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REVIEW

Nicotinamide is an inhibitor of SIRT1 in vitro, but can be a stimulator in cells

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Abstract Nicotinamide (NAM), a form of vitamin B_3, plays essential roles in cell physiology through facilitating NAD^+ redox homeostasis and providing NAD^+ as a substrate to a class of enzymes that catalyze non-redox reactions. These non-redox enzymes include the sirtuin family proteins which deacetylate target proteins while cleaving NAD^+ to yield NAM. Since the finding that NAM exerts feedback inhibition to the sirtuin reactions, NAM has been widely used as an inhibitor in the studies where SIRT1, a key member of sirtuins, may have a role in certain cell physiology. However, once administered to cells, NAM is rapidly converted to NAD^+ and, therefore, the cellular concentration of NAM decreases rapidly while that of NAD^+ increases. The result would be an inhibition of SIRT1 for a limited duration, followed by an increase in the activity. This possibility raises a concern on the validity of the interpretation of the results in the studies that use NAM as a SIRT1 inhibitor. To understand better the effects of cellular administration of NAM, we reviewed published literature in which treatment with NAM was used to inhibit SIRT1 and found that the expected inhibitory effect of NAM was either unreliable or muted in many cases. In addition, studies demonstrated NAM administration stimulates SIRT1 activity and improves the functions of cells and organs. To determine if NAM administration can generate conditions in cells and tissues that are stimulatory to SIRT1, the changes in the cellular levels of NAM and NAD^+ reported in the literature were examined and the factors that are involved in the availability of NAD^+ to SIRT1 were evaluated. We conclude that NAM treatment can hypothetically be stimulatory to SIRT1.

Keywords Nicotinamide (NAM) · NAD^+ · Sirtuin · SIRT1 · NAMPT · Salvage pathway

Abbreviations

NAM	Nicotinamide
NAD^+	Nicotinamide adenine dinucleotide
NAMPT	Nicotinamide phosphoribosyltransferase
SIRT1	Sirtuin 1
PARP	Poly(ADP-ribose) polymerase
eNAMPT	Extracellular NAMPT
NMNAT-1	NMN adenyl transferase
ARTs	Mono-ADP-ribosyl transferases
2-PY	*N*-methyl-2-pyridone-5-carboxamide
4-PY	*N*-methyl-4-pyridone-5-carboxamide

Introduction

Nicotinamide (NAM), an amide form of vitamin B_3, has shown therapeutic effectiveness in a variety of diseases or conditions when administered at a high dose. Its therapeutic potential has been demonstrated either in animals or in clinical trials against a diverse range of diseases such as skin disorders [1], diabetes [2], cancer metastasis [3], cerebral ischemia [4–6], multiple sclerosis [7], Alzheimer disease [8], viral and microbial infection [9], and inflammatory diseases [10]. High-dose tablets (500 or 1000 mg) are currently available on the market, and as a result, NAM is anticipated to be used for clinical and subclinical purposes in the near future. However, to ensure optimal therapeutic strategies and

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to guarantee safety with its use, the mechanisms underlying NAM's diverse beneficial effects and possible side effects should be reviewed and evaluated.

NAM is endogenously found at low quantities in mammalian cells, but when supplemented in therapeutic doses, it is rapidly converted to NAD^+ through a salvage pathway [11, 12]. The effects of NAM are largely mediated by a cellular increase of NAD^+, and these effects are abolished following administration of FK866, which is a potent inhibitor of nicotinamide phosphoribosyltransferase (NAMPT), the first step of the salvage pathway [13]. The effects of increased NAD^+ in cells seem to function in two different ways. First, it results in an increase in the ratio of NAD^+/NADH, a key cellular redox state that plays an important role in redox signaling and bioenergetics. Second, NAD^+ is a substrate for a class of enzymes such as the poly(ADP-ribose) polymerase family proteins (PARPs) and the sirtuin family proteins, and is irreversibly cleaved to an ADP-ribosyl group and NAM. Free NAM is released whereby it exerts feedback inhibition [14, 15] (Fig. 1).

The nature of NAM as a product of these reactions prompted its experimental usage as an inhibitor of non-redox enzymes, in particular sirtuin 1 (SIRT1) [15]. SIRT1 is an important member of the mammalian sirtuin family of proteins that commonly have dual activities as a NAD^+-dependent deacetylase and as a mono-ADP ribosyltransferase [16, 17]. Through deacetylation, SIRT1 modifies the activities of a variety of target proteins, and helps cells survive under various stress conditions, while mediating critical processes such as cell division, differentiation, and senescence. In addition, SIRT1 may also promote longevity [18]. Studies typically demonstrated a SIRT1-mediated biological phenomenon by showing a positive response to treatment with SIRT1 activators such as resveratrol or through overexpression of SIRT1. In many of these studies, the involvement of SIRT1 activation was further proven by demonstrating a loss or reversal of the observed effect following NAM treatment. Similarly, and until very recently, NAM has been the most widely used SIRT1 inhibitor (refer to Table 1 for examples). However,

