Nicotinamide-induced mitophagy: An event mediated by high NAD$^+$/NADH ratio and SIRT1 activation*

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*Running Title: [NAD$^+$/NADH] ratio modulates mitochondria content

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**Background:** Nicotinamide treatment decreases mitochondria content and helps cells maintain high mitochondrial quality.

**Results:** Metabolically-enhanced NAD$^+$/NADH ratio and chemical-induced SIRT1 activation alike decreased mitochondria content, increased autophagy and induced mitochondrial fragmentation.

**Conclusion:** Mitochondria content is modulated by high NAD$^+$/NADH ratio and mechanisms that involve SIRT1 activation.

**Significance:** Elevation of NAD$^+$/NADH ratio may promote cellular health by facilitating mitochondrial autophagy.

**SUMMARY**

Active autophagy coupled with rapid mitochondrial fusion and fission constitutes an important mitochondrial quality control mechanism and is critical to cellular health. In our previous studies, we found that exposure of cells to nicotinamide (NAM) causes a decrease in mitochondrial content and an increase in mitochondrial membrane potential (MMP) by activating autophagy and inducing mitochondrial fragmentation. Here, we present evidence to show that the effect of NAM is mediated through an increase in the [NAD$^+$/NADH] ratio and the activation of SIRT1, an NAD$^+$-dependent deacetylase that plays a role in autophagy flux. The [NAD$^+$/NADH] ratio was inversely correlated with the mitochondrial content, and an increase in the ratio by the mobilization of Malate-aspartate shuttle resulted in autophagy activation and mitochondrial transformation from lengthy filaments to short dots. Further, treatment of cells with SIRT1 activators, fisetin or SRT1720, induced similar changes in the mitochondrial content. Importantly, the activators induced mitochondrial fragmentation only when SIRT1 expression was intact. Meanwhile, MMP did not increase when the cells were treated with the activators, suggesting that the change in MMP is not induced by the mitochondrial turnover per se and that elevation of the [NAD$^+$/NADH] ratio may activate additional mechanisms that cause MMP augmentation.

Put together, our results indicate that a metabolic state resulting in elevated [NAD$^+$/NADH] ratio can modulate mitochondrial quantity and quality via pathways that may include SIRT1-mediated mitochondrial autophagy.

* Cells from aging tissues and late-passage cultures exhibit abnormalities in mitochondrial electron transport chain (ETC) such as cytochrome c oxidase negativity and mtDNA mutations (1). Defective ETCs produce large amounts of reactive oxygen species (ROS), and thereby, play a major role in the induction of cellular senescence and, possibly, tissue aging and are strongly associated with various aging-associated degenerative diseases and cancers as well (2, 3). For this reason, maintenance of mitochondria quality is of upmost importance to body health and longevity (4). Mitochondrial quality control is mediated largely by the removal of dysfunctional ones and biogenesis of new ones. However, during senescence and aging, the removal of damaged mitochondria is attenuated, resulting in an increase of mitochondrial
mass and cellular mitochondrial content (5, 6). Autophagy is the major cellular mechanism removing organelles including mitochondria, and along with coordinated mitochondrial fission and fusion, is believed to selectively remove damaged (depolarized) mitochondria (7). Therefore, the persistence of a high level of autophagy flux and mitochondria structural dynamics may be the key to the maintenance of mitochondrial quality. In fact, the longevity of model organisms has been linked to the efficient maintenance of autophagy, a cellular process that is downregulated during aging (8). The role of autophagy in the maintenance of health and longevity was recently highlighted in studies which showed that calorie restriction exerts its effect by enhancing mitochondrial autophagy (9). However, the mitochondrial status in the cells in which autophagy is activated has rarely been examined in details. Limited information is available as to whether the content of mitochondria decreases or their quality increases in the cells in which autophagy has been induced. Meanwhile, mitochondrial structural dynamics appear to be attenuated during cellular senescence and aging as well (10). This change seems to be a causative event as well as a consequence of cellular senescence. The knock down of mitochondrial fission results in the retention of elongated mitochondria, persistence of high ROS level, and progression into a stage of senescence (11). And, exposure to H₂O₂ or disruption of ETC function, which induce senescence, also cause a reduction in mitochondrial fission activity and formation of elongated or giant mitochondria (12).

Nicotinamide (NAM), an amide form of vitamin B₃, is readily converted to coenzyme β-nicotinamide adenine dinucleotide (NAD⁺) through the salvage pathway involving NAM phosphoribosyltransferase (NAMPT) and NAM mononucleotide adenylyltransferase (NMMNAT) (13, 14). NAM promotes the survival of a variety of cell types, mainly by serving as a source of NAD⁺ (15). One of the known molecular targets of increased NAD⁺ level is SIRT1, a member of the NAD⁺-dependent protein deacetylase family (16). SIRT1 activation has been suggested to promote cell survival and longevity of organisms (17, 18) although this effect has been recently challenged (19). It also exerts protective effects against a number of age-associated disorders and conditions, such as metabolic, cardiovascular, cancer, and neurodegenerative diseases (20). The dependence of SIRT1 on NAD⁺ suggests that SIRT1 fine-tunes cellular metabolism and body physiology according to the availability of cellular NAD⁺. SIRT1 also has been shown to play an essential role in the induction of autophagy during starvation (21, 22). Meanwhile, its absence results in the appearance of abnormally shaped mitochondria (21). However, a direct relationship between SIRT1-mediated autophagy and mitochondria quantity as well as quality has not yet been reported.

