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NAD Metabolism and Sirtuins: Metabolic Regulation of Protein Deacetylation in Stress and Toxicity

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ABSTRACT

Sirtuins are recently discovered NAD^+-dependent deacetylases that remove acetyl groups from acetyllysine-modified proteins, thereby regulating the biological function of their targets. Sirtuins have been shown to increase organism and tissue survival in diverse organisms, ranging from yeast to mammals. Evidence indicates that NAD^+ metabolism and sirtuins contribute to mechanisms that influence cell survival under conditions of stress and toxicity. For example, recent work has shown that sirtuins and increased NAD^+ biosynthesis provide protection against neuron axonal degeneration initiated by genotoxicity or trauma. In light of their protective effects, sirtuins and NAD^+ metabolism could represent therapeutic targets for treatment of acute and chronic neurodegenerative conditions. Our work has focused on elucidating the enzymatic functions of sirtuins and quantifying perturbations of cellular NAD^+ metabolism. We have developed mass spectrometry methods to quantitate cellular NAD^+ and nicotinamide. These methods allow the quantitation of changes in the amounts of these metabolites in cells caused by chemical and genetic interventions. Characterization of the biochemical properties of sirtuins and investigations of NAD^+ metabolism are likely to provide new insights into mechanisms by which NAD^+ metabolism regulates sirtuin activities in cells. To develop new strategies to improve cell stress resistance, we have initiated proof of concept studies on pharmacological approaches that target sirtuins and NAD^+ metabolism, with the goal of enhancing cell protection against genotoxicity.

KEYWORDS: Sirtuins, Sir2, gene silencing, toxicity, genotoxins, longevity, NAD, metabolism, SIRT1

INTRODUCTION

The human genome encodes multiple enzymatic and signaling systems to contend with the toxic effects of compounds and radiation that impinge on cells and tissues. These natural protection systems include multiple detoxifying enzymes, such as manganese superoxide dismutase (MnSOD) and catalase, that catalyze decomposition of reactive oxygen species such as the superoxide anion and hydrogen peroxide. In addition, a DNA damage sensing and repair pathway is activated by genotoxicity to preserve the integrity of cellular DNA. These cell protection systems are subject to regulation by transcription factors, such as the forkhead-box (FOXO) transcription factor family, that transcribe a broad suite of cellular genes, including those encoding enzymes involved in DNA repair and oxygen detoxification.[1] Increased attention has focused on how cell regulatory systems can modify cell stress resistance, DNA repair, and apoptotic signaling.[1,2] It is also of interest to understand how these regulatory systems respond to external and internal signals.[1-3] Importantly, understanding how cells adapt to improve cell survival may provide opportunities to upregulate cell defense mechanisms by pharmacological approaches.[1-4]

Newly identified regulators of cell defense systems are the sirtuin enzymes. The prototypical sirtuin is yeast Sir2p, an NAD^+-dependent deacetylase that removes acetyl groups from the N-terminal tails of histones H3 and H4 to regulate nucleosome and chromatin structure (Figure 1).[5-7] In humans the Sir2p homolog SIRT1 deacetylates transcription factors such as FOXOs,[8-14] p53,[15-19] and nuclear factor Kappa B (NFKB),[20,21] which mediate stress resistance, apoptosis, and inflammatory responses that participate in physiological responses to toxicity. Evidence from unicellular and multicellular organisms indicates that sirtuins have evolved to mediate signaling initiated by stress conditions such as nutrient deprivation to produce adaptation to improve organism survival.[22] Consistent with this concept, extra copies of sirtuin genes increase organism survival in model organisms, including yeast,[23] flies,[24] and worms.[25-27] In addition, sirtuin genes mediate the longevity effects of dietary calorie restriction in these organisms.[22-28] The survival-enhancing characteristics of sirtuins suggest that they influence mammalian responses to toxicity and could be new therapeutic targets for improvement of tissue survival in toxicity-related diseases, particularly neurodegenerative disorders.[3,4,21,22,26,29]

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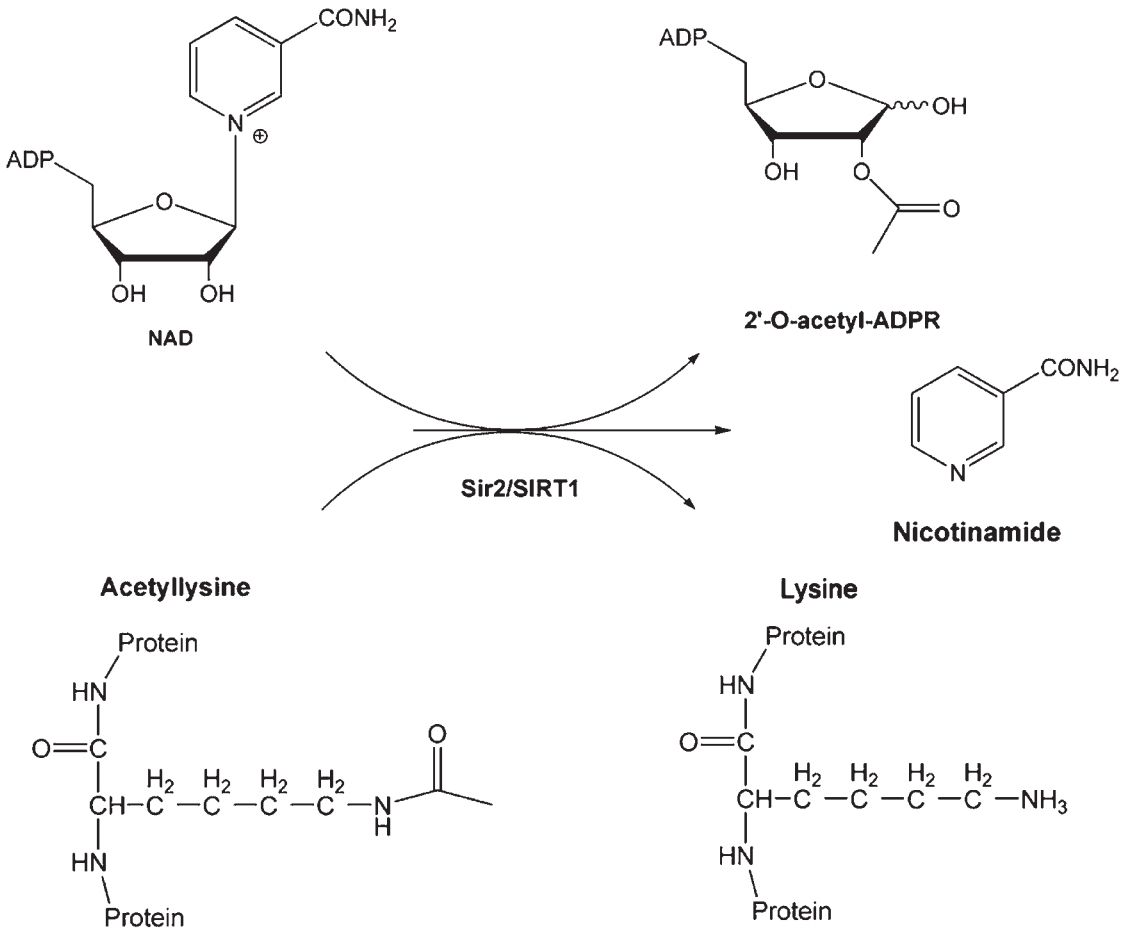