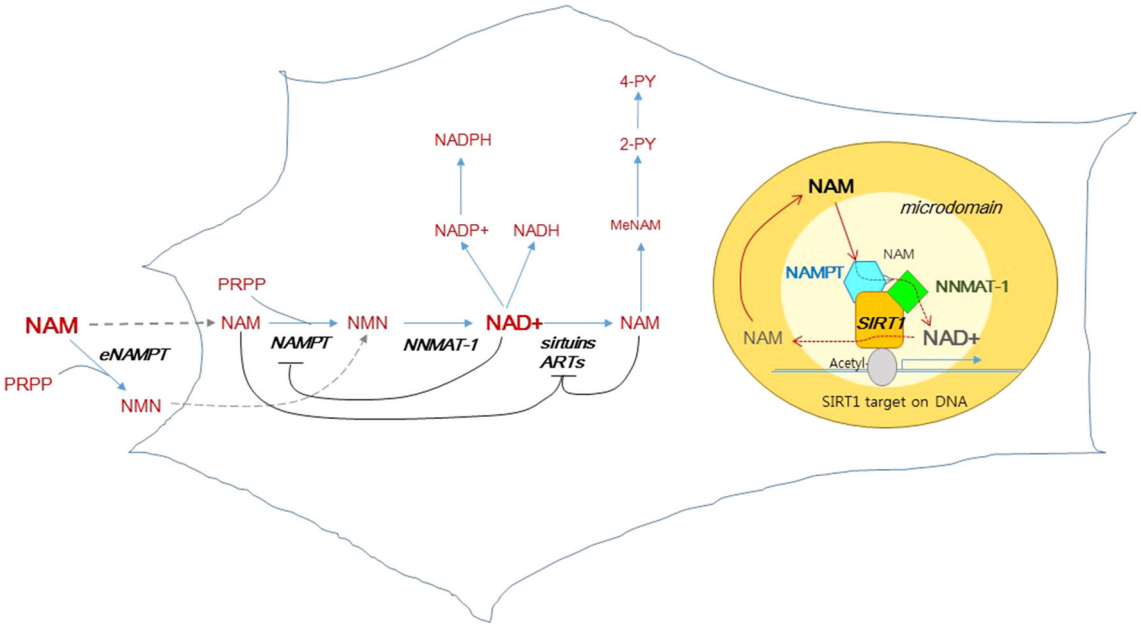

Fig. 1 Conversion of NAM to NAD^+ and other metabolites in cytosol and facilitated NAD^+ production in putative microdomains in the nucleus. Upon administration, NAM transported to the cytosol reacts with PRPP to produce NMN which is, in turn, adenylated to become NAD^+ through the activities of NAMT and NMNAT-1. A small fraction of NAD^+ is reduced to NADH or phosphorylated to $NADP^+$ which can be reduced to NADPH. NAD^+ is also degraded to NAM and ADP-ribose by non-reducing enzymes such as SIRT1 and ARTs. NAM also inhibits the activities of these NAD^+-consuming enzymes. ADP-ribose is removed through poly(ADP-ribosylation) and mono(ADP-ribosylation) (not shown). A limited amount of NAM is removed through conversion to methyl-NAM (MeNAM) which is further metabolized to 2-PY and 4-PY, but these conversions are quite inefficient. Meanwhile, in the nucleus (*brown circle*), NMNAT-1 was found to co-localize with SIRT1 on target chromosomes and facilitate NAD^+ supply to SIRT1 [96]. NAMPT was also found to activate SIRT1 in target gene expression [96], and is expected to localize near SIRT1 forming a microdomain (*beige circle*) in which local concentration of NAM is lowered. These hypothetical microdomains create a condition where SIRT1 is activated within target chromosomes even in the presence of high cellular NAM. In addition, cells were found to secrete eNAMPT which converts NAM in media or body fluids to NMN. This would lower cellular levels of NAM, but the production of NAD^+ may be maintained by the influx of NMN instead of NAM, and thus may facilitate SIRT1 activation. *eNAMPT* extracellular NAMPT, *NMNAT-1* NMN adenyl transferase, *ARTs* mono-ADP-ribosyl transferases, *2-PY* *N*-methyl-2-pyridone-5-carboxamide, *4-PY* *N*-methyl-4-pyridone-5-carboxamide, *NAMPT* NMNAT-1, *acetyl-* acetylated transcription factor, a SIRT1 target

the fact that NAM exerts such a diverse range of beneficial effects yet also inhibits SIRT1 appears contradictory. To reconcile these disparate observations, it is necessary to re-examine NAM-mediated inhibition of SIRT1, particularly in regard to the conversion of NAM to NAD^+ whereby NAM treatment may indirectly result in SIRT1 activation. Such an effect raises the valid need to reconsider the prior interpretation of data in context of the effects of NAM in reactions involving SIRT1. In this review, we examined published literature on SIRT1 modulation by NAM, and re-evaluated the data and interpretations of the observed effects. We found in a number of studies that experimental inhibition of SIRT1 may not have been in effect and the resulting data should be, at the very least, considered inconclusive. Furthermore, because studies found activation in lieu of inhibition of SIRT1, the possibility that in certain circumstances, NAM treatment exerts a stimulatory effect on SIRT1 activity needs a close examination. After analyses of various factors that may affect the availability of NAM and NAD^+ to SIRT1, we posit that NAM is an

Table 1 Examples of studies that used NAM-mediated inhibition of SIRT1

SIRT1-mediated effect	Observed effect with NAM	References
SIRT1 overexpression and resveratrol treatment attenuated TNFα-induced expression of CD40 (a molecule crucial for the onset and maintenance of an inflammation reaction) in 3T3-L1 adipocytes	One-hour pretreatment of 20 mM NAM before TNFα treatment for 8 h caused an increase in CD40 expression in addition to an increase of TNFα-induced NF-κB p65 acetylation	[119]
Proposed that SIRT1 was required for inhibition of apoptosis and inflammatory responses in human tenocytes	Treatment of 10 mM NAM upregulated acetylated NF-κB p65 in tenocytes at 24 h, and abolished the inhibitory effects of resveratrol on inflammatory and apoptotic signaling	[120]
SIRT1 activation enhanced lamellipodium extension and cellular motility, characteristics of metastatic B16F1 melanoma cells	Metastatic properties were suppressed by NAM (concentration not reported). Lifespan was extended and tumor invasion decreased in those mice that received NAM daily (1 mg/g body weight)	[121]
Platelet-activating factor (PAF) signaling is a known trigger of atherosclerosis. SIRT1 activation was found to facilitate PAFR internalization and degradation, and was proposed to contribute to attenuation of atherosclerosis	Treatment of 1 mM NAM or SIRT1-knockdown attenuated SIRT1 activation-induced internalization of PAFR in rat aorta smooth muscle cells when examined 12 h following treatment	[122]
Resveratrol blocked morphological changes of cells under oxidative stress, increased cell proliferation, and inhibited apoptosis	NAM enhanced apoptosis and decreased cell proliferation. NAM also increased p53 acetylation	[123]
Resveratrol ameliorated ER stress and apoptosis induced by doxorubicin treatment, and restored cardiac function in a SIRT1-dependent manner; concluded that SIRT1 attenuates ER stress-induced apoptosis in cardiomyocytes	Pretreatment of 20 mM NAM for 24 h abolished the suppression of ER marker expression by 24 h resveratrol treatment in H9c2 cardiac myoblasts treated with doxorubicin	[124]
Acetylation of α-tubulin is associated with increased microtubule stability [125], and is decreased in tangle bearing neurons in the brain of an animal model of AD. SIRT2 deacetylates α-tubulin [126]	Oral administration of NAM for four months increased acetylation of α-tubulin in brain suggesting inhibition of sirtuin and restored cognitive function of mice with an animal model of AD	[127]
Resveratrol treatment upregulated SIRT1 expression, and increased glycerol release and the expression of adipose triglyceride lipase in porcine preadipocytes (note that in most studies, SIRT1 inhibitors downregulate SIRT1 expression)	Treatment of 100 μM NAM or SIRT1 siRNA downregulated changes at 48 h. In a study on the role of SIRT1 in reduction of triglyceride (TG) levels in differentiating adipocytes, 10 μM NAM attenuated SIRT1-mediated reduction of TG levels in differentiating adipocytes[a]	[105], [128]
SIRT1 downregulates accumulation of oxidized LDL in human umbilical vein endothelial cells (HUVECs). Resveratrol decreased LDL accumulation potentially via activation of autophagy	Pretreatment of 5 mM NAM for 30 min increased Dil-labeled-ox-LDL (Dil-ox-LDL) accumulation in HUVECs with a reduction in autophagy indicators and colocalization of LDL puncta with lysosomes	[129]
NAM protected insulin-producing β-cells from insults such as streptozotocin treatment, which may cause cell death through NAD^+ depletion by PARP activation. The study investigated the underlying mechanism of NAM's protective effect	Treatment of NAM over 10 mM attenuated caspase-3 activation in INS-1 rat insulinoma cells under glucolipotoxicity. Authors speculated that this protective effect was attributed to the inhibitory activity of the mitochondrial sirtuins, SIRT3 and SIRT4	[130]

A representative sample of recent studies that demonstrated the inhibitory effect of NAM on sirtuin is shown

[a] In the majority of studies, NAM was used at doses higher than 5 mM, but in these listed studies, the effects were observed at concentrations of 10 or 100 μM NAM. Whether this response to low doses of NAM is specific to adipocytes is not known

inhibitor of SIRT1 in vitro but its treatment can exert a stimulatory effect on SIRT1 in cells.

