NAD^+ deficiency, prominently affects skin, the gastrointestinal tract, and neurologic function, suggesting that these tissues may be prone to NAD^+ loss. In fact, NAD^+ levels are reported to fall with age in human skin[15]. However, direct measurements of NAD^+ in humans that have or are at risk for pellagra have generally been made only on blood samples, making it difficult to judge the threshold concentration required to prevent dysfunction. As noted above, deletion of *Nampt* in skeletal muscle leads to severe depletion of NAD^+ that is surprisingly well tolerated[8]. Below, we consider evidence from some additional tissues.

Nampt deletion in hepatocytes causes a milder NAD^+ depletion than is seen in skeletal muscle (0-50% loss), probably because the liver is able to generate NAD^+ through the Preiss-Handler pathway and *de novo* synthesis from tryptophan[6,82]. *Nampt* deletion in hepatocytes is sufficient to cause a defect in liver regeneration[82], which requires substantial NAD^+ biosynthesis, but has little effect on basal mitochondrial function or hepatic metabolism in chow or high fat diet fed mice[6,82]. At the same time, the harmful effects of miR-34a in high fat diet fed mice have been attributed to suppression of *Nampt*-dependent NAD^+ biosynthesis, and enhancing hepatic *Nampt* expression has been shown to protect against acetaminophen toxicity and alcoholic steatosis, and to promote liver regeneration[11,82–84]. Finally, the oncogenic effect of unconventional prefoldin RPB5 interactor in hepatocytes has been attributed to suppression of *de novo* NAD^+ biosynthesis, which results in a > 50% loss of NAD^+ and increased DNA damage[85]. Thus, the available evidence suggests that the liver can maintain basal function with up to ~50% NAD^+ depletion, but is more sensitive to NAD^+ loss under stress.

The role of *Nampt* in adipose tissue is more complex because it not only plays a direct role in NAD^+ biosynthesis within adipocytes, but is also secreted and reported to influence NAD^+ concentration in certain tissues including the hypothalamus[58,86]. After

deletion of *Nampt* in adipocytes, 72-93% loss of NAD^+ was reported across adipose tissue depots, whereas in the hypothalamus, but not the hippocampus, NAD^+ was decreased by ~20%. These mice exhibited decreased physical activity that was attributed to the change in hypothalamic NAD^+, and overexpression of NAMPT caused reciprocal phenotypes, increasing hypothalamic NAD^+ (~10%) and physical activity. In addition, these mice exhibited a decline in whole-body insulin sensitivity that was associated with altered phosphorylation of CDK5 and PPARγ in adipose tissue, and was improved by rosiglitazone (a PPARγ agonist)[87]. Similarly generated mice were also found to have impaired mitochondrial respiration in brown fat and to be unable to expand adipose mass in response to a high fat diet[88]. Instead, adipose depots became fibrotic and mice maintained a lower body weight with improved glucose tolerance. In a separate study, intracerebroventricular injection of intact NAD^+ was shown to increase NAD^+ by 30-50% in the mediobasal hypothalamus and to suppress fasting-induced hyperphagia[89]. This NAD^+-mediated anorexic effect was dependent on astrocyte expression of connexin 43, which can transport NAD^+ across the plasma membrane[90]. Contrarily, intracerebroventricular injection of FK866 to deplete the NAD^+ pool was shown to suppress fasting and ghrelin induced food intake[91]. The ability of both increased and decreased NAD^+ to suppress food intake could be due to differences in timing or competing effects of different cell types and/ or hypothalamic regions. Thus, severe depletion of NAD^+ in adipose tissue has mainly negative, but also some positive effects, whereas more subtle changes in hypothalamic NAD^+ appear to have functional consequences. Interestingly, transfer of NAMPT-containing vesicles was reported to be sufficient to improve physical function and longevity in aged mice[58].

In a number of other cell types, including projection neurons, adult neural stem cells, Schwann cells, immune cells, and retinal pigmented epithelial cells, *Nampt* deletion leads to overt dysfunction or death[13,54,92,93]. Inducible deletion of *Nampt* in projection neurons lowers cortical NAD^+ by ~70% at 21 days post deletion (median survival 22 days). Other cell populations are either absent or too small or scattered to allow accurate NAD^+ quantitation after *Nampt* deletion, but the loss of these cells presumably indicates that NAD^+ depletion is more severe than in normal aging, and highlights the need for better fine-tuning of NAD^+ availability to elucidate the relevance of modest declines. Further studies are required to establish the thresholds of NAD^+ depletion that have functional consequences in each tissue and cell type and to determine whether these thresholds change over the lifespan.

How can small decreases in NAD^+ with age matter if large changes are tolerated?