Figure 1. A stoichiometry of the Sir2/SIRT1 reaction. An acetylated protein substrate is reacted with NAD^+ to effect deacetylation. Hydrolysis of NAD^+ and acetyl group transfer to the 2′-OH position of ADP-ribose forms 2′-*O*-acetyl-ADPR and nicotinamide.

We consider in this article ways in which NAD^+ metabolism and sirtuins are involved in cellular responses to toxicity and approaches we and others have taken to understand sirtuins and NAD^+ metabolism. Sirtuins use metabolically expensive NAD^+ to regulate genetic programs that have putative and demonstrated effects on mammalian cell survival. This article also considers pharmacological approaches that target cellular NAD^+ metabolism and sirtuins as a means to improve cell survival upon exposure to genotoxins.

Effects of Toxins on DNA and Involvement of NAD Metabolism in DNA Repair Pathways

Toxicity is an adverse effect of radiation or external chemicals on the cells and the body. Examples of toxins are pharmaceuticals, drugs of abuse, and radiation, such as UV or X-ray light. Both radiative and chemical toxins have the potential to damage biological molecules such as DNA. This damage typically occurs by chemical reaction of the exogenous agent or its metabolites with DNA, or indirectly through stimulated production of reactive oxygen species (eg, superoxide, peroxides, hydroxyl radicals). Repair systems in the cell nucleus excise and repair DNA damage caused by genotoxins. It has been known for some time that NAD^+-using enzymes play an important part in the DNA repair process. Specifically, the poly(ADP-ribose) polymerases (PARPs), particularly PARP-1, are activated by DNA strand breaks and affect DNA repair.[30] The PARPs consume NAD^+ as an adenosine diphosphate ribose (ADPR) donor and synthesize poly(ADP-ribose) onto nuclear proteins such as histones and PARP itself. Although PARP activities facilitate DNA repair, overactivation of PARP can cause significant depletion of cellular NAD^+, leading to cellular necrosis.[30] The apparent sensitivity of NAD^+ metabolism to genotoxicity has led to pharmacological investigations into the inhibition of PARP as a means to improve cell survival.[31,32] Numerous reports have shown that PARP inhibition increases NAD^+ concentrations in cells subject to genotoxicity, with a resulting decrease in cellular necrosis.[31,32] Nevertheless, cell death from toxicity still occurs, presumably because cells are able to complete apoptotic pathways that are activated by genotoxicity.[31,32] Thus, significant cell death is still a consequence of DNA/macromolecule damage, even with inhibition of PARP. This consequence suggests that improvement of NAD^+ metabolism in genotoxicity can be partially effective in improving cell survival but that other players that modulate apoptotic sensitivity, such as sirtuins, may also play important roles in cell responses to genotoxins.

Sirtuin NAD$^+$-Dependent Deacetylase and ADP-Ribosyltransferase Activity

The sirtuin enzymes are a phylogenetically conserved family of enzymes found in diverse organisms, from archaea to humans.[33] These enzymes have highly conserved catalytic domains that catalyze NAD$^+$-dependent protein deacetylation and/or ADP-ribosyltransfer.[5-7,33,34] An increasing body of evidence shows that these enzymes improve cell and organism survival in response to stress, particularly nutrient deprivation.[18,21,23-27] In yeast, the prototypical sirtuin Sir2p deacetylates histones to stabilize heterochromatin.[5-7] By this activity, Sir2p participates in the silencing of genes in the *TEL*, *RDNA*, and *HM* (mating type) loci of the yeast genome.[5-7,35]Sir2p activity is thought to be regulated by stressors such as calorie restriction, osmotic stress, and heat stress.[23,36,37] In response to these stresses, or when extra *SIR2* genes are provided, yeasts have increased replicative survival (measured as cell divisions from a single mother cell).[28,35-37] The extension of yeast replicative survival in response to these stresses has been shown to be sirtuin dependent.[35-37]

The deacetylation activity of sirtuins consumes NAD$^+$ as a substrate to produce deacetylated protein, nicotinamide, and the novel compound 2′-*O*-acetyl-ADP-ribose (Figure 1).[38,39] In humans, 7 sirtuins have been described: SIRT1 through SIRT7.[40] The sirtuin SIRT1 is predominantly nuclear localized and deacetylates multiple transcription factors, the transcriptional coactivators PGC1-α[41] and p300,[42] and histones (H1, H3, and H4).[43] Several sirtuins are localized to other organelles; for example, SIRT3, SIRT4, and >SIRT5 are localized to mitochondria.[44-46] The deacetylation reaction has been difficult to demonstrate for some members of the human sirtuin group. Studies of SIRT6 detected no deacetylase activity, and only protein ADP-ribosyltransfer activity was observed.[47] The SIRT6 knockout (KO) has defects in DNA repair and genome stability,[48] suggesting that this sirtuin could have some convergent enzymatic and biological functions with PARP enzymes that ADP-ribosylate nuclear proteins in response to DNA damage and regulate genome stability.[31,32]

