

NAD⁺ Intermediates: The Biology and Therapeutic Potential of NMN and NR

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Research on the biology of NAD⁺ has been gaining momentum, providing many critical insights into the pathogenesis of age-associated functional decline and diseases. In particular, two key NAD⁺ intermediates, nicotinamide riboside (NR) and nicotinamide mononucleotide (NMN), have been extensively studied over the past several years. Supplementing these NAD⁺ intermediates has shown preventive and therapeutic effects, ameliorating age-associated pathophysiologies and disease conditions. Although the pharmacokinetics and metabolic fates of NMN and NR are still under intensive investigation, these NAD⁺ intermediates can exhibit distinct behavior, and their fates appear to depend on the tissue distribution and expression levels of NAD⁺ biosynthetic enzymes, nucleotidases, and presumptive transporters for each. A comprehensive concept that connects NAD⁺ metabolism to the control of aging and longevity in mammals has been proposed, and the stage is now set to test whether these exciting preclinical results can be translated to improve human health.

Introduction

Recent years have witnessed a resurgence of interest in nicotinamide adenine dinucleotide (NAD⁺) biology. This has been driven in part by the discoveries that two intermediates of NAD⁺ biosynthesis, nicotinamide riboside (NR) and nicotinamide mononucleotide (NMN), effectively increase NAD⁺ concentration in a variety of tissues, in many cases with beneficial or therapeutic effects. The purpose of this review is to summarize recent progress in understanding NAD⁺ biology, with a focus on the effects of these two molecules.

NAD⁺ was originally discovered by Harden and Young in 1906 as a factor that could enhance the rate of fermentation in yeast extracts (Harden and Young, 1906). Over subsequent years, NAD⁺ was determined to be a nucleotide by von Euler-Chelpin and to play a role in redox reactions by Warburg (Berger et al., 2004). Elvehjem demonstrated that nicotinamide is able to prevent canine pellagra (a disease of NAD⁺ deficiency) by contributing to NAD⁺ biosynthesis (Elvehjem, 1949), leading to the eventual designation of the classical NAD⁺ precursors nicotinamide and nicotinic acid as “vitamin B₃.” Since the recognition of NAD⁺ (and the related NADP⁺) as key players in cellular metabolism, there have been two separate occasions when the number of publications including the term “nicotinamide adenine dinucleotide” doubled within a relatively short period. The first occurred just after Chambon, Weill, and Mandel published their historical 1963 paper reporting that NMN activated a new DNA-dependent polyadenylic acid synthesizing nuclear enzyme (Chambon et al., 1963). Their

findings led to a series of phenomenal discoveries of poly-ADP-ribose and poly-ADP-ribose polymerases (PARPs). During this period, Hayaishi and his colleagues began to reveal the NAD⁺ biosynthetic pathway from tryptophan through another NAD⁺ intermediate, nicotinic acid mononucleotide, in mammals (Ikeda et al., 1965). Gholson also proposed the concept of an active turnover cycle of NAD⁺ in 1966, predicting “an important, but as yet unknown, function” of NAD⁺ in cellular metabolism (Gholson, 1966). In 1976, Rechsteiner and his colleagues provided convincing evidence for rapid NAD⁺ turnover, reaffirming that “it seems likely that in eukaryotic cells, NAD has some other major function in addition to the classical cytoplasmic role in oxidation and reduction” (Rechsteiner et al., 1976). This was an exciting era in NAD⁺ biology research, over a decade full of critical discoveries. The elucidation of this “other major function” of NAD⁺ had to wait until 1989 and then 2000. In 1989, Lee and his colleagues discovered a novel NAD⁺ metabolite, cyclic ADP-ribose, after incubation of NAD⁺ with sea urchin egg extracts (Lee et al., 1989). The responsible enzyme, ADP-ribosyl cyclase, was found first in *Aplysia* in 1991 (Lee and Aarhus, 1991) and then in mammals as CD38 (States et al., 1992). The second doubling of papers on NAD⁺ occurred between 2000 and the present. In 2000, Guarente and Imai made the critical discovery that yeast SIR2 (silent information regulator 2) and a mouse ortholog SIRT1 possess NAD⁺-dependent protein deacetylase activity, fueling new interests in NAD⁺ biology (Imai et al., 2000). Indeed, human nicotinamide/nicotinic acid mononucleotide