In previous studies, we demonstrated that the treatment of human fibroblasts with 5 mM NAM activates autophagy and causes a decrease in mitochondrial content and ROS level, while increasing mitochondrial membrane potential (MMP). NAM treatment also induces transformation of mitochondria from filamentous network structures to short-dot structures (23). Furthermore, long-term treatment of NAM resulted in a significant extension of the replicative lifespan of normal human fibroblasts and keratinocytes (24). On the basis of these findings, we hypothesized that, by accelerating mitophagy, NAM may facilitate the maintenance of high quality mitochondria and restrict oxidative stress to low levels. However, molecular mechanisms underlying the acceleration of autophagy and the mitochondrial transformation have not yet been elucidated. In the current study, we examined the involvement of high cellular [NAD⁺]/[NADH] ratio and SIRT1 activation in the NAM-induced decrease of the mitochondrial content and their transformation. Our results show that cellular NAD⁺ metabolism modulates mitochondria content through pathways that might involve SIRT1 activation.

**EXPERIMENTAL PROCEDURES**

**Cell culture and chemicals.** Normal human fibroblasts were cultured in DMEM plus 10% FBS with or without NAM (5 mM), NAD⁺ (5 mM), L-asparagine (20 mM), or indicated amounts of fisetin, resveratrol, (Sigma-Aldrich Co., St. Louis, MO, USA), SRT1720 (Selleck chemical, Houston, US), or FK866 (NIMH Drug Supply Program, [http://nimh-repository.rti.org/](http://nimh-repository.rti.org/)). For long-term treatment, the medium
supplemented with the chemicals was replaced every 2 days.

**Western blot analysis**  Cells were lysed with RIPA buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS) supplemented with NaF, NaVO₄, and a protease inhibitor mixture (Sigma-Aldrich). Typically, 30–40 μg of proteins were separated by SDS-PAGE, transferred to nitrocellulose membrane, and blotted with antibodies against human LC3, acetylated p53 (Cell Signaling Technology, Beverly, MA), p53, SIRT1, or ERK1/2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), acetyl-histone H3 (Millipore), or histone H3 (Abcam, Cambridge, UK). Protein bands were visualized by using horseradish peroxidase-conjugated secondary antibodies and SuperSignal WestFemto substrate (Pierce, Woburn, MA, USA). For histone extraction, the RIPA lysate was pelleted, washed in Tris-EDTA (pH7.4) buffer, and incubated in 200 μl of 0.4 N H₂SO₄ for 90 min on ice. After centrifugation, the supernatant was mixed with 1 ml cold acetone and kept at -20°C overnight. The histones were collected by centrifugation, air-dried, and resuspended in H₂O. Typically 1 μg proteins were applied to Western blotting analysis.

**Measurement of mitochondrial content and MMP** Cells were stained with 50 nM nonyl acridine orange (NAO) or 100 nM MitoTracker Red (MTR) (both from Invitrogen, Carlsbad, CA, USA) for 30 min at 37°C in dark, and were then subjected to flow cytometry with 488 nm excitation/530 nm emission and 488 nm excitation/585 nm emission, respectively. For the measurement of MMP, cells were stained with 50 nM nonyl acridine orange (NAO) or 100 nM MitoTracker Red (MTR) (both from Invitrogen, Carlsbad, CA, USA), acetyl-histone H3 (Millipore), or histone H3 (Abcam, Cambridge, UK). The mixtures were incubated for 30 min at 37°C, and fluorescence was detected as per the supplier’s protocol.

**In vivo SIRT1 activity assay** Cells were incubated with Fluor-de-Lys-SIRT1 deacetylase substrate (BML-K177, Enzo Life Sciences, Farmingdale, NY, USA) and 1 M Trichostatin A for 1 h, and fluorescence was detected as per the supplier’s protocol.

**Measurement of total [NAD⁺]/[NADH] ratio** Cells (1 × 10⁶) were washed in PBS and lysed by the addition of 200 μl of cold HClO₄ solution (0.5 M) and incubated in ice for 15 min. The extracts were neutralized by adding 61 μl 2 M KOH/0.2 M K₂HPO₄ (pH 7.5) and spun at 13000 × g for 3 min. Supernatant or NAD⁺ standard diluents (30 μl) were mixed with 150 μl of the reaction solution. The mixtures were incubated for 30 min at 37°C, and absorbance at 450 nm was measured. The reaction solution was composed of 8 μM WST-1 (Takara Bio Inc, Shiga, Japan), 70 μM 1-methoxy-5methyl-phenazinium methyl sulfate, 20 IU alcohol dehydrogenase (Sigma-Aldrich), 64 mM nicotinamide, and 0.32 M ethanol (Sigma-Aldrich) in 64 mM Gly-Gly buffer (pH 7.4).