Biological Functions of Human Sirtuins

Human sirtuins regulate a variety of processes that alter cell response to genotoxicity, including the detoxification of reactive oxygen species, DNA repair mechanisms (eg, SIRT6), and sensitivity of cells to apoptosis. The sirtuin SIRT1 upregulates stress-protective pathways by deacetylation of FOXO transcription factors,[8-14] leading to increased transcription of *GADD45* (DNA repair)[11] and *MnSOD* (reactive oxygen detoxification).[11]SIRT1 concomitantly downregulates FOXO transcription of the proapoptotic factors Fas[11] and Bcl-2 interacting mediator of cell death (BIM).[11] Interestingly, SIRT1 also interacts with p53 and deacetylates the C-terminal regulatory domain.[17,18] This activity downregulates p53 transcriptional effects,[17,18] which is consistent with evidence that deacetylation of the C-terminal domain of p53 weakens DNA binding activity.[49] Importantly, SIRT1 KO animals have hyperacetylated p53, indicating that SIRT1 typically regulates p53 acetylation levels in wildtype animals.[16]

Several other SIRT1 protein substrates involved in cell stress response signaling have been identified, including ku70, a proapoptotic factor that is downregulated by SIRT1 deacetylation,[50,51] and the transcriptional coactivator PGC1-α[41]. PGC1-α has been shown to regulate a variety of transcriptional activities in various tissues, including transcription of genes that encode uncoupling protein 1 and 2 (UCP1 and 2), and oxygen detoxification enzymes such as catalase and MnSOD.[52,53] In addition, PGC1-α regulates transcription of genes for mitochondrial biogenesis.[53] Rodgers et al recently identified 13 different acetyllysine sites on this protein that are sensitive to SIRT1 deacetylation.[41] Although the full implications of how SIRT1 regulates PGC1-α transcriptional activities are not well understood, SIRT1 has potent effects on PGC1-α activity in the liver,[41] leading to a shift toward gluconeogenesis and a repression of glycolysis in hepatocytes.[41]

Changes in NAD Metabolism as a Regulatory Mechanism for Control of Sirtuin Activity

The involvement of NAD$^+$ as a substrate for sirtuin-mediated deacetylation chemistry has fostered the hypothesis that perturbations in NAD$^+$ metabolism provide a link to connect energy metabolism to signaling in the nucleus.[22,23] In yeast, this view is supported by genetic studies that link the NAD$^+$ biosynthetic pathway to Sir2p activity. For example, deletion of *NPT1,* which encodes a recycling enzyme (nicotinate phosphoribosyltransferase) in the yeast NAD$^+$ biosynthetic pathway (Figure 2), causes a decrease in Sir2p activity in cells, as determined by decreased gene silencing[6] and by a loss of Sir2p-dependent extended longevity.[23] Measurements of NAD$^+$ concentrations of these yeasts show they are 60% depleted in cellular NAD$^+$,[6,54] indicating that Sir2p is sensitive to intracellular NAD$^+$ concentration. In addition, extra copies of *NPT1* cause increases in silencing at Sir2p-sensitive genetic loci, which is consistent with the view that NAD$^+$ concentrations in the wildtype do not typically saturate Sir2p activity.[55] Deletion of *PNC1*, which encodes an enzyme that degrades nicotinamide to nicotinic acid, also causes defects in gene silencing and yeast lifespan,[36,56,57] although *PNC1* deletion does not change global NAD$^+$ concentrations in yeast cells.[56] Instead, the defects suggest a buildup of intracellular nicotinamide as a source

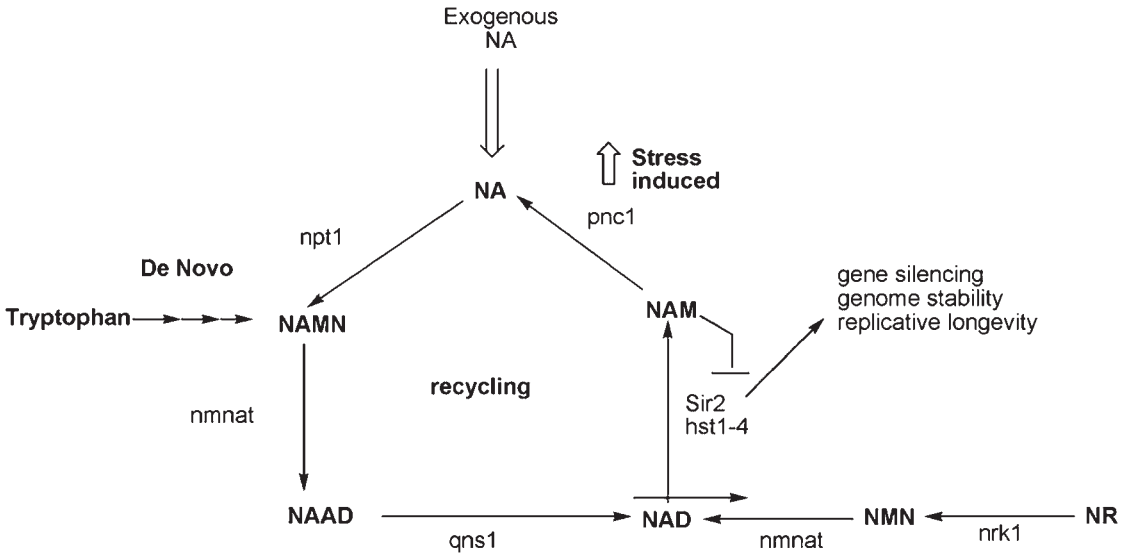

Figure 2. NAD^+ metabolism in yeast. The pathways have a de novo component derived from tryptophan that ultimately forms NAMN. The Sir2 enzyme and its homologs can convert NAD^+ to nicotinamide. Nicotinamide is an endogenous inhibitor of Sir2 functions. Nicotinamide recycling is dependent upon the activity of pnc1, a nicotinamidase enzyme that is constitutively expressed but is subject to inducible transcription linked to stress conditions. Evidence shows that this nicotinamidase can regulate nicotinamide concentrations in cells, thereby regulating Sir2 catalytic function. Genes for nmnat and pnc1 when deleted or overexpressed provide changes in Sir2 biological functions (silencing and longevity). NA indicates nicotinic acid; NAMN, nicotinic acid mononucleotide; NAAD, nicotinic acid adenine dinucleotide; NR, nicotinamide riboside; NMN, nicotinamide mononucleotide; NAM, nicotinamide; pnc1, pyrazinamide/nicotinamide hydrolase; npt1, nicotinic acid phosphoribosyltransferase; nmat, nicotinic acid mononucleotide adenylyltransferase; qns1, glutamine-dependent NAD^+ synthetase; and nrk1, nicotinamide riboside kinase.