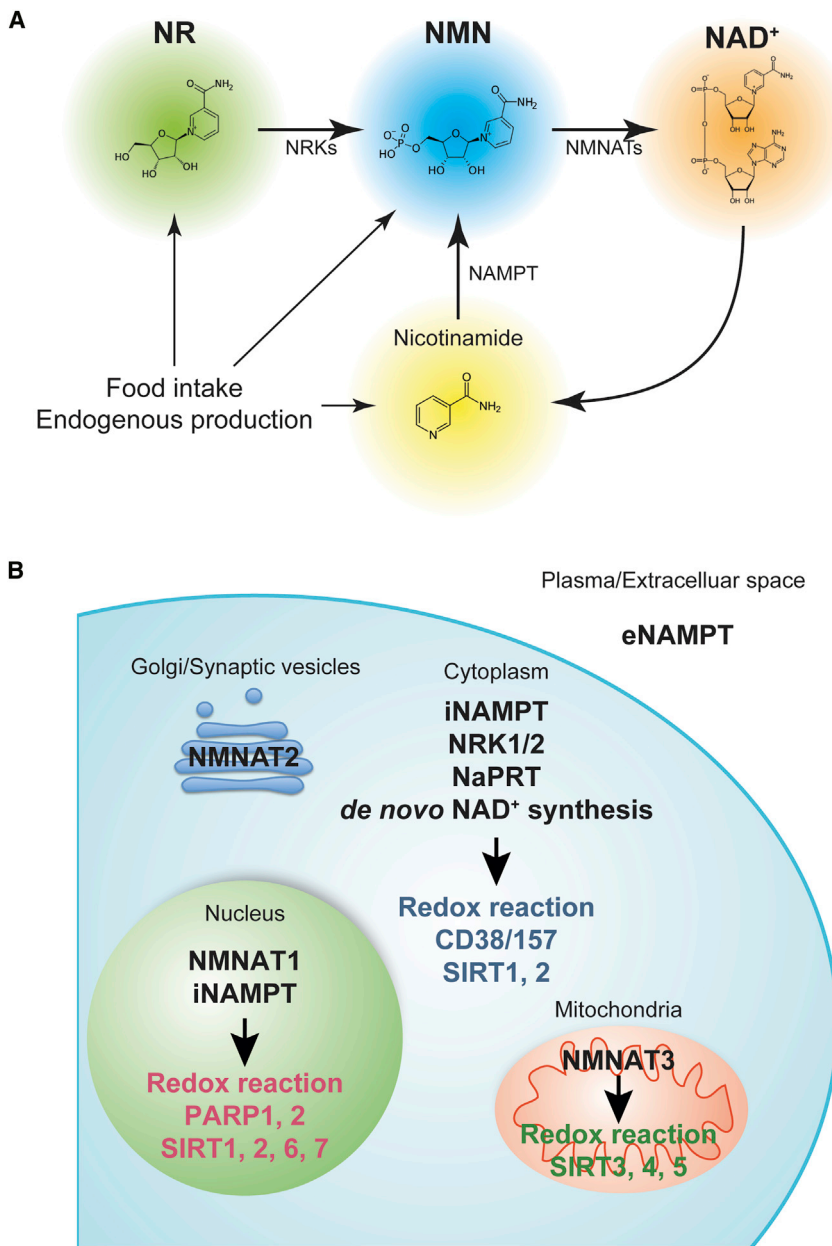


Figure 1. NAD⁺ Intermediates, Biosynthetic Enzymes, and Downstream Mediators

(A) Nicotinamide mononucleotide (NMN) and nicotinamide riboside (NR) are natural compounds that effectively enhance NAD⁺ biosynthesis and have health benefits. In mammals, NMN is synthesized from nicotinamide by the rate-limiting enzyme, nicotinamide phosphoribosyltransferase (NAMPT). NMN is also synthesized from NR via an NR kinase (NRK)-mediated phosphorylation reaction. NMN is then converted into NAD⁺ by NMN adenylyltransferases (NMNATs).

(B) NAMPT has two distinct forms: intracellular and extracellular NAMPT (iNAMPT and eNAMPT, respectively). iNAMPT is present in the cytoplasm and nucleus. eNAMPT is secreted by multiple cell types, including adipocytes and immune cells. eNAMPT is fully active as an NAD⁺ biosynthetic enzyme capable of catalyzing the generation of NMN, whereas it has also been reported to have a role as an inflammatory cytokine that is independent of catalytic activity. Nicotinic acid phosphoribosyltransferase (NaPRT) catalyzes the first step in the Preiss-Handler pathway, which converts nicotinic acid to NAD⁺, and *de novo* synthesis from tryptophan occurs through a complex series of steps ending with conversion of nicotinic acid adenine dinucleotide (NaAD⁺) to NAD⁺ by glutamine-dependent NAD⁺ synthase. NRK1 and NRK2 convert NR to NMN, which can then be acted on by NMNAT1-3. NMNAT1 is exclusively nuclear. NMNAT2 has been reported to localize to the Golgi complex and synaptic vesicles and is predominantly expressed in neurons. NMNAT3 is the only NAD⁺ biosynthetic enzyme shown to be localized in the mitochondrial matrix, except in erythrocytes where it is expressed in the cytosol. NAD⁺ is consumed by NAD⁺-dependent enzymes, such as PARPs, sirtuins, CD38/157, and other NAD⁺ glycohydrolases, and reduction to NADH.

Reflecting such rapid progress in the field, there have already been a number of excellent review articles about general NAD⁺ biology and its relevance to disease (Belenky et al., 2007; Canto et al., 2015; Chini et al., 2016; Fang et al., 2017; Imai and Guarente, 2014; Katsyuba and Auwerx, 2017; Verdin, 2015; Yang and Sauve, 2016). Therefore, in this review article, we particularly focus on the biology of two key NAD⁺ intermediates, NMN and NR, in