Inhibitory effect of NAM on SIRT1 activity

In vitro studies with purified proteins have demonstrated the inhibitory nature of NAM on sirtuins. NAM binds to Sir2p, which is a yeast sirtuin, and inhibits the enzyme in a noncompetitive manner [15, 19]. NAM binding is specific and the protein is insensitive to other related metabolites such as nicotinic acid [19, 20].

In yeasts, Sir2p activities such as transcriptional silencing and suppression of rDNA recombination were abolished by treatment with 5 mM NAM [19, 21, 22]. It was reported that overexpression of PNC1, which is a yeast nicotinamidase that converts NAM to nicotinic acid [23], could reactivate Sir2p and prevent NAM-induced inhibition of rDNA silencing [24, 25], whereas deletion of *PNC1* demonstrated the opposite effect [26]. The deletion of *PNC1* did not change cellular NAD^+ levels, but caused a tenfold increase in the NAM cellular levels. A study by Bitterman's group also found that NAM inhibits human SIRT1 in vitro. An IC_{50} value of NAM for SIRT1 was found to be within a range of 50–180 μM [27–31]. Therefore, 5 mM of NAM could be a sufficiently high concentration to induce inhibition, when supposed that it is readily transported to cells. Furthermore, studies found that NAM was a more potent inhibitor than other inhibitory compounds tested [15]. In addition, the fact that all members of the sirtuin protein family are likely to be inhibited by NAM made it a de facto pan-SIRT inhibitor, which further drove its adoption as a sirtuin inhibitor. In fact, even recent studies have put forth that it is the inhibitor of choice. To illustrate, several studies that demonstrated the use of NAM as a SIRT1 inhibitor are described in Table 1.

Unclear or contradictory findings of NAM as a SIRT1 inhibitor

Despite the pertinacious use of NAM as an inhibitor of SIRT1, studies have reported findings that contradicted this inhibitory effect. For example, a study examining the anti-inflammatory effect of SIRT1 in mesenchymal stem cells [32] evaluated SIRT1 activity through changes in ionizing radiation (IR)-induced IL-1β and NLRP3 expression. NLRP3 is an essential component of the NLRP3 inflammasome that has an important role in the inflammatory response [33]. The authors found that both protein and mRNA levels of IR-induced IL-1β and NLRP3 expression decreased following a 12 h pretreatment with resveratrol. However, in the presented data, a reverse effect was not observed following treatment with NAM. In fact, IL-1β and NLRP3 expression either remained attenuated or showed no effect following pretreatment with NAM, and IL-1β secretion did not increase [32]. Nevertheless, the authors concluded that "NAM pretreatment significantly augmented IL-1β and NLRP3 expression." Also, in another study that intended to elucidate the mechanisms underlying the resveratrol-induced autophagy in endothelial cells under inflammatory stress, NAM and EX527 were used to demonstrate the effect of SIRT1 inhibition [34]. Cells were pretreated with resveratrol along with either NAM or EX527 (another SIRT1 inhibitor [15]) and further treated with TNF-α, and the effect on autophagy marker expression was monitored. Despite the authors' claim that NAM markedly inhibited autophagy, an increase rather than a decrease in the ratio of LC3-type II/type I and LC3 puncta formation, key markers of autophagy activation, was evident. Therefore, this response of cells to NAM did not support the proposed SIRT1 activation-mediated autophagy while the cellular response to EX527 or SIRT1 knockdown did [34]. It is not clear whether there was additional evidence supporting the conclusions that were not presented in these studies, but it appears that the authors were focused solely on the change induced by resveratrol without enough consideration to the outcomes of the NAM treatment. Such contradictory findings and the lack of their interpretation are not unique to these studies; additional examples are described in Table 2. In these studies, NAM treatment showed either null, insignificant, or inconclusive effects on the proposed activity of SIRT1.

SIRT1-stimulatory effect of NAM treatment

Further, several studies have demonstrated that NAM treatment causes SIRT1 activation. NAD^+ levels and the ratio of NAD^+/NADH increased nearly twofold in human fibroblasts in 24 h following treatment with 5 mM NAM accompanying an increase in SIRT1 activity [35]. Studies also found a positive effect of NAM on SIRT1-mediated activation of autophagy [36, 37]. In NAM-treated cells, mitochondrial content decreased with the activation of mitophagy in a manner dependent on NAM conversion to NAD^+ [35, 38, 39]. The SIRT1 activators, resveratrol, fisetin, and SRT1720, all caused an increase in mitophagy. In this study, treatment with 20 mM NAM was unable to induce a decrease in the mitochondria content during the 48-h period post-treatment. Whether a stimulatory effect may have occurred at later time points is not known, as measurements were not taken. NAM treatment-induced autophagy has also been reported by Santidrian et al. [40]. The authors found that the treatment of NAM or nicotinic acid increased the NAD^+/NADH ratio and induced

Table 2 Studies showing unclear or contradictory findings of NAM as a SIRT1 inhibitor