for decreased Sir2p activity.[36,57] Nicotinamide is a potent and general sirtuin deacetylase inhibitor, with inhibition constants measured for sirtuins from yeast and humans in the range of 30 to 200 μM.[58] Nicotinamide added to external media causes similar defects to deletion of *PNC1* in gene silencing and lifespan.[58] In collaboration with another group, our laboratory has confirmed that a *pnc1*Δ strain experiences a more than 10-fold increase in intracellular nicotinamide.[59]

Genetic studies in yeast confirm that perturbations in NAD^+ metabolism profoundly alter sirtuin catalytic function in silencing and longevity assays. However, it has been more difficult to establish that changes to NAD^+ metabolism are the mechanism by which stresses such as calorie restriction increase Sir2p activity. To establish the regulatory significance of NAD^+ metabolism to sirtuin activity it is crucial to demonstrate that wildtype yeasts (or other model organisms) respond to stressful stimuli with appropriate changes in NAD^+, nicotinamide, and/or NADH concentrations. Investigations in yeast show that NAD^+ metabolism is subject to stress-related changes and that these changes are likely to regulate sirtuin activity. For example, protein and transcript levels of the nicotinamidase pnc1 are increased by cell stresses such as salt stress, amino acid starvation, and calorie restriction.[36] Providing extra *PNC1* genes to yeast to determine the effect of increased *PNC1* transcription causes increased silencing and increased longevity,[36] which is consistent with the idea that *PNC1* is a regulator of Sir2p activity. It is also consistent with the concept that nicotinamide concentrations typically limit Sir2p activity. In support of this proposal, Gallo et al showed that addition of pnc1 protein to Sir2p deacetylation reactions could increase Sir2p catalytic function in vitro, by removing the inhibitory effect of nicotinamide.[57] In our own work, we identified a nicotinamide antagonist, isonicotinamide, that increases silencing in yeast cells, to support the conclusion that nicotinamide concentrations normally limit Sir2p function.[59] In addition, we measured nicotinamide concentrations in yeast to be in the range of 10 to 150μM, similar to concentrations that limit Sir2p deacetylation activity in biochemical assays.[59] It remains undetermined whether overexpression of *PNC1* in yeast causes a decline in cellular nicotinamide concentrations; however, it has been shown that additional *PNC1* genes antagonize the inhibitory effects of exogenous nicotinamide on silencing and on longevity.[57]

Calorie restriction in yeast has been determined to elicit additional changes in NAD^+ metabolism in the form of decreases in NADH concentrations.[54,60] In the absence of new NAD^+ biosynthesis, and assuming that NAD^+ and NADH are at equilibrium, this finding indicates that calorie restriction changes overall cell redox potential.[54,60] It has been proposed that the NADH levels affect Sir2p catalytic activity by competitive binding with NAD^+,[54] but there is uncertainty about this mechanism of Sir2p regulation, in

part because it is difficult to accurately and simultaneously determine NADH and NAD^+ concentrations in the nucleus, where these compounds exert their effects on Sir2p activity.

NAD Metabolism in Mammals and Putative Effects on Sirtuin Biological Functions

NAD^+ metabolism in humans is somewhat different from the corresponding metabolism in yeast and microbes (Figure 3). A major difference between the respective metabolic pathways is the lack of a nicotinamidase gene in humans.[61,62] Most microbes (bacteria, yeast, and protozoans) complete an obligatory breakdown of nicotinamide to nicotinic acid to recycle nicotinamide to NAD^+. Humans and mammals compensate with a unique enzyme, a nicotinamide phosphoribosyltransferase enzyme called pre-B-cell enhancing factor (PBEF), that can directly convert nicotinamide to nicotinamide mononucleotide.[61,62] The PBEF activity streamlines nicotinamide recycling from the 4-enzyme process in microbes (nicotinamide, nicotinic acid, nicotinic acid mononucleotide, nicotinic acid adenine dinucleotide NAD^+: Figure 2) to a 2-enzyme process in humans (nicotinamide, nicotinamide mononucleotide, NAD^+: Figure 3).[39,61]

In yeast, nicotinamidase activity (pnc1) is inducible by stress, but it is unclear what the correspondent stress-inducible biochemical activity in humans might be. PBEF appears to be inducible by some stresses,[62-65] particularly in the immune system,[66] suggesting that it could serve as a regulator of nicotinamide concentrations as well as a modifier of NAD^+ levels in mammalian cells.[61,64,66] Recent work indicates that increased transcription of PBEF in mammalian cells causes an increase in cellular NAD^+ concentrations,[61,64] implicating this enzyme as a rate-limiting determinant for NAD^+ production in mammals.[61] In addition, extra copies of PBEF cause upregulation of SIRT1 activity in cells, as measured by microarray analysis and by a SIRT1-sensitive transcriptional reporter system.[61] This work suggests that NAD^+ metabolism and NAD^+ concentrations are regulators of SIRT1 activity in cells and tissues.

Recent biological studies support this view and indicate that NAD^+ metabolism in mammalian cells is far more dynamic and responsive to stress than previously thought. For example, Rodgers et al recently reported that fasting in mice induces an enhanced synthesis of NAD^+ in the liver, resulting in a 50% increase in liver NAD^+ concentrations.[41] The increase in NAD^+ concentration is linked to increased SIRT1 catalytic activity, leading to gluconeogenesis via deacetylation of PGC1-α.[41] Thus, mammalian NAD^+ metabolism is responsive to nutritional stress and can initiate signaling mediated by a sirtuin. In another recent study, Bordone et al determined that NAD^+ concentrations decline in the pancreas of fasted mice.[14] This NAD^+ decrease is linked to increased transcription of *UCP2*. SIRT1 is a negative regulator of *UCP2* transcription,[14,67] and the decrease in NAD^+ and a predicted decrease in SIRT1 activity are hypothesized to explain increased *UCP2* transcription in the pancreas of