an *in vivo* context. As mentioned above, these two NAD⁺ intermediates have long been known and are found in a wide variety of our daily foods, such as vegetables, fruits, and meat (Figure 1A) (Mills et al., 2016). Interestingly, recent studies have documented both at micromolar concentrations in human and cow milk (Bieganowski and Brenner, 2004; Ummano et al., 2017). Nonetheless, it is relatively recent that the importance and the potential of these NAD⁺ intermediates have been extensively investigated in various rodent models. In addition, accumulating evidence suggests that NAD⁺ levels decline with age at a systemic level in diverse organisms, including rodents and humans, contributing to the development of many age-associated pathophysiologicals (Canto et al., 2015; Imai and Guarente, 2014; Verdin, 2015). Therefore, there has been an increasing

adenylyltransferase (NMNAT), an important NAD⁺ biosynthetic enzyme whose activity was originally reported in 1952, was finally isolated and fully characterized in 2001 (Schweiger et al., 2001). Following these discoveries, nicotinamide phosphoribosyltransferase (NAMPT), the rate-limiting enzyme that initiates NAD⁺ biosynthesis from nicotinamide in mammals, was also isolated and characterized (Revollo et al., 2004; Rongvaux et al., 2002). In addition, nicotinamide riboside, another key NAD⁺ intermediate, was shown to be incorporated into NAD⁺ via nicotinamide riboside kinases (NRKs) by Bieganowski and Brenner in 2004 (Bieganowski and Brenner, 2004). In this early 21st century, almost half a century after the first wave of enthusiasm on NAD⁺ biology, we are now in another exciting era of this classical but newly revisited field of science.

interest in using NAD⁺ intermediates as effective interventions to ameliorate or even prevent certain aspects of age-associated functional decline. In this review article, we first summarize the current knowledge on these two NAD⁺ intermediates including their pharmacokinetic and pharmacological features. We also discuss the physiological importance of these NAD⁺ intermediates in mammalian systems and their applications for aging and disease.

Nicotinamide Mononucleotide

NMN is synthesized from nicotinamide, a form of water-soluble vitamin B₃, and 5'-phosphoribosyl-1-pyrophosphate (PRPP), by NAMPT, the rate-limiting NAD⁺ biosynthetic enzyme in mammals (Figure 1A) (Imai and Yoshino, 2013; Revollo et al., 2004; Wang et al., 2006). NMN is also synthesized from NR via an NRK-mediated phosphorylation reaction, which is discussed further in the next section. Conversion of NMN into NAD⁺ is catalyzed by NMNATs (Figure 1B). Evidence accumulating from studies conducted in rodents has demonstrated that systemic NMN administration effectively enhances NAD⁺ biosynthesis in various peripheral tissues, including pancreas (Yoshino et al., 2011), liver (Peek et al., 2013; Yoshino et al., 2011), adipose tissue (Stromsdorfer et al., 2016; Yoshino et al., 2011), heart (Karamanlidis et al., 2013; Martin et al., 2017; North et al., 2014; Yamamoto et al., 2014), skeletal muscle (Gomes et al., 2013), kidney (Guan et al., 2017), testis (North et al., 2014), eyes (Lin et al., 2016), and blood vessel (aorta) (de Picciotto et al., 2016), under normal and pathophysiological conditions. Although it remains unclear whether NMN can cross the blood-brain barrier (BBB), intraperitoneal NMN administration rapidly (within 15 min) increases NAD⁺ levels in brain regions such as hippocampus and hypothalamus (Stein and Imai, 2014; Yoon et al., 2015), suggesting that NMN could pass through the BBB and contribute to NAD⁺ biosynthesis in the brain. Finally, it was recently reported that long-term (1-year) oral administration of NMN (up to 300 mg/kg) is safe and well tolerated and does not cause any obvious deleterious or toxic effects in normal wild-type C57BL/6 mice (Mills et al., 2016).

These results suggest that NMN could offer broad applications and therapeutic potential. Indeed, there is a growing body of evidence showing that NMN has beneficial effects on a diverse array of key physiological functions and therapeutic implications in various disease models (Table 1). Of particular note are the remarkable metabolic benefits from NMN administration. Pioneering studies have suggested that pancreatic β cells are very sensitive to systemic NAD⁺ decline and NMN administration. A single bolus injection of NMN (500 mg/kg) enhances glucose-stimulated insulin secretion and thus improves glucose tolerance in age- and diet-induced diabetic mice (Caton et al., 2011; Yoshino et al., 2011), *Nampt* heterozygous knockout mice (Revollo et al., 2007), and aged wild-type and β cell-specific *Sirt1*-overexpressing (BESTO) mice (Mills et al., 2016; Ramsey et al., 2008; Yoshino et al., 2011). NMN enhances not only insulin secretion but also insulin action: NMN treatment ameliorates high-fat-diet-induced hepatic insulin resistance by restoring NAD⁺ biosynthesis, SIRT1 activity, and gene expression related to inflammation, oxidative stress, and circadian rhythms (Yoshino et al., 2011). Oral NMN administration also increases adipose tissue NAD⁺ biosynthesis and SIRT1 activity and normalizes