SIRT1 activity assayed	Effect of NAM treatment	References
Resveratrol has protective effects in dopaminergic neurons against neurotoxins. Either antioxidative activity or sirtuin-activation was speculated to have a potential role in the effects of resveratrol	The protective effects of resveratrol against neurotoxin-induced loss of neurons and whole tissue injury were not significantly affected by concurrent application of 0.3 and 3 mM NAM	[131]
A separate study proposed a SIRT1-dependent neuroprotective effect of resveratrol based on the attenuation of the effect by NAM treatment [132]	Authors speculated that 2 mM NAM attenuated resveratrol-induced upregulation of Msr and Foxo3a expression. However, western blots were the only data presented to assess the effect of NAM, the quality of which made confirming the finding challenging	[132]
In a study on the anti-inflammatory effects of SIRT1, authors examined the effect of SIRT1 on IR-induced expression of IL-1β and NLRP3 (a major component of the NLRP3 inflammasome). Resveratrol pretreatment attenuated expression of IL-1β and NLRP3	Pretreatment of 20 mM NAM for 1 h augmented IR-induced IL-1β and NLRP3 expression. However, the presented data in fact showed that expression levels of both protein and mRNA of IL-1β and NLRP3 remained attenuated. Pretreatment with NAM did not increase IL-1β secretion	[32]
Fenofibrate, an anti-cholesterol drug, was found to exert an anti-inflammatory effect via SIRT1 activation. TNFα-induced expression of CD40 mRNA and NF-κB p65 protein was suppressed by fenofibrate treatment	Fenofibrate-induced suppression of TNF-α was proposed to be attenuated by pretreatment of 20 mM NAM or 10 μM sirtinol. However, NAM's inhibitory effect appeared only marginal and markedly less significant than that of sirtinol. It cannot be concluded that NAM alone inhibited the effect of fenofibrate	[133]
To elucidate the mechanisms underlying the resveratrol-induced autophagy in endothelial cells under inflammatory stress, a variety of chemicals including those of SIRT1	Resveratrol was added along with 5 mM NAM or EX527 to the cells treated with TNF-α, and effect on autophagy marker was monitored. Despite the authors' documentation that NAM markedly inhibited autophagy, an increase rather than a decrease in the levels of autophagy markers was shown	[34]
To determine whether resveratrol modulates the effects of TNF-α on nucleus pulposus (NP) cells, the expression of metalloproteinase-3 upon TNF-α was examined	The effect of 5 mM NAM on resveratrol-induced autophagy was examined. The authors claimed that NAM caused a decrease in the level of an autophagy marker, but a decrease did not appear to occur in the presented western blot	[134]
SIRT1 was proposed to prevent the progression of atherosclerosis by regulating lipid metabolism, promoting endothelial survival, and inducing autophagy. Expression of autophagy markers increased upon resveratrol treatment in the cell line tested	The study proposed that NAM treatment worked contrary to the effects of resveratrol causing a decrease in autophagy markers. Only a marginal effect was found with 5 mM NAM as compared to that found using 10 mM NAM	[135]
Oocytes arrested at metaphase II undergo postovulatory aging [136], in which mRNA levels of SIRT1 ~ 3 were reduced. This appearance of aging phenotypes included an increase in the ROS level	The aging phenotypes were reproduced by treatment with 5 mM NAM when assayed 6 or 12 h post-treatment. However, at 24 h, the phenotypic differences between the NAM- and mock-treated groups were diminished	[57]
In a study investigating the molecular mechanism of transcriptional repression by COUP-TF-interacting protein 2 (CTIP2), SIRT1 was shown to potentiate transcriptional repression of CTIP2 by interacting with it and causing histone deacetylation	NAM treatment at 10 mM resulted in a marginal change in CTIP2-mediated transcriptional repression and histone deacetylation. The authors speculated that "this may be because of the relative lack of efficacy of NAM as an inhibitor" of SIRT1 and/or "insufficient intracellular levels of NAM" reached	[137]

Cases with poor inhibitory outcomes by NAM are presented. Note that the list is not exhaustive

changes indicative of enhanced autophagy, while decreasing cancer cell metastasis. These changes were reproduced by activation of mitochondrial complex I through transduction of the yeast NADH dehydrogenase, Ndi1, which causes an increase in NAD^+/NADH that is NAM independent. Notably, SIRT1 knockdown blocked NAM-mediated induction of autophagy, at least in certain cell types. SIRT1 activation via elevated NAD^+ is supported by other studies. Treatment with 5 mM of NAM increased the levels of NAD^+ and NAD^+/NADH by 40% in 8 h, and increased SIRT1 expression and activity in a dose-dependent manner in hepatocytes [41]. Furthermore, it was demonstrated that this effect contributed to the ameliorative effect of NAM on palmitate-triggered endoplasmic reticulum (ER) stress. In addition, increased SIRT1 expression has been proposed to be attributed to NAM-mediated cAMP/PKA/CREB activation [42].

These example studies do not support NAM being simply the SIRT1 inhibitor. The cellular levels of NAM are subject to regulation by various factors, as are those of NAD^+ (Fig. 1). A close examination of these factors would yield better understanding of the reasons for the

discordance in the observed effects of NAM treatment. For this reason, the information in the literature on the changes in the levels of NAD^+ and NAM was examined. In addition, factors that determine the availability of NAM and NAD^+ to SIRT1 were also re-evaluated.

Changes in cellular levels of NAM and NAD^+ upon NAM administration

Changes in cellular levels of NAD^+

The absence of an inhibitory effect of high-dose NAM administration may not be because of an insufficiency of NAM per se, but rather because of the rapid conversion of NAM to NAD^+. This also can explain the appearance of a stimulatory effect of the NAM treatment in some studies. Indeed, an immediate increase in cellular levels of NAD^+ upon NAM administration has been found in studies both on animals and cultured cells. Intraperitoneal administration of NAM at a dose of 500 mg/kg, which was estimated to be close to a concentration of 0.9 mM, caused a linear increase of NAD^+ by eightfold in 8–12 h in the liver and two- to threefold in the kidneys, spleen, and leukemia cells, and 1.5-fold in the brain. The levels then dropped and reached near basal level in 24 h [43]. A similar pattern was observed in rats with a small variation in the extent and the kinetics [44, 45]. NAD^+ levels in the tissues were determined at single time point post-NAM injection and might not represent the peak level. The tissue variation in the degree of the increase of NAD^+ levels may be due to different efficiencies in salvage conversion of NAM or its cellular transport. Increase of NAD^+ was also observed in cultured cells in similar patterns. In human fibroblasts and cancer cell lines, NAM treatment at 5–20 mM typically caused a 50–100% increase in NAD^+ levels in 8–12 h [41, 46]. The levels, thereafter, decreased and were maintained to be approximately 20% higher than basal levels for the tested period (72 h in [46]). A rapid increase of the NAD^+/NADH ratio was also reported; a near twofold level in 5 h in rat liver [44] and a 1.6-fold level in human fibroblasts and hepatoma cells [35, 41]. Overall, an increase of cellular NAD^+ levels occurred in 12 h commonly in animal tissues and cultured cells. Endogenous NAD^+ levels in human cells were estimated to be 360–540 μM [47–49], and a twofold higher level would be well above K_M for SIRT1, which is estimated at 34–171 μM [29, 50, 51]. According to the kinetics reported by Smith et al. [51], deacetylation rate of SIRT1 reaches near maximum at 700 μM NAD^+. Therefore, NAD^+ levels, when considered alone, become stimulatory to SIRT1 after the treatment of NAM at the doses of near or above 1 mM.

Changes in cellular levels of NAM

NAM cellular levels after administration has rarely been studied, but a pattern of an immediate and steep increase right after administration and a slow decline thereafter was reported in animal studies. In a study by Collins and Chaykin [52], after intraperitoneal injection in mice, the levels of the radio-labeled NAM increased rapidly in the tested organs during the initial 10 min and then declined with varying rates during the chase of 50 min. Concordantly, during this period, radioactive NAD^+ increased linearly in all the organs with wide difference in the rates (four representative examples are shown in Fig. 2). At the final 60-min point, more radioactivity was found in NAD^+ in certain organs (kidney and heart) while more was still in NAM in others (spleen and skeletal muscle) reflecting different efficiencies of the salvage activity (represented in Fig. 2). A largely similar pattern was found in a recent study, where NAD^+ and its metabolomics in mouse liver were traced [53]. After NAM administration through gavage, hepatic [NAM] increased acutely by three- to fourfold in 15 min and then dropped and maintained at levels close to near twofold of the basal level (which, in this study, appeared at near 230 μM) for the tested 12 h. The high basal- and NAM-chased levels may be a phenomenon specific to hepatocytes which produce large amounts of NAM to provide it to other organs [52, 54]. Meanwhile, hepatic [NAD^+] increased steadily to two- to threefold levels for the first 8 h and then dropped, but still remained above the basal levels. From these animal studies, NAM administration at high doses is certainly expected to cause a strong inhibition of SIRT1 immediately and briefly. However, thereafter, [NAM] declines but is still maintained at levels higher than basal levels for a certain duration that differs in different organs. During this period, SIRT1-inhibitory effect may be exerted at varying degrees. Meanwhile, there is no report on the fate of NAM administered to cultured cells. It is expected to change similar to the patterns in tissues. Still, in cultures, NAM supply would be sustained longer and, therefore, the high levels may be sustained for a longer period than in animal peritonea where NAM may be lost in circulation.