Another important question to consider is whether the small decreases in NAD^+ reported to occur during aging (generally < 30%) are likely to matter, given that much larger changes can have subtle consequences. This is particularly puzzling in the case of skeletal muscle, where a 20-65% decline in NAD^+ content has been reported with age and functional benefits have been reported from supplementation of the NAD^+ pool, yet genetically altered mice initially tolerate a greater degree of NAD^+ loss[2,8,94]. Skeletal muscle specific *Nampt* KO mice have an approximately 85% loss of NAD^+ and have almost no observable phenotypes as young adults[8]. Although they develop myopathy by 5-7 months of age, this can be reversed by a nicotinamide riboside supplementation regimen that only modestly increases skeletal muscle NAD^+ content, leaving the rescued animals with much less NAD^+ than is

present in aged wild type mice. This might suggest that the "rescue" comes from effects of NR in another tissue, however benefits are observed in *ex vivo* contraction assays and even in mitochondria isolated from the muscles. Moreover, preventing the age-related decline in skeletal muscle NAD^+ content by overexpression of *Nampt* was sufficient to attenuate the age-related decline in endurance, supporting the hypothesis that NAD^+ decline is functionally relevant[8]. One possibility is that something else changes in aged muscle that renders it more reliant on NAD^+-dependent processes. Interestingly, genetic loss of ~95% of mitochondrial respiratory capacity in skeletal was reported to have almost no consequences until about 4 months of age, a time course that is reminiscent of the effect of severe NAD^+ depletion and suggests a fundamental metabolic switch[95]. Moreover, aging skeletal muscle was reported to exhibit an NAD^+-dependent "pseudohypoxic state" that was not observed in young muscles depleted of NAD^+, again suggesting age- or time-dependent differences in the consequences of NAD^+ deficiency[9]. A second possible way to reconcile consequences of age-related NAD^+ decline with the lack of consequences of more severe depletion in young animals is to hypothesize that the NAD^+ in aged tissues is not distributed evenly; NAD^+ may be heterogeneous such that some cells have normal NAD^+ content while others have severe deficiencies. To date, it has been very difficult to measure NAD^+ in tissues with cellular resolution, but new technologies such as imaging mass spectrometry may soon change this. In addition, NADH autofluorescence can be measured at high resolution (e.g.,[96,97]), and the generation of mice using recently developed florescent sensors[66] may even allow subcellular compartment-specific information on NAD^+ content to be obtained. These types of experiments represent and important frontier in NAD^+ research.

Will NAD^+ supplementation delay aging?

There is now an extensive body of literature supporting health benefits of NAD^+ supplementation in rodents, although the number of studies examining aging per se is much more limited and human data are generally absent or negative for most indications. Nicotinamide riboside initiated at 2 years of age improved stem cell function in C57BL/6 mice and modestly extended their remaining lifespan[94]. Nicotinamide mononucleotide administered between the ages of 5 and 17 months attenuated multiple age-related phenotypes in the same strain; the treated mice exhibited improvements in body composition, insulin sensitivity, mitochondrial respiration, eye function, and bone mineral density[98], although the effect of NMN on lifespan has not been reported to date. NAM supplementation beginning at one year of age reduced hepatic steatosis and improved glucose homeostasis in high fat diet fed mice without extending lifespan[99]. To-date 46 clinical trials are registered for NR, and five for NMN (ClinicalTrials.gov, accessed December 2019) to evaluate the safety and potential benefits of NAD^+ supplementation in metabolic, cardiovascular, and neurological diseases. Most have yet to report results. However, a study of 12 weeks of NR supplementation did not detect any improvement in insulin sensitivity or skeletal muscle mitochondrial function in obese, insulin resistant men[100–102]. Another trial with NR also failed to find evidence of improvements in glucose homeostasis or exercise performance, but provided preliminary data that are suggestive of modest benefits on blood pressure and arterial stiffness[103]. Three weeks of NR treatment boosted NAD^+ level in the blood but not skeletal muscle of aged individuals[104].

However, the levels of NAM catabolites, N-methyl nicotinamide, N1-methyl-2-pyridone-5-carboxamide, and N1-methyl-2-pyridone-5-carboxamide were increased in skeletal muscle, blood and urine. Unexpectedly, NR supplementation decreased the expression of genes involved in energy metabolism in skeletal muscle, although mitochondrial bioenergetics remained unchanged. NR also appears to decrease circulating levels of several pro-inflammatory cytokines in humans, which could have long-term benefits[104]. Confirmation of these effects in longer studies with greater statistical power would indicate mechanisms by which NR could potentially have a positive impact on human lifespan. In addition, a preliminary study on ALS, a progressive age-related disease, indicated improvement in patients treated with a proprietary combination of NR and pterostilbene[105]. Larger studies will be required to confirm these effects and elucidate whether NR per se is playing an important role. Nicotinamide mononucleotide has lagged behind NR in terms of human trials, but at least one study has been completed (NCT03151239) and is expected to report results in the near future. On the whole, it is simply too early to state with confidence whether NAD^+ supplementation will have a beneficial effect on human lifespan, or which of the findings from rodent models will translate into clinically relevant changes in human patients. In addition, it is important to keep in mind that while the available studies with NR uniformly support its safety, longer-term studies with large populations have not been performed.