severe hypoadiponection and multi-organ insulin resistance in adipocyte-specific *Nampt* knockout (ANKO) mice (Stromsdorfer et al., 2016). In addition, long-term NMN administration suppresses age-associated adipose tissue inflammation and improves whole-body insulin sensitivity independently from effects on body weight in regular chow-fed wild-type C57BL/6 mice (Mills et al., 2016). Given that adipose tissue NAD⁺ biosynthesis is severely impaired in obese and aged mice (Camacho-Pereira et al., 2016; Yoshino et al., 2011), these findings suggest that adipose tissue NAD⁺ could be a good therapeutic target for insulin resistance, which is an important risk factor of type 2 diabetes and cardiovascular disease (DeFronzo and Ferrannini, 1991; Reaven, 1988; Yamaguchi and Yoshino, 2017). NMN has also been reported to improve mitochondrial function in various metabolic organs, including skeletal muscle (Gomes et al., 2013; Mills et al., 2016), liver (Peek et al., 2013; Uddin et al., 2016), heart (Lee et al., 2016), and eyes (Lin et al., 2016). NMN-treated mice have increased mitochondrial oxidative phosphorylation in skeletal muscle (Gomes et al., 2013; Mills et al., 2016), likely contributing to weight loss by increasing whole-body energy expenditure (Mills et al., 2016). NMN increases SIRT3 activity and hepatic mitochondrial lipid oxidation in circadian mutant mice (Peek et al., 2013) and hepatic citrate synthase activity in high-fat-diet-fed obese mice (Uddin et al., 2016). An important aspect of the above findings is that aged animals appear to be more responsive to NMN treatment, compared with young animals. NMN improves high-fat-diet-induced glucose intolerance and dyslipidemia (Yoshino et al., 2011), skeletal muscle mitochondrial oxidative metabolism (Gomes et al., 2013), and endothelial function (de Picciotto et al., 2016) better in aged mice than young mice. It is conceivable that age-associated decline in NAD⁺ availability (Braidly et al., 2011; Camacho-Pereira et al., 2016; Frederick et al., 2016; Gomes et al., 2013; Masudi et al., 2012; Stein and Imai, 2014; Yoshino et al., 2011) could sensitize aged mice to NAD⁺ replenishment by NMN administration. Therefore, it will be of great interest to carefully compare the metabolic effects of NMN in aged mice with those in young mice.

Recent studies have suggested that NMN improves numerous neuronal functions in the brain. NMN administration improves cognition and memory in mouse and rat models of Alzheimer's disease (Long et al., 2015; Wang et al., 2016; Yao et al., 2017). NMN protects neurons from cell death after ischemia (Park et al., 2016) or intracerebral hemorrhage (Wei et al., 2017a) and ameliorates the loss of BBB integrity and tissue plasminogen-activator-induced hemorrhagic transformation in brain ischemia (Wei et al., 2017b). NMN also restores severe retinal degeneration, through mitochondrial sirtuins SIRT3 and SIRT5, in rod or cone photoreceptor-specific *Nampt* knockout mice (Lin et al., 2016). Furthermore, long-term NMN administration prevents age-associated loss of the neural stem/progenitor pool in the dentate gyrus in wild-type C57BL/6 mice (Stein and Imai, 2014). Interestingly, data obtained from dose-response studies have indicated that lower doses of NMN may be more beneficial, compared with larger doses for some neuronal outcomes. For example, 100 mg/kg of NMN improves physical activity better than 300 mg/kg (Mills et al., 2016); 62.5 mg/kg of NMN more effectively treats ischemia-induced brain damage than higher doses (125–500 mg/kg) (Park et al., 2016). Therefore, it is possible that local NMN accumulation, due to administration of

Table 1. Effects of NMN Administration *In Vivo*

Model	Gender	Dose	Duration	Outcome	Reference
<i>Nampt</i> heterozygous KO mice	female	500 mg/kg (IP)	single	improved glucose tolerance and insulin secretion	Revollo et al., 2007
β cell-specific <i>Sirt1</i> overexpressing (BESTO) mice	female	500 mg/kg (IP)	single	improved glucose tolerance and insulin secretion	Ramsey et al., 2008
High-fat diet (HFD)-induced obese mice	female male	500 mg/kg (IP)	7–10 days	improved glucose tolerance and insulin sensitivity (female); improved glucose tolerance and insulin secretion (male)	Yoshino et al., 2011
Age-induced diabetic mice	female male	500 mg/kg (IP)	11 days single	improved insulin sensitivity and plasma lipids profile (HFD-induced diabetic female mice); improved glucose tolerance and insulin secretion (naturally occurring diabetic male mice)	Yoshino et al., 2011
Fructose-rich-diet-fed mice	male	500 mg/kg (IP)	single	improved insulin secretion and inhibited inflammation	Caton et al., 2011
C57BL/6J	unspecified	500 mg/kg (IP)	7 days	enhanced skeletal muscle mitochondrial oxidative metabolism in aged mice	Gomes et al., 2013
<i>Bmal1</i> KO mice	unspecified	250 mg/kg (IP), 500 mg/kg (IP)	single 10 days	increased hepatic mitochondrial respiration	Peek et al., 2013
Cardiac-specific <i>Ndufs4</i> KO (cKO) mice	unspecified	500 mg/kg (IP)	3 days	decreased the mitochondrial protein acetylation and improved the permeability transition in mitochondria (mPTP) sensitivity	Karamanlidis et al., 2013
Adenovirus vector overexpressing miR-34a injected mice	male	500 mg/kg (IP)	10 days	improved glucose tolerance and increased expression of fatty acid β -oxidation genes	Choi et al., 2013
C57BL/6N mice	male	100, 300 mg/kg (drinking water)	12 months	maintained the neural stem/progenitor cells pool	Stein and Imai, 2014
Ischemia/reperfusion heart injury in C57BL/6J mice	unspecified	500 mg/kg (IP)	once or 4 times	reduced the infarct area after ischemia/reperfusion injury	Yamamoto et al., 2014
Alzheimer's disease-relevant (AD-Tg) mice	female and male	100 mg/kg (SC)	28 days (every other day)	decreased amyloid precursor protein (APP) levels and increase mitochondrial function	Long et al., 2015
Adipocyte-specific <i>Nampt</i> KO (ANKO) mice	female	500 mg/kg (IP)	single	restored physical activity	Yoon et al., 2015
Cerebral ischemia in C57BL/6 mice	male	31.25–500 mg/kg (IP)	single	reduced cell death of neuron and improved neurological outcome (noted 62.5 mg/kg induced the best outcome)	Park et al., 2016
C57BL/6 mice	male	300 mg/kg (drinking water)	8 weeks	improved carotid artery endothelium-dependent dilation	de Picciotto et al., 2016
Rod-specific <i>Nampt</i> KO mice, light-induced retinal dysfunction (129S1/SvImJ)	unspecified	150 mg/kg (IP) 300 mg/kg (IP)	4 weeks 10 days	improved retinal degeneration and protected retina from light-induced injury	Lin et al., 2016