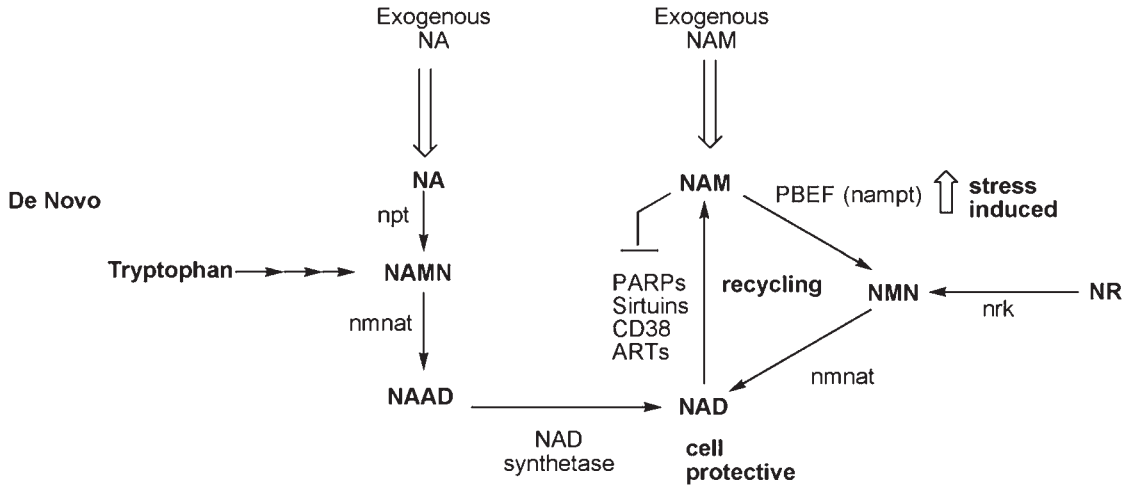

Figure 3. NAD^+ metabolism in humans. The human NAD^+ biosynthetic pathway lacks a gene encoding nicotinamidase activity. The salvage of nicotinamide back to NAD^+ is achieved by an enzyme called PBEF, a nicotinamide phosphoribosyltransferase, which can react 5-phospho-ribose1-pyrophosphate with nicotinamide to form NMN and inorganic pyrophosphate. NMN and ATP can be reacted by nmnat to form NAD^+. The PBEF activity means that human NAM recycling (2 steps) is much simpler than the corresponding pathway in microbes (4 steps). NAM is an endogenous inhibitor of a variety of enzymes that use NAD^+ as a substrate, including SIRT1, PARP-1, and CD38. Regulation of both NAD^+ and NAM concentrations in cells may depend upon the expression levels of PBEF. NA indicates nicotinic acid; NAMN, nicotinic acid mononucleotide; NAAD, nicotinic acid adenine dinucleotide; NR, nicotinamide riboside; NMN, nicotinamide mononucleotide; NAM, nicotinamide; npt, nicotinic acid phosphoribosyltransferase; nmnat, nicotinic acid/nicotinamide mononucleotide adenylyltransferase; nrk, nicotinamide riboside kinase; nampt, nicotinamide phosphoribosyltransferase; PBEF (nampt), pre-B-cell enhancing factor; PARPs, poly(ADP-ribose) polymerases.

fasted animals.[14] Collectively these data support the concepts that mammalian NAD^+ metabolism is regulated during stress and that perturbations in NAD^+ metabolism provide cues for sirtuin-initiated signaling. The dominant biochemical mechanisms controlling NAD^+ metabolism have yet to be identified clearly, and it is still unclear whether PBEF expression levels are generally instrumental in regulating NAD^+ levels in various tissues.

In consideration of these recent findings, it had been known that NAD^+ metabolism is subject to dynamic effects as a consequence of genotoxic stress. PARP enzymes are activated by genotoxicity as part of the normal DNA repair pathway. NAD^+ levels can moderately or seriously decline as a consequence of genotoxicity. This raises the question of how sirtuin activities are linked to PARP activation. This line of thinking suggests that even moderate genotoxicity can downregulate sirtuin biological activity and thereby decrease cell stress resistance.

Biochemical Properties of Sirtuins and Sensitivity of Catalytic Activity to NAD and Related Metabolites

Perturbations of NAD^+ metabolism alter sirtuin catalytic activity in yeast and in human cells and implicate NAD^+ and related metabolites as regulators of genetic events in the cell nucleus. NAD^+ is an abundant metabolite, and recent data indicate that concentrations of this metabolite are normally in the range of 400 to 700μM in human cells.[61] K_m's for NAD^+ of human and yeast sirtuins are in the range of 100 to 300μM.[26,39] Given that the average cellular NAD^+ concentration exceeds this K_m range, it is not clear that fluctuations in NAD^+ concentrations would be expected to significantly affect intracellular sirtuin biochemical function. On the other hand, a large number of proteins bind NAD^+ and NADH such that the unbound NAD^+ concentration in cells could be significantly lower than the determined intracellular NAD^+ concentrations. It is difficult to measure the unbound NAD^+ concentration in cells, so this question remains unanswered. It remains to be determined whether a significant fraction of cellular NAD^+ is bound to proteins such that the unbound NAD^+ concentrations in cells are insufficient to saturate the sirtuin catalytic rate. If the free NAD^+ concentrations are insufficient to cause rate saturation, then fluctuations in NAD^+ concentrations in cells could regulate sirtuin biochemical functions.

Lin et al have shown that in yeast cells the amount of NADH varies with caloric intake.[54] They have proposed that NADH provides control of sirtuins as a function of nutrient availability and have published kinetic studies to show that NADH is an inhibitor of yeast Sir2p and the human sirtuin SIRT1 in the sub-mM range.[54] However, the inhibitory properties of NADH have been independently determined to be in excess of the mM range for yeast Sir2p and for the human sirtuin SIRT2 by Schmidt et al,[68] who have challenged the assertion that NADH can inhibit these sirtuins under physiological conditions, since it is unlikely that NADH concentrations ever reach the relevant mM concentrations in cells.[68] In spite of these discrepant measurements, it remains plausible that NADH concentrations can regulate sirtuin functions even in the absence of competitive binding of NADH to sirtuins if NADH content significantly affects the amount of NAD^+ in cells. In principle this scenario seems possible, since NAD^+ and NADH can be formed from each other by metabolism.

Nicotinamide is a potent biochemical inhibitor and a biological regulator of sirtuins.[36,57,58] The inhibitory constants for nicotinamide have been determined to be in the range of 30 to 200μM for a variety of different sirtuin enzymes.[58,69,70] Physiological nicotinamide concentrations are not widely reported, but measurements from our lab, to be described in the next section, establish that intracellular nicotinamide concentrations are typically within this range for both yeast and mammalian cells, consistent with the proposal that nicotinamide is a relevant negative regulator of sirtuin biochemical function in cells.

New Methods to Quantitate NAD Metabolism in Cells and Tissues

Our laboratory has developed new methodologies to determine NAD^+ and nicotinamide concentrations in cells,[59] with an emphasis on understanding the dynamics of NAD^+ metabolism in a variety of different physiological situations, such as nutritional stress and in toxicity. In addition, we are interested in evaluating the effectiveness of different pharmacological strategies that can alter NAD^+ metabolism.