Factors that may affect the cellular levels of NAD^+, NAM and their availability to SIRT1

Time when the effect is assayed

NAM is a noncompetitive inhibitor of SIRT1 and a marginal elevation of [NAM] would be inhibitory, but the significance would depend on its level. NAM is known to exist endogenously in human fibroblast within the range of

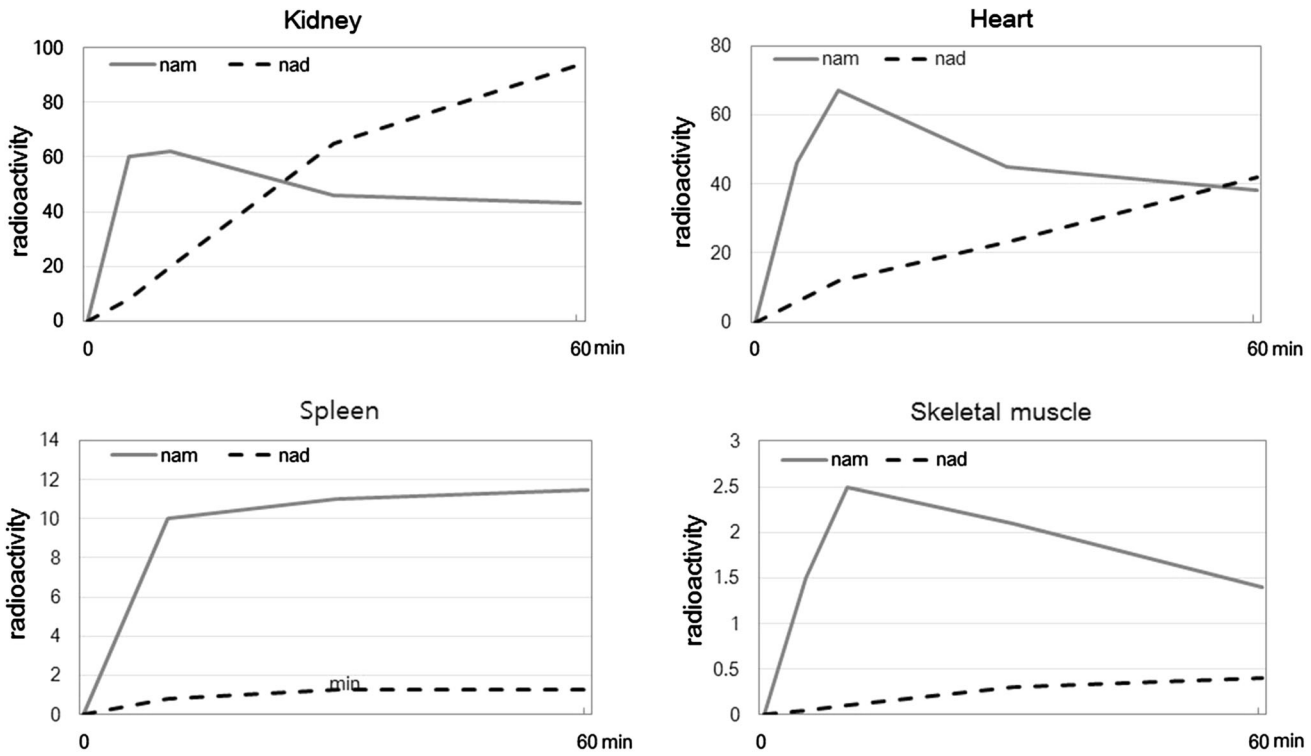

Fig. 2 Observed change in the levels of NAM and NAD^+ in the tissues administered with high-dose NAM. The data reported by Collins and Chaykin (in Table 1, Fig. 3, and Fig. 4 of [52]) were adopted to show representative changes in the levels of NAM and NAD^+ after NAM administration in mice. The numbers in the *y*-axes of the graphs are the radioactivity in NAM or NAD^+, which were measured in the tissues isolated at intervals from 5 to 60 min (*x*-axis) after intraperitoneal administration of radioactive NAM (0.9 μmol). In all the organs, the levels of NAM increased rapidly during the initial 10 min and then declined with varying rates for the 50 min of chase. During this chase period, radioactive NAD^+ increased linearly in all the organs but with a wide variation in the rates. As a consequence, the degree of NAD^+ conversion at the final 60-min point appeared quite different from tissue to tissue. A large amount of NAM was converted to NAD^+ in the kidneys and heart while only a small amount was in the spleen and skeletal muscles. This difference in the conversion rates appears to be independent of the amount of NAM-entered cells (note that the radioactivity in the *y*-axis is different in all four graphs). In the report, the conversion that occurred in other tissues fell between the two extremes of the cases of the kidneys and skeletal muscles

0.7–13.7 μM [55]. [NAM] of twofold higher levels than the cellular basal levels (as seen to be maintained in the study of Trammell et al. [53]) may be still lower than IC_{50} level for SIRT1, which is known to be 50–180 μM [28–31], and so the inhibition may easily become marginal or disappear by a slight drop in the levels. Therefore, the time when the effect is examined would determine whether one sees an inhibition of SIRT1 after NAM treatment. NAM levels have been chased for only up to 12 h in these animal studies, and no information is available on how fast the levels drop. Meanwhile, most studies which intended to show SIRT1 inhibition by NAM did assay the inhibitory effect at early time points after the treatment. For example, NAM treatment was indeed found to attenuate the effects caused by SIRT1 activation (AMPK activation and NF-kB p62 downregulation) when examined 1 h after the treatment [56]. The importance of the time when an effect is experimentally measured is exemplified in the study of Zhang et al. [57]. The oocyte aging process, which is a time-dependent deterioration following ovulation, is accompanied by decreases in SIRT1, SIRT2, and SIRT3 levels. This was acutely reproduced by treatment with 5 mM NAM when examined at 6 or 12 h post-treatment. The finding indicates that SIRT proteins may play a protective role against oocyte aging. However, oocyte aging was either not reproduced or less prominent when examined at 24-h post-NAM treatment. This difference may be because a substantial conversion of NAM to NAD^+ had occurred by the 24-h time point. Additionally, a study using NIH3T3 cells found that [NAD^+] was not increased following treatment with 5 mM NAM [12], but it was not noted at what time following NAM treatment [NAD^+] was measured. The findings may have been different if measurements were taken at later time points. In general, studies usually report the inhibitory effect of NAM within 12 h, or at the latest, 24 h of treatment. Maybe, after such points, the level of NAM enters a stage of a marginally high level where the inhibitory effect is barely noticeable.