(Continued on next page)

Table 1. Continued

Model	Gender	Dose	Duration	Outcome	Reference
Adipocyte-specific <i>Nampt</i> KO (ANKO) mice	female	500 mg/kg, (drinking water)	6-8 weeks	Improved multi-organ insulin sensitivity, increased adiponectin production, and decreased free fatty acids production	Stromsdorfer et al., 2016
High-fat-diet-induced obese mice	female	500 mg/kg (IP)	~17 days	improved glucose tolerance, liver citrate synthase activity, and triglyceride accumulation	Uddin et al., 2016
Transverse aortic constriction (TAC)-stressed mice, cKO mice	male	500 mg/kg (IP)	33 days (every 3 days)	improved mitochondrial function and protected mice from heart failure	Lee et al., 2016
C57BL/6NTac wild-type mice and <i>Nrk1</i> KO, or <i>Nrk1/2</i> double KO mice	male	50, 500 mg/kg (IP)	single	failed to increase NAD ⁺ concentrations in kidney and brown adipose tissue of KO mice	Ratajczak et al., 2016
C57BL/6N mice	male	100, 300 mg/kg (drinking water)	12 months	inhibited age-induced weight gain, improved insulin sensitivity and plasma lipids, and increased physical activity, energy expenditure, and muscle mitochondrial function	Mills et al., 2016
Intracerebroventricular infusion of A β ₁₋₄₂ oligomer in Wister rats (Alzheimer's disease model)	male	500 mg/kg (IP)	10 days	improved learning and memory	Wang et al., 2016
APP ^{swe} /PS1 ^{dE9} transgenic mice (Alzheimer's disease model) and wild-type mice	unspecified	100 mg/kg (SC)	28 days (every other day)	improved cognitive function	Yao et al., 2017
Collagen-induced intracerebral hemorrhage (ciCH) in CD1 mice	male	300 mg/kg (IV) 300 mg/kg (IV and IP)	single 7 days	reduced brain edema and cell death (one bolus) and promoted the recovery of body weight and neurological function (7 days)	Wei et al., 2017a
Old C57BL/6J mice and young irradiated C57BL/6J mice	unspecified	500 mg/kg (IP) 2000 mg/kg (PO)	7 days 8 days	reduced DNA damage and protected against irradiation-induced alterations in white blood cell counts, lymphocytes, and hemoglobin	Li et al., 2017
Ischemia reperfusion or cisplatin-induced acute kidney injury in <i>Sirt1</i> heterozygous KO (+/-) mice, C57BL/6 mice, and 129S2/Sv mice	unspecified	500 mg/kg (IP)	4 days	Protected renal function from cisplatin-induced injury in wild-type mice but not in <i>Sirt1</i> (+/-) mice	Guan et al., 2017
Cardiac-specific <i>Fxn</i> KO mice (FXN-KO) (Friedreich's ataxia cardiomyopathy model) and <i>Sirt3</i> -KO/FKO-KO mice	male	500 mg/kg (IP)	4-5 weeks (twice a week)	Improved cardiac functions and reduced energy waste and improves energy utilization in FXN-KO mice but not in <i>Sirt3</i> -KO/FKO-KO mice	Martin et al., 2017
Tissue plasminogen-activator-treated cerebral ischemia CD1 mice	male	300 mg/kg (IP)	single	improved mortality, brain infarction, edema, apoptosis, hemorrhage, and BBB integrity	Wei et al., 2017b

IP, intraperitoneal; IV, intravenous; KO, knockout; PO, per oral; SC, subcutaneous.

high doses of NMN, has negative impacts in a brain region- or function-specific manner. Indeed, data obtained from *in vitro* experiments suggest that NMN promotes Wallerian degeneration, although this notion was recently challenged (Di Stefano et al., 2015; Gerds et al., 2016). Future studies are awaited to fully elucidate the pharmacokinetics of NMN in the brain and determine the optimal dosing regimen of NMN in each key neuronal function and brain region.