Conclusion

NAD^+ plays fundamental roles in the metabolic reactions that underlie all life. The observations that: 1) NAD^+ concentration declines with age in multiple tissues, and 2) NAD^+ precursor supplements are beneficial in rodents, create an attractive narrative that restoring NAD^+ concentration to youthful levels might stave off some of the damaging effects of age. Thus, NAD^+ biology is of interest for identifying therapeutics and/or nutraceuticals that promote healthier aging. However, much remains to be understood about the causes and consequences of age-related NAD^+ decline. Human clinical data on NAD^+ restoration remain sparse, but have provided weak evidence for beneficial effects on vascular health and inflammation while providing relatively robust evidence against a major benefit for insulin sensitivity or muscle performance. Further studies are clearly needed and improving our understanding of NAD^+ metabolism in specific tissues and subcellular compartments, as well as the potential for cross talk with other pathways may offer new opportunities for more targeted therapeutic interventions.

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Highlights:

- NAD^+ is an essential metabolite that declines with age
- Changes in metabolism and tissue composition may contribute to NAD^+ decline
- Lack of cellular/subcellular resolution is a limitation of most studies on NAD^+
- Supplementation of NAD^+ is beneficial in aged rodents

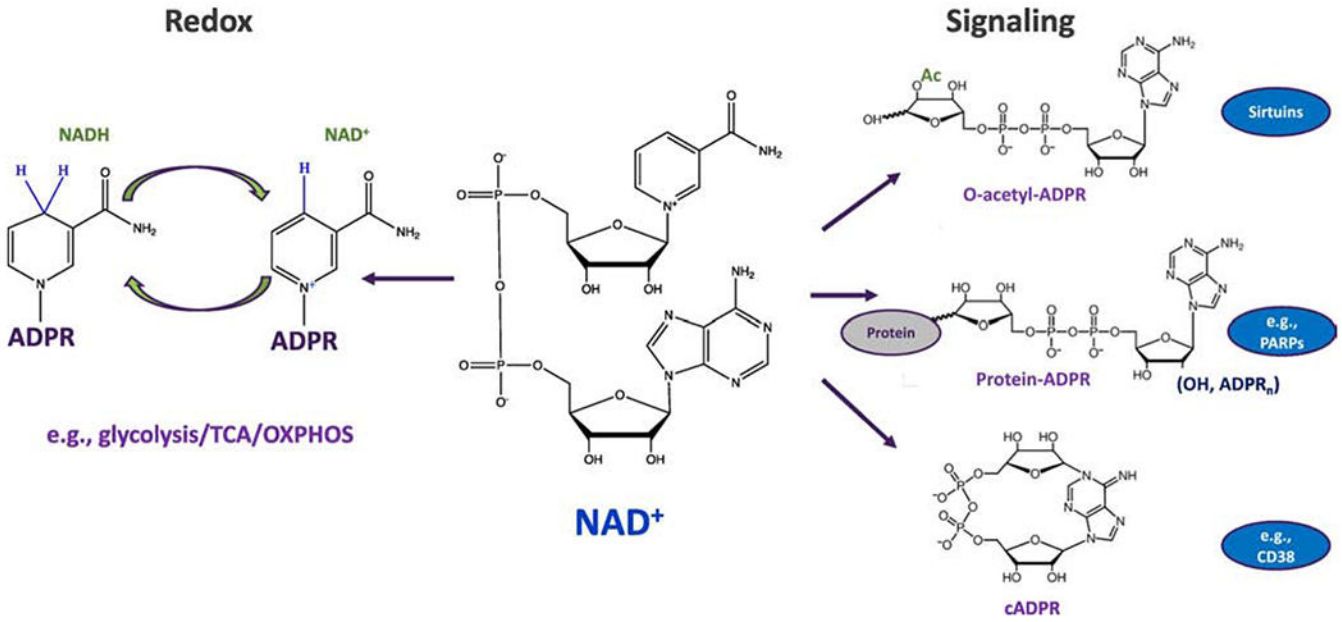

Figure 1: NAD^+ as a redox cofactor and cosubstrate for signaling enzymes.