To achieve NAD^+ and nicotinamide quantitations in cells, we decided upon an "isotope dilution" approach. This method requires synthesis of isotopically labeled nicotinamide and NAD^+ for use as internal standards for mass spectrometer measurements of nicotinamide and NAD^+ in biological samples. The syntheses of these molecules are shown in Figure 4. First, 3-cyano-pyridine was converted to the corresponding amide in the presence of ^{18}O water to effect the synthesis of ^{18}O nicotinamide. Mass spectrometry measurements established that the ^{18}O incorporation was 96.5% starting from ^{18}O water of similar ^{18}O enrichment (Cambridge Isotope Laboratory, Andover, MA). The synthesis of ^{18}O-NAD^+ required the exchange of ^{18}O nicotinamide into NAD^+ catalyzed by the enzyme CD38 (Figure 4B). The conditions of this exchange equilibrated the nicotinamide to achieve an ^{18}O incorporation of 90%, as measured by matrix-assisted laser desorption ionization mass spectrometry (MALDI) (Figure 5).

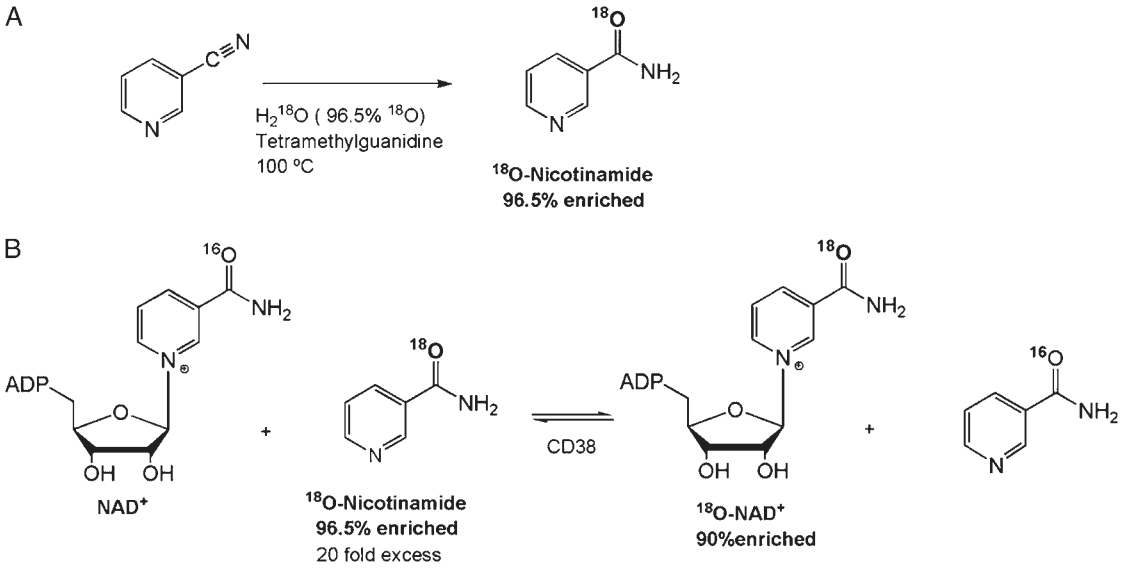

Figure 4. Synthesis of ^{18}O-labeled NAM and NAD^+. (A) The synthesis of ^{18}O-NAM is achieved by base-catalyzed hydrolysis of 3-cyano-pyridine to ^{18}O-nicotinamide by a reaction conducted in the presence of 96.5% purity ^{18}O water. This reaction can be conducted on multi-milligram quantities of 3-cyano-pyridine without great expense. The ^{18}O-nicotinamide product is isolated by HPLC. (B) The synthesis of ^{18}O-NAD^+ is achieved by base exchange of ^{18}O-nicotinamide and NAD^+ catalyzed by the NAD^+ glycohydrolase/cyclase CD38. The reaction is equilibrated to achieve 90% ^{18}O isotopic incorporation into NAD^+. The NAD^+ is purified by HPLC and assayed by MALDI (Figure 5).

In a typical experiment, a flash-frozen biological sample (50 mg) is homogenized with 1 nmole each of ^{18}O nicotinamide and NAD^+ standards in 1M perchloric acid. After insolubles are pelleted, the sample is separated by high-performance liquid chromatography (HPLC) and fractions containing the nicotinamide and NAD^+ are dried and then analyzed by either electrospray ionization (ESI) or MALDI mass spectrometry (MS) (Figure 6). The amount of NAD^+ and nicotinamide in a sample is obtained by the ratio of the labeled standard to the unlabeled metabolite. This protocol provides powerful quantitation because all losses to the sample are proportionately shared by the isotopically labeled standard and the unlabeled metabolite. Consequently, the ratio of the 2 isotope-related molecules remains fixed throughout the sample preparation process. It is assumed that the isotopes have a negligible effect on the physical properties of the 2 types of molecules except their mass behavior in the mass spectrometry analysis.

An initial application of this methodology was provided in our study of yeast nicotinamide contents. We pulverized wild-type and *pnc*Δ yeasts with ^{18}O nicotinamide and determined that nicotinamide concentrations in wildtype yeasts exceed 10 μM.[59] However, in a *pnc*Δ strain the concentrations of nicotinamide were found to increase more than 10-fold, to 150 μM. This nicotinamide concentration is well above the determined K_i for nicotinamide inhibition of Sir2p and explains well the defects in gene silencing observed for *pnc1*Δ strains.[59]

We have increasingly sought to apply this method to address questions of how mammalian cell NAD^+ metabolism can change under different physiological, pharmacological, and genetic conditions. For example, we have shown by this method that NAD^+ concentrations in cultured mouse embryonic stem cells are 500 to 700μM and the corresponding nicotinamide concentrations are typically ~100 μM (A.A. Sauve and T. Yang, unpublished data, July 2005). The values for nicotinamide are higher than the few published values in the literature and provide evidence that nicotinamide concentrations are likely to regulate sirtuins in mammalian cells. Exposure to a genotoxin (methyl methane sulfonate [MMS] 0.01%) causes rapid loss of up to 80% of cellular NAD^+ in mouse embryonic stem cells within 4 hours after treatment (A.A. Sauve and T. Yang, unpublished data, July 2005). Surprisingly, nicotinamide concentrations in cells do not increase as a result of NAD^+ degradation but rather decrease along with NAD^+ (A.A. Sauve and T. Yang, unpublished data, July 2005). This decline suggests that nicotinamide is either degraded to 1-methylnicotinamide and its downstream metabolites or converted to an intermediary metabolite such as nicotinamide mononucleotide. We are currently attempting to address these possibilities experimentally. Alternatively, it is possible that nicotinamide, which has a low pK_a of ~3 and is unprotonated and therefore neutral at physiological pH, diffuses out of cells passively and rapidly. Nicotinamide leaching from cells may be partly responsible for the necrosis observed in various states that produce significant genotoxicity, such as ischemia and acute chemical toxicity, and may help to explain efficacies observed for nicotinamide treatments in preventing tissue damage in conditions of ischemia[71] and in alcohol exposure to the fetus.[72]