In addition to its anti-diabetic and neurological effects, NMN administration inhibits acute renal injury in a SIRT1-dependent manner (Guan et al., 2017), heart failure (Karamanlidis et al., 2013; Lee et al., 2016; Martin et al., 2017; Yamamoto et al., 2014), and radiation-induced DNA damage (Li et al., 2017), further demonstrating pleiotropic benefits of NMN. Given that NMN is effective in aged mice and suppresses age-associated physiological decline (Mills et al., 2016), it will be of great importance to evaluate the effects of NMN administration on other age-associated diseases such as cancer and sarcopenia, and aging and lifespan *per se* in rodents.

Nicotinamide Riboside

The direct contribution of NR to NAD⁺ metabolism was first recognized by Bieganowski and Brenner in 2004 (Bieganowski and Brenner, 2004). This report described a class of enzymes known as NRKs that can convert NR directly to NMN, bypassing the need for NAMPT in the salvage pathway (Figure 1). Since the reaction catalyzed by NAMPT is rate limiting, requires the use of energetically costly PRPP, and is subject to feedback inhibition by NAD⁺ (Dietrich et al., 1968), NR also offers an interesting opportunity to boost NAD⁺ levels beyond what is achievable through conventional B vitamin metabolism. There is a substantial body of evidence supporting metabolic benefits for NR, as well as efficacy in a variety of disease models and a modest lifespan increase in aged mice (Table 2).

The first major study of NR in mice examined metabolic health after high-fat-diet feeding. Mice receiving NR at a dose of 400 mg/kg/day in the diet were protected from weight gain, were more insulin sensitive, and had increased mitochondrial content in skeletal muscle and brown adipose tissue compared with untreated controls (Canto et al., 2012). Accordingly, NR was found to confer increased endurance and improved cold tolerance in the high-fat-diet-fed mice. Whether NR has significant benefits in lean, healthy muscle is less clear, as only a nonsignificant trend toward increased endurance was observed in regular chow-fed mice (Canto et al., 2012), and a subsequent study in rats showed a trend toward decreased endurance in a forced swim test (also not significant) (Kourtzidis et al., 2016).

NR has been shown to have therapeutic effects in a number of muscle disorders. Despite not correcting the underlying genetic defects, NR improved mitochondrial abundance and function in two different mitochondrial myopathies (Cerutti et al., 2014; Khan et al., 2014). NR also increased survival time, induced autophagy, and decreased the mitochondrial unfolded protein response in a model of heart failure induced by cardiac-specific deletion of the transferrin receptor (Xu et al., 2015). Mice lacking *Nampt* in skeletal muscle exhibit a progressive wasting syndrome that is reversed by providing NR in the drinking water, with endurance restored in as little as 1 week of treatment (Fredrick et al., 2016). These mice histologically and transcriptionally

resemble the *mdx* model of Duchenne's muscular dystrophy, which has also been reported to display decreased NAD⁺ content in skeletal muscle (Chalkiadaki et al., 2014). Intriguingly, NR treatment has since been shown to improve stem cell function and partially ameliorate the muscle wasting phenotype in *mdx* mice, leading to hope for a rapidly translatable therapy for the human condition (Ryu et al., 2016; Zhang et al., 2016). Improvement in stem cell function appears to be a general phenomenon during NR treatment and has been suggested to underlie a small, but significant extension of lifespan in mice treated beginning from 2 years of age (Zhang et al., 2016).

NR improves liver health in a variety of contexts. It potently reduces fat accumulation through a mechanism that involves induction of the mitochondrial unfolded protein response (Gariani et al., 2016). It further reduces inflammation at least in part through decreased activity of the NLRP3 inflammasome and lessens the development of fibrosis (Lee et al., 2015). Moreover, NAD⁺ biosynthesis was found to be impaired in a mouse model of hepatocellular carcinoma, and both DNA damage and tumorigenesis were prevented when NR was provided in the diet to restore NAD⁺ (Tummala et al., 2014). In the regenerating liver (following partial hepatectomy), NR reduces lipid accumulation, promotes hepatocyte replication, increases hepatic ATP content, and leads to faster regain of liver weight (Mukherjee et al., 2017). Together, these observations suggest that enhancing NAD⁺ content by NR is a promising therapeutic strategy to improve liver health.

Like NMN, NR has further been reported to have a number of intriguing benefits in the nervous system. One of the first studies to examine the effects of NR *in vivo* revealed a striking improvement in the progression of Alzheimer's disease pathology in the Tg2576 model of the disease (Gong et al., 2013). NR has been shown to prevent noise-induced hearing loss and neurite retraction from hair cells in the inner ear through a SIRT3-dependent mechanism (Brown et al., 2014). NR also protects against diabetic and chemotherapy-induced neuropathy in mice (Hamity et al., 2017; Trammell et al., 2016b), raising hopes that it could have a role in the management of chronic pain. It has recently been recognized that PARP-mediated NAD⁺ depletion plays a major role in the pathogenesis of neurodegenerative disorders that involve DNA repair defects, including Cockayne syndrome, xeroderma pigmentosum, and ataxia telangiectasia. While NR is unable to correct the primary DNA repair deficiencies, it dramatically improves phenotypes in mouse models of each of these conditions, most remarkably more than tripling survival time in ataxic mice (Fang et al., 2014, 2016; Scheibye-Knudsen et al., 2014).