**Measurement of free [NAD⁺]/[NADH] ratio** For the lactate oxidase assay, 10 μl of the culture medium or lactate standard solution was mixed with 196 μl of the assay buffer (0.1 M citrate, 1 mg/ml BSA, 0.1% CaCl₂, 0.02% NaN₃, adjusted to pH 6.5 with 1 M Na₂HPO₄), 1 μl of 2 μM/lactate oxidase stock (lactate oxidase enzymes
(Sigma-Aldrich) were dissolved in the enzyme dilution buffer (10 mM KH$_2$PO$_4$, 10 μM FAD, adjusted to pH 7.0 with KOH), 1 μl of 0.5 U/μl peroxidase stock (peroxidase (Sigma-Aldrich) dissolved in distilled water), and 2 μl of 5 mM Amplex UltraRed stock (Invitrogen). Assay mixtures were incubated for 30 min at 37°C and fluorescence was read at excitation/emission = 535/590 nm. For the pyruvate oxidase assay, 10 μl of the culture medium or pyruvate standard solution was mixed with 196 μl of the assay buffer (50 mM KH$_2$PO$_4$, 1 mg/ml BSA, 0.2 mM TPP, 10 μM FAD, 0.97 mM EDTA, 9.8 mM MgCl$_2$, 0.02% NaN$_3$, adjusted to pH 6.5 with 1 M NaOH), 1 μl of 2 μM/μl lactate oxidase stock (pyruvate oxidase diluted in buffer [10 mM KH$_2$PO$_4$, 10 μM FAD, adjusted to pH 7.0 with KOH]), 1 μl of 0.5 U/μl peroxidase stock, and 2 μl of 5 mM Amplex UltraRed stock. The assay mixtures were then incubated for 30 min at 37°C and fluorescence was read at ex/em = 535/590 nm. For the calculation of the ratio of free \([NAD^+] / [NADH]\), the following equation was used (25).

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\frac{NAD^+}{NADH} = \frac{[pyruvate]}{[lactate]} \times \frac{1}{K}, \quad K = 1.11 \times 10^{-4}
\]

Statistical analysis All quantitative measurements were made at least in triplicate, and mean ± S.E.M values were presented. Intergroup comparison of the mean values was performed by one-way analysis of ANOVA using InStat 3.06 (GraphPad software Inc., San Diego, CA, USA). A P-value of ≤0.05 was considered to be statistically significant.

RESULTS

NAM-induced decrease of mitochondrial content requires NAM conversion to NAD$^+$

Fig. 1A shows an example of the change in the mitochondrial content that is commonly observed in various tested human cells (including normal fibroblasts and MCF-7, H460, and HCT116 cancer cell lines) after supplementation of 5 mM NAM in culture media. The mitochondrial content as determined by flow cytometry using two different mitochondria-specific dyes, was substantially decreased on the 1st day, and further decreased until the 3rd day, and thereafter, remained at a level about 70% of that in the untreated cells as far as the cells were fed with 5 mM NAM in every two to three days, as reported previously (23).

In mammalian cells, NAM is readily converted to NAD$^+$ through the salvage pathway; the first step of this pathway is the conversion of NAM to niacinamide mononucleotide (NMN), which is mediated by NAM phosphoribosyltransferase (NAMPT) (26). In the cells pulsed with 5 mM NAM, cellular NAD$^+$ concentration increased by near 40% within 12 h, and this increase was maintained throughout the 21 days of the investigation (Fig. 1B and Fig. 1C (-), grey bar; and Supplemental figure 1).

The NAM-induced change in the mitochondrial status appears to be mediated by the increase in the NAD$^+$ level. First, treatment of cells with 1 mM NMN, which caused an increase in the level of basal [NAD$^+$] by nearly 40–50% (Fig. 1C (NMN)), resulted in a decrease in the mitochondrial content to the level achieved by NAM treatment, although NAM co-treatment did not cause any further decrease (Fig. 1D (NMN)). Next, the suppression of NAMPT by treatment with either FK866, a potent inhibitor of NAMPT (27), or NAMPT-specific siRNA resulted in a substantial decrease in the basal as well as the NAM-raised levels of NAD$^+$ (Fig. 1C, (FK866) and (siNAMPT) (FK8866)). Further, these treatments resulted in a significant increase in the basal as well as the NAM-suppressed levels of the mitochondrial content. Importantly, this increase in mitochondrial content was attenuated by NMN supplementation, which enables cells to bypass the FK866- or siNAMPT RNA-induced inhibition of NAMPT (Fig. 1C and 1D, (FK866+N Mn) and (siNAMPT+N Mn)). Overall, these results suggest that the NAD$^+$ level determines the cellular mitochondrial content.

Lactate treatment alters both the [NAD$^+$]/[NADH] ratio and mitochondrial content

We further questioned if the downregulation of mitochondrial content by NAM is caused by an increase in the cellular ratio of [NAD$^+$]/[NADH] rather than the increase of [NAD$^+$] _per se_. In fact, the ratio of free [NAD$^+$]/[NADH] increased near two fold on day 1 and remained elevated thereafter (Interestingly, it further increased after day 3.) (Fig. 2A). The ratio of the total [NAD$^+$]/[NADH] also increased, albeit to a slightly lesser extent (Fig. 2B, compare NAM(-) and NAM(+)). The [NAD$^+$]/[NADH] ratio is reciprocally regulated by the [pyruvate]/[lactate] ratio through lactate fermentation, and when elevated, downregulates the conversion of pyruvate to lactate. Indeed, in the NAM-treated cells, the cellular lactate production, as determined by its concentration in

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\text{Ratio of [NAD$^+$]/[NADH]} = K = 1.11 \times 10^{-4}
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\begin{align*}
\text{[NAD$^+$]} & = K \times \text{[pyruvate]} / \text{[lactate]}, \\
\text{[NADH]} & = K \times \text{[pyruvate]} / \text{[lactate]}
\end{align*}
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the culture medium, decreased with an inverse correlation to the change in the \([\text{NAD}^+]/[\text{NADH}]\) ratio (Fig. 2A). Meanwhile, the addition of 10 mM lactate to the medium resulted in a decrease in the ratio in the control as well as the NAM-treated cells to the levels well below those of the mock-treated cells (Fig. 2B). And, surprisingly, lactate treatment resulted in an increase in the mitochondrial contents of both the control and NAM-treated cells in a dose-dependent manner (Fig. 2C). This indicates that lactate itself or the lactate-induced decrease in the \([\text{NAD}^+]/[\text{NADH}]\) ratio increases the mitochondrial content.