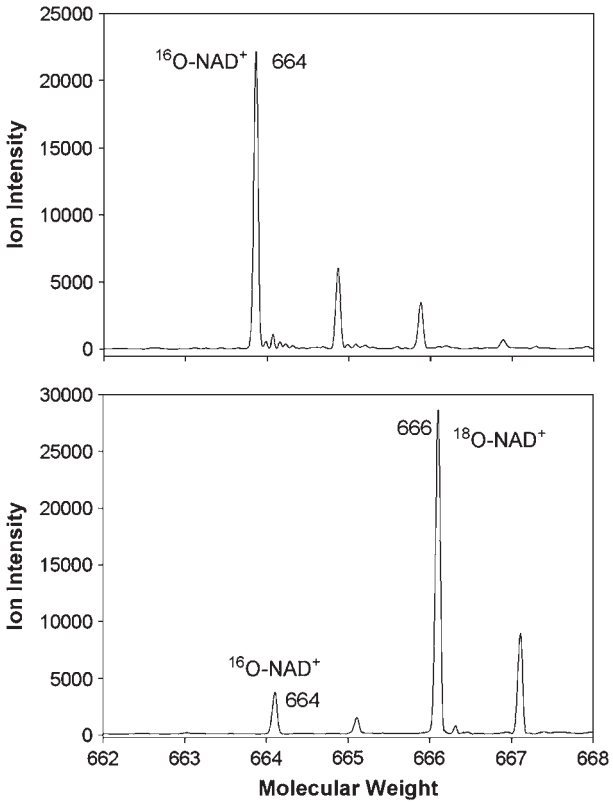

Figure 5. MALDI (positive mode) of ^{16}O (top panel) and ^{18}O-labeled NAD$^+$ (bottom panel). Isotopically labeled ^{18}O NAD$^+$ (90% ^{18}O) is obtained by the synthetic method described in Figure 4.

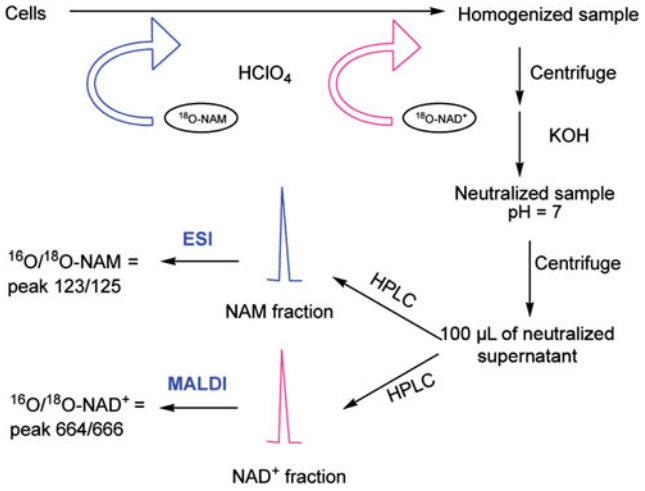

Figure 6. A schematic of procedure to determine NAD$^+$ and NAM concentrations in biological samples. ^{18}O-labeled metabolites are added in 1M perchloric acid in fixed amounts (typically 1000 pmoles) to 50 mg of biological sample. The sample is homogenized, pelleted, neutralized, repelleted, and fractionated by HPLC. Fractions containing NAM or NAD$^+$ are assayed by mass spectrometry. The ratio of ion intensities 123/125 (^{16}O/^{18}O) quantitates the NAM amounts in the samples after correction for isotopic abundances. Similarly, the ratio of ion intensities 664/666 (^{16}O/^{18}O) quantitates the NAD$^+$ amounts after correction for isotope abundances. This methodology automatically corrects for sample losses, and total recovery of metabolite from a biological sample is unnecessary to obtain accurate quantitation of metabolites in the sample.

Pharmacological Approaches to Enhance Sirtuin Activity

The activity identified for sirtuins in upregulating stress adaptation pathways and in reducing cell sensitivity to apoptosis has led to attempts to increase the catalytic rate of these enzymes in cells. Howitz et al[73] identified a family of molecules from a library screen designed to detect compounds causing sirtuin activation. The screen identified a family of the well-known plant polyphenols, such as quercetin and the *trans*-stilbenoid resveratrol.[73] These sirtuin-activating compounds (STACs) increase sirtuin biochemical functions by decreasing the apparent K_m for the peptide substrate.[73] These compounds have potent effects in decreasing apoptosis caused by ionizing radiation, trauma, or genotoxicity.[29,73] The published biochemical activation effects of STACs appear to require the presence of a nonphysiologic fluorophore, complicating the understanding of the molecular mechanism by which these compounds provide sirtuin activation in cells.[74]

An alternative activation strategy pioneered by Sauve et al focuses on derepression of nicotinamide inhibition of sirtuins.[59] Nicotinamide inhibition of sirtuins is caused by a chemical process, called base exchange, that occurs at the active site of sirtuins. This base exchange mechanism competes for an enzyme-bound intermediate called the peptidylimidate, which is formed from the acetylated substrate and NAD$^+$. The imidate intermediate links the base exchange and the deacetylation reaction pathways.[38,68] We found that nicotinamide exchange and deacetylation reaction mechanisms compete for this intermediate, leading to nicotinamide inhibition of deacetylation. We hypothesized that a small molecule that could bind to the nicotinamide binding pocket within the active site would cause antagonism of nicotinamide inhibition of deacetylase activity.[59] Accordingly, a small-molecule isostere of nicotinamide called isonicotinamide (pyridine-4-carboxamide) that binds competitively with nicotinamide to inhibit base exchange was identified. This competitive effect does not inhibit deacetylation and causes antagonism of nicotinamide inhibition of Sir2p deacetylation catalysis. Consequently, isonicotinamide can reduce nicotinamide inhibition to increase the deacetylation reaction rate.[59] In yeast silencing assays, isonicotinamide is a potent agonist for stabilizing heterochromatin formation, and it stimulates Sir2p-dependent gene silencing.[59] Although a nicotinamide derepression strategy has yet to be proven pharmacologically effective in activating human sirtuin biological functions, our recent measurements of nicotinamide concentrations in mammalian cells and tissues (typically in the 50-150 µM range) indicate that SIRT1 and other mammalian sirtuins are likely to be inhibited by intracellular

nicotinamide. We are currently exploring the effectiveness of small molecules related to isonicotinamide in activating mammalian sirtuins in vitro and in vivo.