Limitations of Our Current Understanding and Possible Detrimental Effects of Enhancing NAD⁺ Biosynthesis

As outlined above, it is clear that both NMN and NR have beneficial effects in multiple conditions in rodents. Indeed, there are a number of pathophysiological conditions that show significant decreases in tissue NAD⁺ levels (Table 3). Although both compounds have been tested in some models, no side-by-side comparisons have been conducted between NMN and NR. Therefore, even though both compounds are capable of enhancing NAD⁺ biosynthesis, there might be certain interesting differences in their effects on these pathophysiological conditions. In addition, in the vast

Table 2. Effects of NR Administration In Vivo

Mouse Model	Gender	Dose	Duration	Outcome	Reference
Chow-fed or high-fat-diet-induced obese C57BL/6 mice	male	~400 mg/kg (diet)	12 weeks	decreased weight gain, improved insulin sensitivity	Canto et al., 2012
Tg2576 mice (Alzheimer's disease model, C57BL/6 background)	unspecified	~250 mg/kg (diet)	3 months	slowed cognitive decline	Gong et al., 2013
"Deletor" mice (Twinkle mutant causing mitochondrial myopathy, C57BL/6 background)	male	~400 mg/kg (diet)	4 months pre- or post-manifestation	induced mitochondrial biogenesis and stimulated mitochondrial unfolded protein response	Khan et al., 2014
<i>Sco2</i> knockout E129K knockin mice	unspecified	~400 mg/kg (diet)	4 weeks	improved endurance and mitochondrial respiratory capacity in muscle	Cerutti et al., 2014
Human unconventional RBP5 interactor knockin mice (inducible, liver-specific)	unspecified	~500 mg/kg (diet)	9–57 weeks	prevented DNA damage and tumor development in livers when given early, tumor regression when given late	Tummala et al., 2014
4- or 18-month-old C57BL/6 WT and <i>Csb^{mm}</i> mice	male	500 mg/kg (IP)	1 week	improved the function of mitochondria isolated from cerebellum	Scheibye-Knudsen et al., 2014
3-month-old C57BL/6 wild-type or <i>Xpa^{-/-}/Csa^{-/-}</i> (CX) mice	unspecified	500 mg/kg (SC)	14 days	lowered mitochondrial membrane potential and ROS (also noted that CX genotype is lethal if mice are weaned to hard chow and NR in the drinking water of mothers failed to rescue)	Fang et al., 2014
129/SvEv mice with cardiac-specific deletion of the transferrin receptor (causing heart failure)	unspecified	750 mg/kg (IP)	P5 death (up to 10 days)	increased survival time, decreased mitochondrial unfolded protein response, enhanced autophagy	Xu et al., 2015
KK/HIJ mice	male	100 mg/kg (osmotic pump)	7 days	improved glucose homeostasis, increased adiponectin, and lowered hepatic cholesterol	Lee et al., 2015
C57BL/6, BALB/c, CBA mice, C57BL/6 <i>Sirt3</i> KO	unspecified	1,000 mg/kg (IP)	5 days before and/or 14 days after noise injury (twice daily injection)	protection against transient or permanent noise-induced hearing loss in all WT strains, but not in <i>Sirt3</i> KO	Brown et al., 2014
C57BL/6 wild-type and liver-specific <i>Sirt1</i> KO fed high-fat high-sucrose diet, <i>ApoE</i> KO fed high-fat high-cholesterol diet	male	~400 mg/kg (diet)	2–18 weeks	prevented or reversed fatty liver and induced the mitochondrial unfolded protein response with diminished effects in <i>Sirt1</i> KO	Gariani et al., 2016
C57BL/6 fed chow, high-fat diet, or high-fat diet + streptozotocin	male	3,000 ppm in diet	8 weeks	improved glucose tolerance, reduced weight gain, reduced hepatic steatosis, protected against neuropathy	Trammell et al., 2016b
C57BL/6 young, aged, muscle stem cell-specific <i>Sirt1</i> KO, mdx on C57BL/10SnJ background	male	400 mg/kg (diet)	6–8 weeks or 2 years old; death	improved endurance, grip strength, and recovery from cardiotoxin-induced muscle injury in aged mice, induced the mitochondrial unfolded protein response and delayed senescence in stem cells, increased lifespan	Zhang et al., 2016

(Continued on next page)