Cellular transport of NAM

Uptake of NAM in human erythrocytes occurred in a manner suggestive of facilitated diffusion at an estimated rate of 0.25 nmol/min/10^6 cells with 5 mM NAM [29]. At this rate, 125 μM NAM would be transported in a human cell in a minute, and this appears to support the observed initial increase of cellular [NAM] with the consideration of a slight gap due to the conversion to NAD^+ in the meantime. In CHO cells, the rate appeared to be higher [29], but no cell difference has been reported. Regulation of NAM transport has not been reported either. Meanwhile, when cells are cultivated in NAM-supplemented medium, [NAM] would remain in the medium in vast excess for awhile and the influx would not decrease quickly.

The level of NAMPT

NAMPT produces nicotinamide mononucleotide (NMN) by combining NAM and 5-phosphoribosyl-1-pyrophosphate (PRPP). It is known to be a slow-working enzyme with an estimated k_{cat} of 0.085/min [12] and has been thought of as a rate-limiting enzyme for NAD^+ salvage synthesis. However, the activity was initially determined with bacteria-purified protein, and likely underestimated. At a physiological level of cellular ATP, a majority of NAMPT in human cells is in a phosphorylated form which better converts NAM by virtue of drastically lowered K_M (from 877 to 5 μM) [58]. Importantly, its level is different in different tissues [59], and it is subject to change upon various stresses [18]. Further, its level in human cells was found to increase upon NAM treatment [Kang and Ok, personal communications]. The underlying mechanism is not yet known.

Meanwhile, NAMPT reaction is subject to competitive inhibition by NAD^+. The increased [NAD^+] is known to cause an elevation of K_M up to 21.7-fold for NAM [60], but this may not matter much, since in NAM-treated cells, [NAM] would be far higher. Meanwhile, [PRPP] has been reported in human cells to vary widely at 5–1300 μM (it was determined in cancer-derived cells and may be overestimated) [61, 62]. If the cellular level of PRPP is a low estimate, the rate of the conversion to NMN could be limited despite the abundance of NAM as the PRPP availability was also shown to determine purine biosynthesis via salvage pathway [63]. Overall, these suggest a possible variation in cellular rate of the salvage conversion of NAM in different cells and conditions, and the levels of NAMPT and PRPP would play important roles in the change in cellular levels of NAM and NAD^+.

eNAMPT and its possible contribution

The extracellular form of NAMPT (also known as eNAMPT or visfatin) was shown to have even higher NMN synthetic activity than its intracellular form with higher k_{cat}, and the exogenous NMN was proposed to help cells maintain cellular NAD^+ levels [64]. The activity of eMAMPT would make NAM treatment more easily stimulatory to SIRT1 since the intracellular NAM level would get lower while NAD^+ production is still driven at a high level (Fig. 1). In the body, adipocytes are a key source of circulating eNAMPT [64–66], but a constitutive secretion was found to occur in a variety of cell types examined including hepatocytes, melanoma cells, COS-7, and CHO cells [64, 67–70]. Upregulated expression of eNAMPT was also reported in other types of cells [71, 72]. Whether the expression of eNAMPT is ubiquitous is not known. Extracellularly produced NMN may be diluted out to body fluids or culture media and, therefore, its contribution in the increase of cellular [NAD^+] may not be high. Further, the extracellular level of PRPP would not be high in culture media if present at all. Nonetheless, the physiological role of eNAMPT in SIRT1 activation is gaining support. For example, monocyte-derived eNAMPT was shown to play a role in maintaining [NAD^+] and NAD^+-dependent SIRT1 activity and survival of cardiomyocyte in pressure-overloaded mice by providing circulating NMN [73]. Therefore, at least in the body, eNAMPT may play a role in the SIRT1 stimulation upon NAM administration.

Interference by PARP-1

The activity of PARP-1 is the most important factor in the maintenance of cellular NAD^+ levels. Following DNA damage, cellular [NAD^+] drops to 20% of the normal levels within 5 min [74, 75]. Thereupon, SIRT1 activity lowers in DNA-damaged cells [76, 77]. PARP-1 has K_M and k_{cat} for NAD^+, not very different from those of SIRT1 [27, 78, 79]. However, poly(ADP-ribosyl)ation increases by more than 100-fold upon DNA damage, as does NAD^+ consumption [80]. Dominance of PARP-1 over SIRT1 in NAD^+ consumption was demonstrated in HeLa cells, where PARP-1 inhibitors partially reduced FK-866-induced NAD^+ depletion while SIRT1 inhibitors did not [81]. In addition to PARP-1, cyclic ADP-ribose hydrolase also cleaves NAD^+ to NAM and ADP-ribose and can influence cellular NAD^+ levels [82] but its expression appears to be limited to certain tissues [83]. Overall, at normal physiology or in cultured cells, the NAD^+-consuming activity is not high enough to affect SIRT1 activity much. At the same time, PARP-1 activity is also inhibited by NAM with IC_{50} of 31 μM [84], which appears to be lower than that of SIRT1 and, therefore, in the cells with

high [NAM], its activity may be more easily inhibited than SIRT1.

Metabolites of NAD^+ and NAM

Both NAD^+ and NAM are degraded or converted to other molecules that play roles in NAD^+ redox state and metabolism, and function as toxins (Fig. 1). They may also affect SIRT1 activity by lowering cellular [NAD^+] or [NAM]. NAD^+ is phosphorylated to $NADP^+$ by NADP kinase (NADK). $NADP^+$ and NADPH are present in cells in about 1/10 concentration of NAD^+ pool, and human NADK has a very high K_M for NAD^+ (1.07 mM) [85] and, therefore, does not easily lower NAD^+ levels. Meanwhile, NAM is converted to *N*-methylnicotinamide (MeNAM) by nicotinamide *N*-methyltransferase (NNMT) [86]. This activity would, thereby, lower the cellular levels of both NAM and NAD^+. However, human NNMT also has high K_M for NAM (of 0.4 mM range) and its expression seems to be restricted mostly to the liver [87]. Further, the levels of MeNAM and its two oxidized forms, *N*-methyl-2-pyridone-5-carboxamide (2-PY) and *N*-methyl-4-pyridone-5-carboxamide (4-PY), were shown to increase sharply upon NAM administration but only to levels lower by one or two orders of magnitude than that of NAM remaining in the liver [53]. Therefore, they may not be significant players in determining the cellular levels of NAM. Meanwhile, 2-PY has been proposed to inhibit PARP-1 [88], but its effect on SIRT1 has not been known. After all, NAM is slowly removed from cells through these metabolites [89].