High \([\text{NAD}^+]/[\text{NADH}]\) ratio lowers mitochondrial content and increases MMP
The importance of the \([\text{NAD}^+]/[\text{NADH}]\) ratio as a determining factor of the cellular mitochondrial status was confirmed in the cells where the ratio was altered through the modulation of the malate-aspartate shuttle. In this shuttle, malate dehydrogenase oxidizes NADH to \(\text{NAD}^+\) by transferring a hydrogen atom and an electron to oxaloacetate to produce malate, which crosses the mitochondrial membrane through the malate/\(\alpha\)-ketoglutarate antiporter and becomes oxaloacetate again by reducing mitochondrial \(\text{NAD}^+\) to NADH. And, the mitochondrial oxaloacetate returns to cytosol in the form of aspartate (28). The cytosolic conversion of NADH to \(\text{NAD}^+\) can be promoted by an increase in the concentration of aspartate, which can be achieved by the addition to the culture medium of asparagine that is readily converted to aspartate by cytosolic asparaginase (29). We treated cells with 20 mM asparagine (Asn) or \(\text{NAD}^+\) (treatment included as a positive control), and effects on the mitochondria content and MMP were monitored. Treatment of Asn caused a decrease in the mitochondrial content, with the kinetics being similar to that induced by \(\text{NAD}^+\) or NAM. For 3 days, nearly 40% reduction in the mitochondrial content was observed in both the cases of Asn and \(\text{NAD}^+\) (Fig. 2D). The change in the MMP induced by Asn treatment was also similar to that induced by NAM treatment - an increase of nearly 50% in 3 days (23)(Fig. 2E). These results strongly indicate that the cytosolic \([\text{NAD}^+]/[\text{NADH}]\) ratio indeed plays a determinative role in the regulation of the mitochondrial content and MMP.

Both \(\text{NAD}^+\) and asparagine induce autophagosome formation and mitochondrial transformation
Our previous studies showed that NAM-induced changes in the mitochondrial status are dependent on the activation of autophagy and the transformation of mitochondria from long filaments to short dots (23). Whether Asn treatment also activates autophagy and induces mitochondrial transformation was determined. First, the effect of \(\text{NAD}^+\) or Asn treatment on autophagy activation was determined by examining the formation of LC-3 punctae through immunofluorescence for the endogenous LC-3 proteins (Fig. 3A). The numbers of prominent LC-3 punctae was significantly higher, and the punctae appeared to be more prominent in the cells treated with either \(\text{NAD}^+\) or Asn. The individual punctae were as big as those in the cells treated with resveratrol, which is known to induce autophagy through the activation of either SIRT1 (30) or AMPK (31). Overall, these results suggest that autophagy flux is indeed increased by the treatment of Asn.

Next, a change in mitochondrial structure was examined through confocal microscopy. In mock-treated fibroblasts, most mitochondria appeared as long thread-like structures, as represented in Fig. 3B (-). However, in most of the NAM-treated cells, they seemed to be fragmented and appeared as short filaments or dots, many of which were shorter than 2 μm in length (23, and Fig. 3B (NAM)). Importantly, in the \(\text{NAD}^+\)- or Asn-treated cells, a decrease in mitochondrial length was also noted (Fig. 3B (\(\text{NAD}^+\)) and (Asn)). Overall, all the three chemicals, NAM, \(\text{NAD}^+\), and Asn increased autophagy flux and induced mitochondrial fragmentation, which strongly suggests that the increased \([\text{NAD}^+]/[\text{NADH}]\) ratio accelerates mitochondrial turnover via the induction of autophagy which is accompanied with mitochondrial fragmentation.

NAM at 5 mM but not at higher concentrations causes the activation of SIRT1 and the decrease of mitochondria content
SIRT1 plays an essential role in autophagy by deacetylating the proteins involved in autophagosome formation (21). And, SIRT1 requires \(\text{NAD}^+\) as a substrate, and its activity is enhanced by an increase in the \(\text{NAD}^+\) level or \([\text{NAD}^+]/[\text{NADH}]\) ratio (32, 33, 34). However, SIRT1 activity is also inhibited by NAM itself (35), which makes it difficult to directly point to SIRT1 as a key mediator of the effect of NAM. In our previous study, knocking down SIRT1 substantially increased the level of the mitochondrial content both in the control and the
NAM-treated cells, which rather demonstrates the importance of SIRT1 in the basal level autophagy (23). To determine if the effect of NAM is mediated by the activation of SIRT1, we first checked if SIRT1 activity is indeed elevated in cells treated with 5 mM NAM. In the fibroblasts treated with 5 mM NAM, the acetylation level of histone H3, a known SIRT1 substrate (36), was apparently decreased from the 1st day and maintained at the low levels thereafter, indicating an elevation of SIRT1 activity (Fig. 4A). Interestingly, SIRT1 protein level itself was substantially higher in the NAM-treated cells (Fig. 4A). Although the underlying mechanism is not known yet, this increase in the protein level would certainly help cells maintain high SIRT1 activity. Furthermore, the results from the western blots where p53 acetylation level appeared to be lower at least from 12 hr post treatment (Fig. 4B) and the in vivo assay in which the deacetylation of a p53-based artificial substrate (37) similarly increased suggest an activation of SIRT1 upon the treatment of 5 mM NAM (Fig. 4C). Meanwhile, NAM at 10 or 20 mM dose caused an increase of [NAD⁺]/[NADH] to the levels almost identical to that in the cells treated with 5 mM NAM (Fig. 1B), but resulted in an inhibition of SIRT1 activity during the early time period (SY Jang, data not shown). Importantly, the mitochondrial content was not downregulated but rather increased in the cells treated with 20 mM NAM (Fig.4D). This suggests that SIRT1 activities including that induces autophagy, are indeed inhibited when cells were fed with higher dose of NAM (especially at the early time period when a large quantity of NAM has not been converted to NMN and remains as NAM). In this regard, it is noteworthy that, in contrast to NAM, the treatment of NAD⁺ at a wide range of concentrations (1 μM – 5 mM) all alike resulted in a decrease in the mitochondria content, and not a single high dose caused an increase over the level that was maintained in the control cells (Supplemental figure 2). This difference between NAM and NAD⁺ quite likely points to the inhibitory effect of NAM exerted at high doses.