Pharmacological Approaches to Enhance NAD Metabolism

Prevention of NAD^+ breakdown by inhibition of PARP enzymes has proven to be a valuable intervention to rescue NAD^+ metabolism from complete depletion under conditions of genotoxic stress.[30,31] NAD^+ also potentiates sirtuin biological effects. Although these effects are just now being investigated in mammalian cells, it is apparent that sirtuins confer survival advantages to cells. For example, a recent report indicates that sirtuins are required for protection against traumatic stress and genotoxic stress in neurons.[29] In addition, sirtuin activity has been shown to increase resistance of cells to apoptosis[18,50,51] and to prevent at least 1 form of cell senescence.[19] In addition, sirtuins are known to downregulate the NFKB signaling pathway,[20,21] suggesting that they could attenuate inflammatory responses, which can play adverse roles in toxicity and increase tissue damage.[21]

The possible benefits of enhanced sirtuin function and the vulnerability of NAD^+ metabolism to depletion have led our lab and others to consider pharmacological approaches that can support or augment NAD^+ metabolism during toxicity. These interventions include the use of nicotinamide or nicotinic acid as a source of increased NAD^+. In addition, the small molecule nicotinamide riboside was recently described as an attractive natural compound that could enhance NAD^+ levels in cells.[75] The human genome encodes a nicotinamide riboside kinase[75] that can convert nicotinamide riboside to nicotinamide mononucleotide, the precursor to NAD^+ in the nicotinamide recycling pathway (Figure 7).[75] Consistent with the proposed model shown in Figure 7, treatment of cells with either nicotinamide or nicotinamide riboside increases cellular NAD^+ content, as determined by mass spectrometry assay (A.A. Sauve and T. Yang, unpublished data, October 2005). We also recently showed that nicotinamide riboside can provide some protection against cell death caused by MMS genotoxicity (A.A. Sauve and T. Yang, unpublished data, October 2005).

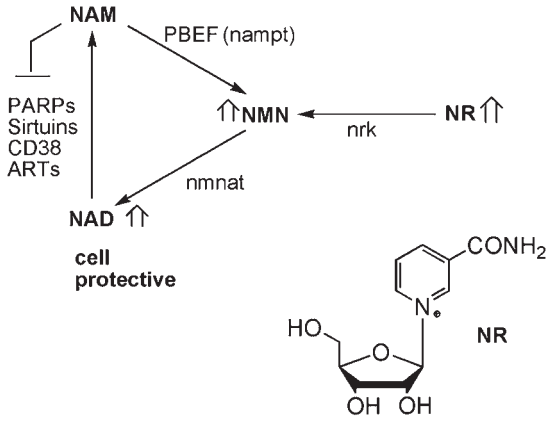

Figure 7. Strategy to enhance NAD^+ concentrations via nicotinamide riboside (NR). NR can enter cells and be phosphorylated to NMN by a nicotinamide riboside kinase (nrk). Nicotinamide and NR provide protection in genotoxic conditions, as described in the main article text. These findings suggest that pharmacological targeting of the NAM recycling pathway can be an effective strategy of improving cell survival during genotoxic stress.

Conclusion

Physiological and biochemical mechanisms that determine the effects of chemical and radiation toxicity in tissues are complex, and evidence indicates that NAD^+ metabolism is an important player in cell stress response pathways.[30,31] For example, upregulation of NAD^+ metabolism, via nicotinamide/nicotinic acid mononucleotide (*NMNAT*) overexpression, has been shown to protect against neuron axonal degeneration,[29] and nicotinamide used pharmacologically has been recently shown to provide neuron protection in a model of fetal alcohol syndrome[72] and fetal ischemia.[71] Such protective effects could be attributable to upregulated NAD^+ biosynthesis, which increases the available NAD^+ pool subject to depletion during genotoxic stress. This depletion of NAD^+ is mediated by PARP enzymes, which are activated by DNA damage and can deplete cellular NAD^+, leading to necrotic death.[30,31] Another mechanism of enhanced cell protection that could act in concert with upregulated NAD^+ biosynthesis is the activation of cell protection transcriptional programs regulated by sirtuin enzymes.

Examples of cell and tissue protection linked to NAD^+ and sirtuins include the finding that SIRT1 is required for neuroprotection associated with trauma and genotoxicity.[29] SIRT1 can also decrease microglia-dependent toxicity of amyloid-beta through reduced NFKB signaling.[21] A recent study also showed that SIRT1 and increased NAD^+ concentrations provide neuroprotection in a model of Alzheimer's disease.[76] Sirtuins are NAD^+-dependent enzymes that have protein deacetylase and ADP-ribosyltransferase activities that upregulate stress response pathways. Evidence indicates that SIRT1 is upregulated by calorie restriction and in humans could provide cells with protection against apoptosis via downregulation of p53[18,19] and Ku70 functions.[50,51] In addition, *SIRT1* upregulates FOXO-dependent transcription of proteins involved in reactive oxygen species (ROS) detoxification, such as MnSOD.[11] The sirtuin *SIRT6* has been shown to participate in DNA repair pathways and to help maintain genome stability.[48]

It seems likely that pharmacological agents that target both NAD^+ metabolism and sirtuins could provide tools to elucidate the involvement of these factors in modulating toxicity-induced tissue damage. Moreover, it seems possible that new therapeutic options for treatment of acute and chronic tissue-degenerative conditions could emerge if sirtuins and NAD^+ metabolism can be validated as providing enhanced tissue protection. Agents such as the plant polyphenols (eg, resveratrol), the niacin vitamins, and the compound nicotinamide riboside may enhance cell survival outcomes by increasing NAD^+ biosynthesis, reducing NAD^+ depletion, and/or activating sirtuin enzymes. This area will require additional investigation before the cell survival mechanisms of these compounds are fully understood.

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