NADH is believed to be an inhibitor to sirtuins. However, its IC_{50} to SIRT1 and other sirtuin family proteins was found to lie between 1.3 and 68 mM [31], values too high to impact SIRT1 by itself. Rather, the rise of NADH levels appears to work by altering the NAD^+/NADH ratio. A decrease in the cytosolic NAD^+/NADH ratio (from 235 at basal level to 129) caused a decrease in SIRT1 activity in human cells [90], indicating a functional role of NAD^+ redox in SIRT1 activity modulation. Inversely, an increase in the NAD^+/NADH ratio by more than 50%, which has generally been found to be reached upon NAM administration [35, 46], would activate SIRT1. For the NAD^+ redox level, mitochondrial activity is likely the most important factor. While the cytosolic NAD^+/NADH ratio ranges between 60 and 700 in a typical eukaryotic cell, the ratio in mitochondria is maintained at 7–8 [91, 92]. Although NAD^+ and NADH cannot move through the mitochondrial membrane, a change of the mitochondrial NAD^+/NADH ratio seems to be transmitted to cytosol and thereby changes the cytosolic NAD^+/NADH ratio and can alter SIRT1 activity. At the same time, the NAD^+/NADH redox state varies in a wide variety of different cell physiologies. In addition, NAD^+ redox states in both the cytosol and mitochondria are also subject to change along with the activities of glycolysis, pyruvate transporters and dehydrogenases, TCA cycle, and the respiratory chain (ETC), which are, in turn, determined by the cellular ATP demand and uncoupling in electron transport chain [93]. Therefore, cellular levels of ATP and oxygen supply can affect the outcome of NAM treatment. However, NAD^+/NADH ratio is not expected to vary much in normal conditions of cell cultures and the body. Meanwhile, the conversion of NAD^+ to NADH in active glycolysis or TCA cycle would lower [NAD^+]. But, the effect is not likely significant since the decrease of [NAD^+] would not be high since the cellular level of NADH is usually maintained at 1/00 level of [NAD^+] and, therefore, the decrease will occur at this range.

Free NAD^+ levels in the nucleus

Since SIRT1 functions dominantly in the nucleus, the nuclear levels of NAD^+ and NAM should be of importance. NAMPT and NMNAT-1, the two constituent enzymes of the NAD^+ salvage pathway, are present in the nucleus [94–96] as well as in the cytosol [97, 98], and thereby NAD^+ production occurs in both compartments. Importantly, most NAD^+ is bound to proteins especially in the nucleus and, therefore, actual concentration of NAD^+ that is freely available to SIRT1 is expected to be far lower than the measured cellular NAD^+ level. Determination of free NAD^+ is difficult and may vary depending on model systems and methods of measurement. However, in one study, the nuclear free NAD^+ level was estimated at 10–100 μM [99], which is similar to or slightly lower than the estimate of K_M for SIRT1 (34–171 μM). The close proximity between these parameters suggests that SIRT1 activity is regulated more sensitively by the changes in the levels of NAD^+ than expected.

Possible sub-compartment of facilitated conversion of NAM to NAD^+

A model that suggests accelerated conversion of NAM to NAD^+ at special locations in the nucleus has been proposed. SIRT1 is recruited to the promoters of certain genes by the interaction with target transcription factors and modulates the activities of these factors and associated histones through deacetylation [100, 101]. In the model, the salvage conversion of NAM near SIRT1 in action on target chromosomes occurs at high rates and thereby SIRT1 activation is better facilitated than in other parts in the cell. Zhang et al. [96] observed that NAMPT and NAMAT-1 modulate the expression of certain genes in a SIRT1- and NAD^+-dependent manner. Also, they noticed that knocking down NAMPT and NMNAT-1 induced a small

decrease in total cellular NAD^+ levels but resulted in a huge decrease in SIRT1 activity. They speculated that NAMPT and NMNAT-1 may activate SIRT1 through enhancing local NAD^+ levels in excess of that in the cells. Indeed, NMNAT-1 was shown to bind to SIRT1 at gene promoters. As proposed by Zhang et al. [96], there it may promote NAD^+ supply to SIRT1 through a mechanism of substrate channeling [102]. NAMPT was also postulated to localize to certain microdomain in the nucleus where it removes NAM and thereby facilitates SIRT1 activation [103]. This microdomain hypothesis is difficult to prove, but it easily explains the observed stimulatory effect of NAM administration on SIRT1 activity. This hypothesis predicts a localized concentration of PRPP. Presence of a complex that contains NAMPT and SIRT1 as well as ribose-phosphate diphosphokinase, which produces PRPP, on or in close proximity to chromosome, if found, will strongly support this microdomain hypothesis.

Susceptibility of SIRT1 level and activity for modulation

The amount of SIRT1 in cells may matter in the apparent susceptibility to NAD^+ and NAM. In a study, treatment of 5 mM NAM in 293T cells was shown to inhibit the deacetylation of PGC-1α mediated by the endogenous SIRT1 proteins. However, when SIRT1 protein level got enhanced by adenoviral transduction of the gene, NAM, even at 50 mM, was unable to bring in PGC-1α acetylation [104]. SIRT1 expression is regulated by endogenous proteins involved in signal transduction and transcription including itself. In a study, resveratrol treatment enhanced the SIRT1 mRNA level by 1.5-fold. Meanwhile, NAM treatment (of 0.1 and 0.15 mM) caused a near 30% decrease in the mRNA level [105]. However, the change was too subtle and did not correlate with the doses of NAM. In contrast, NAM treatment (of 5 mM), while increasing NAD^+ levels, upregulated Sirt1 expression (over 2.5-fold) and activity in hepatocytes in a dose-dependent manner [41]. SIRT1 activity is also subject to regulation by modifications of phosphorylation, methylation, SUMOylation, and nitrosylation [106], some of which may sensitize SIRT1 to different levels of NAD^+ or nullify the effect. NAD^+ levels in pelvic tissues of human donors were found to decline with aging in both males and females, but SIRT1 activity did not show a significant decrease in aging females. Further, no correlation was found between NAD^+ levels and SIRT1 activity either [107]. Although the consistency of these observations needs to be established, this may be an example of a possibility that NAD^+ availability, though required, may not be the most sensitive modulator of SIRT1 activity.

Tissue variation

There certainly are cellular and tissue differences in the efficiency of NAM conversion and the availability of NAD^+. For example, in the study of Collins and Chaykin [52], the intraperitoneally injected radioactive NAM disappeared at different rates in different tissues, as did the increase in the radioactive NAD^+ as shown in Fig. 2. NAMPT activity exhibits a wide variation in tissue distribution. Liver has the highest activity; kidney, spleen, heart, muscle, brain, and lung have about 1/10 or 1/20 of that in the liver; intestine and pancreas have only 1–2% of that in the liver [59]. Thereby, cells from these organs may have different rates of NAM conversion to NAD^+. In addition, NAD^+ levels appeared to be fourfold higher in certain cancer cells harboring mutations in *BRCA1*, a tumor suppressor protein gene [109]. BRCA1 was shown to downregulate NAMPT levels and thereby lower cellular NAD^+ levels. This suggests a possibility of cellular conditions where NAD^+ levels are quite differently maintained. Meanwhile, in our study, no consensus could be made on cell-type specificity regarding the difference in the effect of NAM treatment on SIRT1 activity due to the small sample sizes of the studies with non-inhibitory effects in Table 2. In addition to tissue variation, NAMPT protein and activity levels have been shown to change upon stress and glucose restriction [18, 108]. However, these different conditions would not play significant roles in determining the outcome of NAM treatment in cultured cells and bodies of normal physiology.