SIRT1 activation induces the decrease of the mitochondrial content The results above suggest that SIRT1-mediated autophagy activation may be at least one of the underlying mechanisms for the decrease in the mitochondrial content in the NAM-treated cells. This possibility was verified by examining the effect of the two chemicals that activate SIRT1; fisetin (3,7,3′,4′-tetrahydroxyflavone), which is structurally and functionally similar to resveratrol and therefore suffers from the problem of off-target effects (38, 39)), and SRT1720, which is known to be highly specific to SIRT1 (40). Treatment with either chemical caused a decrease of the mitochondrial content to levels similar to that achieved by NAM treatment (Fig. 5A-4C). In the case of fisetin, the effect tended to be rapid, with the minimum level being attained on the 1st day, even at the lowest dose (10 μM). At the 2nd day, the content was restored a little bit, but remained at nearly 80% of the mock-treated cells. Resveratrol also caused a similar change in the mitochondria content (S Jang, data not shown). Meanwhile, the treatment of SRT1720 caused a gradual decrease in the content, which reached nearly 60% of that in the mock-treated cells on day 2, resembling that of the NAM treatment. Moreover, the doses of 80 and 800 nM caused almost identical changes, possibly reflecting a high specificity of the chemical. Together, the finding that different SIRT1 activators cause a decrease in the mitochondrial content to largely similar extents suggests that SIRT1 activation indeed causes downregulation of cellular mitochondrial content. In addition, the mitochondrial content was maintained at the low levels as far as the cells were continuously fed with fresh activators as was the case with NAM (Fig. 5C).

SIRT1 activation induces mitochondrial fragmentation Next, we examined whether SIRT1 activation induces mitochondrial fragmentation as well. Treatment of 10 μM fisetin or 160 nM SRT1720 indeed resulted in the appearance of severed mitochondria in a large population of the cells on day 1 (Fig. 6A, b and c, respectively). Interestingly, mitochondria were severed but appeared to be still aligned to the location of the previous filaments in some cells treated with SRT1720 (Fig. 6A, c). Importantly, the mitochondrial fragmentation was blocked when SIRT1 mRNA was knocked down (Fig. 6B). As shown in Fig. 6A, (e and f), mitochondria remain as long filaments in the presence of fisetin or SRT1720 in the cells transfected with siRNA to SIRT1. These findings imply that mitochondrial transformation to dot structures is an event dependent on SIRT1 activation.

NAM-induced MMP change is not dependent on SIRT1 activation Finally, we sought to
[\text{NAD}^+]/[\text{NADH}]$ ratio modulates mitochondria content

determine whether the increase in MMP observed in the cells treated by NAM, NAD$^+$, or Asn is also induced by SIRT1 activation. For this purpose, cells were treated with the SIRT1 activators, stained with JC-1, and subjected to flow cytometry for MMP determination. Surprisingly, none of the SIRT1-activators induced a significant increase in MMP (Fig. 7A and 7B). This indicates that the increase in MMP by the treatment of NAM, NAD$^+$, or Asn is mostly not because of SIRT1 activation or SIRT1-induced autophagy activation, but due to certain unknown mechanisms induced by the elevation of the $[\text{NAD}^+]$ or $[\text{NAD}^+]/[\text{NADH}]$ ratio. Finally, fisetin treatment, rather, induced a substantial decrease in MMP. This suppressive effect was not significant in the SRT1720-treated cells, and therefore, was likely caused by the off-target effect.

**DISCUSSION**

Removal of dysfunctional mitochondria requires the activation of autophagy coupled with on-going mitochondrial fission (7). Our current and previous studies show that both of these processes can be induced by NAM treatment. And, the earlier part of the current study showed that the effect of NAM was exerted through an increase of $[\text{NAD}^+]/[\text{NADH}]$ ratio. The decrease of the mitochondrial content was not only dependent on the conversion of NAM to NAD$^+$ but was also inversely proportional to the cellular levels of lactate, which is tightly linked to the $[\text{NAD}^+]/[\text{NADH}]$ ratio via lactate fermentation. More importantly, treatment of Asn, which mobilizes the aspartate-malate shuttle toward an increase in the $[\text{NAD}^+]/[\text{NADH}]$ ratio in cytosol, caused a decrease in the mitochondrial content and an increase in MMP with the kinetics and extent of change being similar to those induced by the NAM treatment. Furthermore, NAM, NAD$^+$, and Asn commonly induced mitochondrial fragmentation, an essential prerequisite for mitochondrial autophagy.