NAD^+ (center) can accept electrons (in the form of a hydride anion, H^-), converting the cofactor to its reduced form, NADH, and facilitating the oxidation of substrates. Subsequently, the electrons can be donated to facilitate reduction reactions with concomitant oxidation of NADH back to NAD^+. This process is critical to hundreds of reactions, including those of central carbon metabolism, driving energy production (i.e., glycolysis, the TCA cycle and oxidative phosphorylation). NAD^+ also serves as a co-substrate for several families of enzymes that regulate key biological processes via changes in protein modification or the generation of signaling molecules (e.g., Sirtuins, PARPs and CD38/CD157).

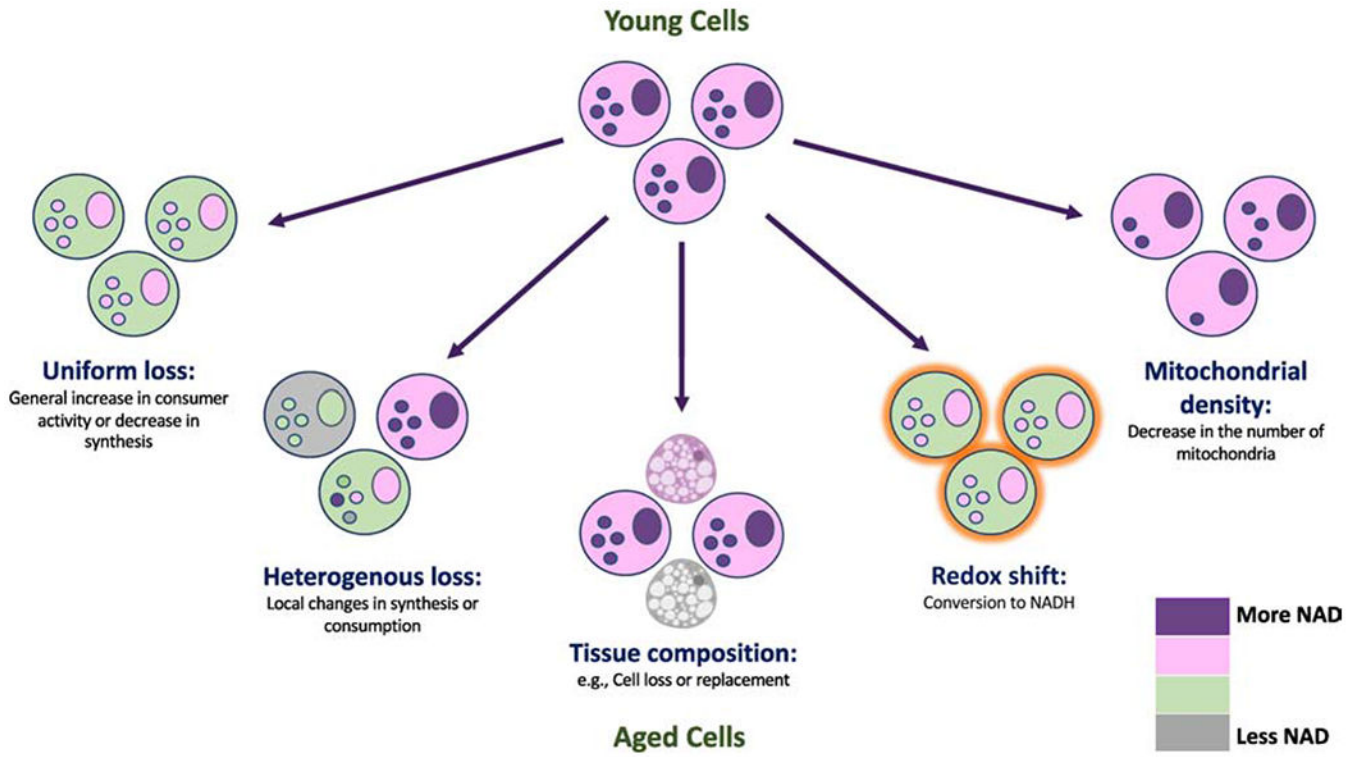

Figure 2: Potential mechanisms resulting in lower tissue NAD^+ concentrations with age.

Schematic illustrating distinct scenarios that could result in lower measured NAD^+ in extracts of tissues from aged animals. Uniform loss involves all cells experiencing a similar NAD^+ deficit. Heterogenous loss suggests local defects resulting in impaired synthesis or excess consumption that could affect a subset of cells disproportionately. Tissue composition may also change with age, resulting in decreased cellularity or the appearance of cells with less NAD^+ (e.g., adipocytes). A shift in the redox balance could lower NAD^+ without any change in the total (NAD^+ + NADH) pool. A decrease in the number of mitochondria (or other NAD^+-rich organelles) could decrease the whole-tissue NAD^+ concentration and apparent redox state (i.e., whole tissue NAD^+:NADH ratio) without actually changing NAD^+ concentration or redox state in any given compartment.