Discussion

Our review found that the effects of NAM on SIRT1 may have been generally assumed without due consideration of a more complex range of effects. For example, the suppressive effects of SIRT1 in HCV replication were investigated in human hepatocytes. Cells were treated with 5 mM NAM for 1 h, and HCV gene expression was determined at 24, 48, and 72 h post-treatment. The authors found consistent increases in HCV gene expression and proposed that NAM treatment abolished SIRT1-mediated suppression of HCV replication [110]. However, it is unlikely that 5 mM NAM remained at SIRT1-inhibitory levels without being converted to NAD^+ during this long-time course. We believe that this result as it is presented cannot be accepted as supportive evidence for HCV suppression without demonstrating SIRT1 inhibition by NAM treatment. In a different study examining differentiation-associated changes in cellular levels of diverse deacetylases, NAM was used as a sirtuin inhibitor. To gauge selectively the deacetylase activity of sirtuin proteins

(which are classified as class III HDAC), cellular deacetylation activity was assayed in the presence of TSA, which is a generic class I/II/IV HDAC inhibitor. Conversely, to determine the activities of class I/II/IV HDACs, deacetylase activity was assayed in the presence of NAM. The authors found that, in contrast to the decreased activity associated with TSA, NAM treatment resulted in a minimal change in deacetylase activity during differentiation. Based on these findings, the authors concluded that the level of sirtuin deacetylases decreases but that of the other HDACs does not change during differentiation [111]. However, the absence of a change in deacetylase activity following NAM treatment may be because of an insufficient inhibitory effect by NAM. For a phenomenon that appears upon NAM treatment to be credited to a result of SIRT1 inhibition, it should be accompanied with evidence that proves SIRT1 as inactive in the treated cells. Demonstration of increased acetylation levels of one or two SIRT1 substrates such as Ku70 or p53 would be confirmative.

These two studies are examples of possible misinterpretations of the findings because of the assumption of the inhibitory effect of NAM on sirtuins. These misinterpretations may stem from an incomplete understanding of the differences between in vitro conditions and cellular conditions. The finding and interpretation on NAM as a sirtuin inhibitor is correct in an in vitro setting where NAMPT is absent and conversion of NAM to NAD^+ does not occur, but in cells, it may not. However, even if the difference between in vitro and in vivo situations was recognized, it would not be easily acceptable that NAM treatment is stimulatory to SIRT1. Although it has been shown that the level of NAM drops after a high peak, it appears to remain higher than the basal level for a period of time. Also, the duration of this state is likely longer in the case of cultured cells compared to the case of tissues of the administered animal. Still, there exist possibilities that allow cellular situations to be favorable for SIRT1 activation. First, NAM levels that are maintained after the peak may not be too high to exert strong inhibitions to SIRT1 activity in the presence of the elevated levels of NAD^+. It is noteworthy that the treatment of NMN, nicotinamide riboside (NR) or nicotinic acid (NA), other NAD^+ precursors [112], caused SIRT1 activation by raising cellular content of NAD^+ to 1.5- to 1.8-fold levels (depending on cell types) [113], which are quite similar to that achieved by NAM treatment. Although a direct comparison of NAM and these precursors for the effects on the levels of SIRT1 activation and cellular NAD^+ has not been made, this raises a possibility that the inhibitory effect of NAM may indeed become marginal shortly after its administration. Second, considering the estimated endogenous level of NAM at the range of 10 μM and the administered doses of 5 mM or above (so, a difference of 1/500), the increase in the cellular NAM level appears to be small (in the study of Trammell et al., the peak was only fivefold higher than the basal level). This small increase may reflect the ongoing activity of eNAMPT, which allows the influx of NAD^+—producing power in the form of NMN. Third, the microdomain hypothesis provides a possibility of a way to activate SIRT1 in the presence of high cellular [NAM]. Localized facilitation of the salvage conversion would lower [NAM] and enhance [NAD^+] in the vicinity of SIRT1 *en act* (Fig. 1). Considering the importance of the maintenance of SIRT1 activity, especially for gene expression in diverse physiological and pathological conditions, this hypothesis appears quite plausible, and its feasibility deserves vigorous examination. Cells would need this machinery to better maintain SIRT1 activity in response to frequent danger of NAD^+ depletion caused by PARPs upon DNA damage.

Finally, it is important to note that most findings regarding the effects of NAM have been made at a concentration range of 5–10 mM. NAM administered at higher doses results in cell death potentially via apoptosis [114]. At these high doses, regardless of whether cell death is caused by prolonged inhibition of SIRT1 or by changes in critical cell metabolic processes, a physiological change may easily be hidden by the death of cells. Therefore, NAM at doses over 10 mM may not be adequately used to inhibit or activate SIRT1 in most cells.

Conclusion

Of the several different forms of vitamin B_3, NAM alone can both stimulate and inhibit the activity of sirtuins, and this capacity provides a compound capable of fine-tuning key life activities. However, this dual opposing nature has likely confounded the interpretation of findings in previous studies and will do so in future investigations as well. Although this kind of misinterpretation has been found in a rather minor number of studies, the actual number of cases that unexpectedly encountered such null or rather stimulatory effects on SIRT1 activity might be underestimated since such findings might have been simply regarded as an experimental failure and discarded. Therefore, we propose that NAM is no longer used as a SIRT1 inhibitor. In fact, NAM is not particularly potent compared to other synthetic inhibitors such as sirtinol (IC_{50} of 131 μM) [115] and EX527 (IC_{50} of 38 nM) [116]. NAM may be considered as a pan-sirtuin inhibitor (i.e., inhibitory effects have been demonstrated for SIRT1, SIRT2, and SIRT3 with IC_{50} values in the range of 100 μM) [27]. However, there are also inhibitory chemicals with a competitive potency in the same set of sirtuins such as 2-anilinobenzamide derivatives [117]. If NAM is intended to be used, it should be assayed in a very short time frame, such as within 1 or 2 h

following treatment. Finally, maybe of greater importance, the inverse effect of NAM, a stimulant to pan-sirtuin activity, which would be manifested in a longer term treatment, may deserve more studies and usage.

The nature of NAM binding to the SIRT1 protein and exerting feedback inhibition led Taylor et al. [118] to propose development of new compounds that would specifically block the NAM-binding site in sirtuins. This antagonism would thereby abrogate inhibition by NAM, and if such a compound was used in concert with NAM, it may result in the enhanced or prolonged activity of sirtuins that would better realize their therapeutic potential in treating degenerative diseases.

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