The plausibility of SIRT1 being involved in the effect of the elevated $[\text{NAD}^+]/[\text{NADH}]$ ratio was examined in the later part of the study. In our previous study, knocking down SIRT1 mRNA attenuated the effect of NAM on both mitochondrial content and MMP (23). However, SIRT1 appears to be an essential element in the basal level autophagy (21). If this is the case, its absence would nullify cellular autophagy activity, and the results of this SIRT1 knock-down experiment may emphasize the requirement of SIRT1 for the effect of NAM but do not prove that NAM exerts its effect through the activation of SIRT1. In our current study, the possibility for the involvement of SIRT1 activation is better supported. SIRT1 activation by fisetin, resveratrol, or SRT1720 caused a decrease in the mitochondrial content, with the extents being similar in all the cases and almost equivalent to those induced by NAM, NAD$^+$, and Asn. More importantly, the treatment of the activators, similar to the treatment of NAM, NAD$^+$, and Asn, caused mitochondrial fragmentation, and this effect required SIRT1 expression.

One important message of our study is that the activation of SIRT1 causes a decrease in the cellular content of mitochondria through the induction of autophagy and mitochondrial fragmentation. Previous studies showed that SIRT1 overexpression or its activation by resveratrol (although the effect of resveratrol may also be mediated by AMPK activation (31)) induces autophagy (21, 41). SIRT1 has also been shown to play an essential role in the induction of autophagy in fasting cardiac myocytes (42). However, in these studies, it was not determined whether the cellular mitochondrial status is affected by the SIRT1-activated autophagy. Active autophagy does not necessarily mean on-going mitophagy or a change in the status of mitochondria. Increased autophagy should be accompanied by the transformation of mitochondria to the structures that are suitable for autophagosome encirclement. In fact, a recent study showed that mitochondria are spared during starvation-induced autophagy by acquiring an elongated structure (43).

With regard to reasons for the decrease in the mitochondria content, the possibility of downregulation of mitochondria biogenesis was ruled out in the previous study on the basis of the finding that the mRNA levels of PGC-1$\alpha$, NRF-1 TFAM, as well as the ETC proteins were by and large unaffected by NAM treatment, at least soon after the treatment (23). On the contrary, SIRT1 activation has been reported to induce mitochondrial biogenesis, which would result in an increase in the mitochondrial content. In these studies, treatment with either resveratrol (44) or SRT1720 (45) activated PGC-1$\alpha$ and thereby induced mitochondrial biogenesis, which, in turn, results in an increased aerobic capacity of primary cultured cells or mice. If both mitochondrial autophagy and biogenesis occur simultaneously in a cell, these seemingly-
NAM is the end product of SIRT1 activity, and noncompetitively inhibits the activity in vitro (35). Therefore, NAM has been widely used as an inhibitor of SIRT1. Meanwhile, NAM, when added to a cell culture, is readily converted to NMN by the intracellular and extracellular NAMPT enzymes (48). In fact, NAD^+ level increased rapidly and was maintained at elevated levels for 21 days of experiment (Fig. 1 and Supplemental figure 1). Further, in our studies thus far, it appears that SIRT1-stimulation rather than SIRT1-inactivation is the outcome of the treatment with 5 mM NAM. In MCF-7 and H460 human cancer lines, NAM treatment at 5 mM (but, not at 20 mM) caused a similar decrease in the mitochondria content (HT Kang, unpublished data). Meanwhile, to induce SIRT1 inhibition in vivo, NAM is frequently used at concentrations higher than 5 mM (For example, 40 mM was used by 49). With regard to this contradictory effect of NAM, it is noteworthy that 5 mM NAM inhibited SIRT1-mediated PGC-1α deacetylation in 293T cells, but even at 50 mM was unable to increase PGC-1α acetylation when SIRT1 protein level was increased by the adenosiral-transduction of the gene (47). This indicates that NAM’s inhibitory effect is sensitive to the cellular level of the SIRT1 protein. An apparent increase of SIRT1 protein level occurred, and this, in the presence of the high concentration of NAM, would also enable cells maintain high level SIRT1 activity (Fig. 4A). Meanwhile, during the course of the NAM-induced change in the mitochondria content, a brief increase in the content occurred within 12 hr post treatment suggesting a possibility that SIRT1 might be transiently inactivated at early period of NAM treatment (Fig. 4D). This raises a possibility that the SIRT1 inhibitory effect of NAM last only transiently, and therefore, has to be sought rather immediately after the treatment. This also emphasizes the importance of measuring the intracellular concentration of NAM itself at various time points after treatment in this as well as other studies that may examine the cellular effect of NAM. Meanwhile, there is a possibility that SIRT1 may function indirectly in the NAM-induced autophagy activation. For example, SIRT1 may work as a sink for the incoming NAM. Activity of certain unknown molecule may benefit from this SIRT1-modulated [NAM] or NAD^+ /NADH ratio and cause mitochondrial degradation or mitophagy. Despite being appeared to be rather remote considering the molecular ratio of SIRT1 and NAM in cells, such a possibility needs to be experimentally ruled out.

Mitochondria frequently undergo change in morphology which is mediated by continuous and rapid fusion and fission (7). And, mitophagy coupled with this structural dynamics functions in removing the depolarized parts of mitochondria. Mitochondria dynamics appears to be implicated in many cellular processes including proliferation, apoptosis (50), and senescence (51). For example, fission activity declines during the course of cellular senescence (10), and conversely, blocking mitochondrial fission leads to senescence (11). Mitochondria dynamics is also implicated in cardiac and neuronal development (52, 50) and in a variety of neurodegenerative diseases and metabolic disorders (reviewed in 50). Our results suggest a possibility that these physiological and pathological processes may be affected by the status of [NAD^+]/[NADH] ratio through its effect on the mitochondria dynamics. Furthermore, certain beneficial effect of calorie restriction may be driven by this metabolic modulator. In fact, calorie restriction is associated with an increase in mitochondrial turnover (41). Our results support the previous notion by other reports that, under conditions of calorie restriction, the maintenance of a high [NAD^+]/[NADH] ratio elevates the rate of mitochondrial turnover through the activation of SIRT1. It would be interesting to see whether the beneficial effects of calorie restriction and SIRT1 activation on various age-related disorders are accompanied by a change in mitochondrial content and morphology.

Finally, fragmented punctiform mitochondria have been found in cells undergoing apoptosis (53), while long filamentous form appears to be
associated with better energy metabolism (54, 55). And, failure in mitochondrial fusion has been shown to cause accumulation of defective mitochondrial DNA (56). The continued presence of the fragmental mitochondria during the long term culture in the presence of NAM did not cause an increase of ROS or shorten cellular lifespan (24). Therefore, such transformation of mitochondria by NAM treatment does not seem to be associated with apoptosis or functional deterioration of mitochondria. Still, for a practical application of NAM, it may be important to further examine the functional significance of this fragmental transformation and its maintenance in energy metabolism.

REFERENCES

[NAD\(^+\)]/[NADH] ratio modulates mitochondria content

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CONFLICT OF INTEREST
The authors declare no conflict of interest.

ABBREVIATIONS
NAM, nicotinamide; MMP, mitochondrial membrane potential; ROS, reactive oxygen species; ETC, electron transport chain; NAMPT, nicotinamide phosphoribosyltransferase; NAO, nonyl acridine orange; MTR, MitoTrack Red; CCCP, carbonyl cyanide 3-chlorophenylhydrazone; Asn, asparagine

FIGURE LEGENDS

FIGURE 1. The importance of NAM conversion to NAD\(^+\) for the decrease of mitochondria content. (A) Time course change in cellular mitochondria content. Cells cultured in the medium supplemented with 5 mM NAM (replaced in every 3 or 4 days) were collected and stained with NAO or MTR and analyzed in flow cytometry. The mean fluorescence of three biological
repeats of the NAM-treated cells was divided by that of the mock-treated cells, and the relative values were plotted. (B) Total [NAD⁺] was determined in the cells collected at 3, 6, 12, 24, and 48 hr after the addition of 5 or 20 mM NAM, and the values relative to the concentration in the mock-treated cells were plotted. (C) and (D) Cells were cultured in the presence (grey bars) or absence (black bars) of 5mM NAM (or 1 mM NMN) for 3 days. For FK866 treatment, 10 nM FK866 was added at the same time of NAM treatment. For siNAMPT expression, cells were first transfected with the siRNA two days prior to NAM addition. Cells were collected for analysis of [NAD⁺] (C) or stained with MTG for quantification of mitochondria content. Nonspecific RNA (siNeg) was used as a transfection control (D). The mean values from triplicate experiments were used to make the plots. (*P <0.05, **P <0.01 (compared to the mock-treated cells (without NAM)), ##P <0.01 (compared to the mock-treated cells (with NAM)), by ANOVA test (Dunnett’s test)).

**FIGURE 2.** NAM-mediated change in [NAD⁺]/[NADH] ratio and lactate production, and the effects of lactate and asparagine on the mitochondria content. (A) Cells were cultured in the presence of 5 mM NAM, and at the indicated time points, cultured medium was collected and applied to lactate/pyruvate oxidase assay to measure the ratio of free [NAD⁺]/[NADH] or to quantify lactate production per cell per hour. (B) and (C) Cells cultured in the presence of 20 mM L-lactate (5 mM or 10 mM in (C)), and further incubated for 3 more days prior to collection for the analysis for [NAD⁺]/[NADH] ratio (B) or for staining with MTG to quantify mitochondria content (C). The mean values from triplicate experiments were used to make the plots. (P <0.05 for all the comparisons to the mock-treated cells (-).) (D) and (E) The effects of NAD⁺ and asparagine on the mitochondria content. Fibroblasts cultured in the presence of 5 mM NAD⁺ (—●—) or 10 mM Asn (---○---) for 1, 3, or 7 days were collected, stained with MTG and JC-1 and applied to flow cytometry for quantification of mitochondria content (D) and MMP (E), respectively. The mean values from triplicate experiments were used to make the plots. (*P <0.05, **P <0.01 (compared to the day 0 control) by ANOVA test (Dunnett’s test)).

**FIGURE 3.** Increase in LC3 punctae and mitochondria fragmentation in the cells treated with NAD⁺ or Asn. (A) Cells cultured on a cover slip were either mock treated (-) or incubated in the presence of 5 mM NAM, 5 mM NAD⁺, 10 mM Asn, or 10 μM resveratrol for 2 days, and immunostained with antibody against LC3B protein (green) and counter-stained with Hoechst33258 for nuclear DNA (blue). Representative confocal-microscopic image of a cell is presented. Number of the prominent LC3 punctae (bigger than 0.5 μm in diameter) in a cell was counted using Image J program (http://rsbweb.nih.gov/nih-image/) and the mean numbers are presented in the table. (B) Cells cultured in the absence (-) or the presence of NAM, NAD⁺, or Asn for 2 days as above were stained with MTR, fixed, and visualized in confocal microscopy. (Magnifications; top row: x 252, the bottom row, x 1000)

**FIGURE 4.** SIRT1 activity and the mitochondria content in NAM-treated cells. (A & B) Cells incubated with 5 mM NAM for indicated time periods were applied to Western blotting for acetylated or total H3, or SIRT1 protein (A) or acetylated or total p53, or Erk protein. The protein bands were quantified by densitometry, and the values relative to those in the control cells (0 hr) were averaged (from 3 different blots) and plotted. (C) *In vivo* SIRT1 activity measured using Fluor-de-Lys assay kit. Each bar represents the mean ± SEM of 6 independent experiments. *P is < 0.02 compared to the 0 hr control (-). (D) Cells cultured in the presence of 5 or 20 mM NAM were collected at the indicated time points, stained with NAO, and applied to flow cytometry to determine mitochondria content. The quantities relative to those of the 0 hr control from three biological repeats were averaged and plotted. (*, #P <0.05 compared to the day 0 control) by ANOVA test (Dunnett’s test)).

**FIGURE 5.** The mitochondria content in the cells treated with fisetin or SRT1720. (A & B) Cells cultured for 1 or 2 days in the medium supplemented either with 10, 50, 100, or 250 μM fisetin (A) or 80, 160, 800 nM SRT1720 (B) were fixed and stained with MTG, and applied to
flow cytometric quantification of mitochondria. In (C), the mitochondria contents in the cells treated with either 10 μM fisetin or 160 nM SRT1720 for 1, 3, or 7 days were measured. The fluorescence values relative to those in the 0 day control cells were averaged from two biological repeats and plotted. (All the points are of $P < 0.01$ compared to the 0 day control cells (-) by ANOVA test.)

**FIGURE 6.** Mitochondria structure in the cells treated with fisetin or SRT1720. (A) Cells cultured in DMEM (a and d), DMEM containing 10 μM fisetin (b and e) or 160 nM SRT1720 (c and f) for 1 day were stained with MTG, fixed, and microphotographed through confocal microscopy. Cells in a, b, c were transfected with siNegative RNA, and cells in d, e, f were transfected with siRNA to SIRT1 two days prior to the chemical treatment. (Magnifications; x 1000). In (B), the status of SIRT1 protein in the cells transfected with siRNA to SIRT1 and mock treated (c) or treated with fisetin (f) or SRT1720 (s).

**FIGURE 7.** Effect of fisetin or SRT1720 treatment on MMP. Cells cultured in the presence of 10, 50, 100, 250 μM fisetin (A) or 80, 160, 800 nM SRT1720 (B) for 1 or 2 days were collected, stained with JC-1 and applied to flow cytometry for quantification of MMP. Values were normalized by those from the 0 day control cells, and an average from three different biological repeats was plotted. In addition to the activators, the values from the cells treated with carbonyl cyanide 3- chlorophenylhydrazone (CCCP), which induces mitochondria depolarization, were added as a control showing a very low level of MMP. (In (A), all the values are $P < 0.05$ compared to the 0 day control cells by ANOVA test.)
FIGURE 1.

(A) Relative fluorescence intensity (% control) over treatment days.

(B) Fold change over treatment hours.

(C) NAD content (% control) over treatment conditions.

(D) Mitochondria content (% control) over treatment conditions.
FIGURE 2.

(A) Free NAD+/NADH ratio modulates lactate production (µmol/mg cell protein).

(B) NAD+/NADH ratio changes in response to lactate treatment.

(C) Relative mitochondrial content in control and lactate-treated cells.

(D) Fold change in mitochondrial content over treatment days.

(E) Fold change in mitochondrial content over treatment days.
[NAD\(^+\)]/[NADH] ratio modulates mitochondria content

FIGURE 3.

(A) 

(B) 

<table>
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<tr>
<th></th>
<th>Control</th>
<th>NAM</th>
<th>NAD(^+)</th>
<th>Asn</th>
<th>Resveratrol</th>
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<td># punctae/cell</td>
<td>23.8</td>
<td>33.9</td>
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FIGURE 4.
FIGURE 5.

(A) [Graph showing fold change vs treatment days for different concentrations of a substance.]

(B) [Graph showing fold change vs treatment days for different concentrations of a substance.]

(C) [Graph showing fold change vs treatment days for different substances.]
[NAD$^+/NADH$ ratio modulates mitochondria content

FIGURE 6.

(A)

siNEG

siSIRT1

(B)

siNEG

siSIRT1
[NAD⁺]/[NADH] ratio modulates mitochondria content

FIGURE 7.
Supplemental figure 1.
Supplemental figure 2.
Supplemental figure 1. Status of \([\text{NAD}^+]\) during the 21 days of NAM treatment. Cells cultured in the medium supplemented with 5 mM NAM (replaced in every 3 days.) were collected at 4, 12, or 21 days and total \([\text{NAD}^+]\) was determined, and the values relative to the concentration in the mock-treated cells were plotted.

Supplemental figure 2. Cellular response in mitochondrial content upon treatment of a wide range of \(\text{NAD}^+\). Fibroblasts were treated with \(\text{NAD}^+\) ranging from 1, 10, 100, 500 μM, or 1 or 5 mM for 1, 2, or 3 days. The cells were stained with NAO and applied to flow cytometry.
Nicotinamide-induced mitophagy: An event mediated by high NAD+/NADH ratio and SIRT1 activation

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