Table 2. Continued

Mouse Model	Gender	Dose	Duration	Outcome	Reference
C57BL/6J mice fed HFD	male	200 mg/kg (diet)	4 weeks	reduced lipid accumulation and fibrosis in liver	Zhou et al., 2016
C57BL/6 wild-type or muscle-specific <i>Nampt</i> KO	male and female	~400 mg/kg (drinking water)	6 weeks	restored muscle mass, force generation, endurance, and mitochondrial respiratory capacity in <i>Nampt</i> KO	Frederick et al., 2016
C57BL/6 mice, healthy humans	male (mice), males and females (human)	185 mg/kg (gavage), 500 mg/kg (IP), 100 mg, 300 mg, 1,000 mg (PO)	Single, 6 days (mice), single, 7 days (human)	direct incorporation of intact NR into hepatic NAD ⁺ (mice), no detection of phosphorylated NAD ⁺ metabolites in plasma, increased NAD, NaAD, MeNAM, Me2PY, in PBMCs (human)	Trammell et al., 2016a
Sprague-Dawley rats	male and female	5,000 mg/kg (gavage), 750–5,000 mg/kg (gavage), 300–3,000 mg/kg (gavage)	Single, 14 days, 90 days	toxicity profile similar to nicotinamide, affecting liver, kidneys, ovaries, and testes with no adverse effects observed below 1,000 mg/kg/day	Conze et al., 2016
Wild-type and <i>mdx</i> C57BL/10SnJ, <i>mdx/utrophin</i> KO	male	400 mg/kg (diet)	8–13 weeks	improved muscle function and reduced heart pathology	Ryu et al., 2016
C57BL/6 wild-type or liver-specific <i>Nampt</i> KO mice	male	400 mg/kg (drinking water)	14 days prior to and 2 days after partial hepatectomy	improved liver regeneration, reduced hepatic steatosis, reversal of the poor regeneration phenotype in liver-specific <i>Nampt</i> KO mice	Mukherjee et al., 2017
C57BL/6 wild-type and <i>Atm</i> ^{-/-} mice	male	12 mM (drinking water)	14 days	improved motor coordination, behavior, Purkinje cell number, and dramatically improved lifespan (<i>Atm</i> ^{-/-})	Fang et al., 2016
Human unconventional RBP5 interactor knockin mice (inducible, liver-specific), C57BL/6 mice fed a high-fat diet	unspecified	~500 mg/kg (diet)	5 weeks (3–8 weeks of age)	decreased DNA damage and inflammatory cell infiltration in liver	Gomes et al., 2016
Wistar rats	male	300 mg/kg (gavage)	21 days	nonsignificant decrease in swimming performance	Kourtzidis et al., 2016
C57BL/6NTac wild-type and <i>Nrk1</i> KO, or <i>Nrk1/2</i> double KO mice	male	500 mg/kg, 50 mg/kg (IP)	1 hr	some degradation of NR to NAM detectable in plasma	Ratajczak et al., 2016
C57BL/6JRcc mice fed a medium-/high-fat diet	male	5–900 ppm (diet)	30 ppm for 4 weeks, then stratified to test groups for 15 weeks	improved metabolic flexibility	Shi et al., 2017
Sprague-Dawley rats injected with paclitaxel or vehicle	female	200 mg/kg (gavage)	7 days prior to and 24 days post-paclitaxel, or 21 days beginning 14 days post-paclitaxel	prevention or reversal of paclitaxel-induced hypersensitivity to pain, no significant effect on basal locomotor activity (rotarod or open field)	Hamity et al., 2017
ICR wild-type mice fed a high-fat diet for 18 weeks	male	300 mg/kg (gavage)	2 weeks	reduced hepatic ER stress induced by the high fat diet	Wang et al., 2017

ER, endoplasmic reticulum; IP, intraperitoneal; KO, knockout; PO, per oral; SC, subcutaneous.

Table 3. Conditions in Which NAD⁺ Decline Has Been Documented

Disease/Condition	Tissue	NAD ⁺ Concentration (% of Normal)	Reference
Aging	muscle	~30%–35% (22 months), ~40%–45% (30 months)	Gomes et al., 2013
		~60%–65% (32 months)	Camacho-Pereira et al., 2016
		~80%–85% (25–31 months)	Yoshino et al., 2011
		~65%–70% (24 months)	Frederick et al., 2016
		~50% (24 months)	Mouchiroud et al., 2013
	liver	~85%–90% (25–31 months)	Yoshino et al., 2011
		~85%–90% (12 months), ~75%–80% (20 months), ~70%–75% (<45 versus >60 humans)	Zhou et al., 2016
		unchanged (12 months), ~40%–50% (24 months)	Braidy et al., 2011
		55%–60% (32 months)	Camacho-Pereira et al., 2016
		~60% (24 months)	Mouchiroud et al., 2013
	adipose	55%–60% (32 months)	Camacho-Pereira et al., 2016
		70%–75% (25–31 months)	Yoshino et al., 2011
	brain	63%–66% (12 months), ~90% (at age 70 versus 20 based on regression)	Stein and Imai, 2014; Zhu et al., 2015
	pancreas	~80%–85%	Yoshino et al., 2011
	spleen	35%–40% (32 months)	Camacho-Pereira et al., 2016
heart	unchanged (12 months), ~30%–40% (24 months)	Braidy et al., 2011	
kidney	unchanged (12 months), ~15%–20% (24 months)	Braidy et al., 2011	
lung	unchanged (12 months), ~20%–25% (24 months)	Braidy et al., 2011	
cerebrospinal fluid	~91% (<45 versus ≥45 humans)	Guest et al., 2014	
Obesity	liver	~50%–60%	Wang et al., 2017
		Unchanged	Escande et al., 2010
		~30%–35%	Gariani et al., 2016
		~80%–85%, ~50%–55%	Trammell et al., 2016b Yoshino et al., 2011
	adipose	~15%–20%	Yoshino et al., 2011
	muscle	unchanged	Yoshino et al., 2011
		~80%–85%	Frederick et al., 2015
mdx (Duchenne muscular dystrophy model)	muscle	~70%–75% ~45%–50%	Chalkiadaki et al., 2014 Ryu et al., 2016
hURI overexpression (oncogenic transgene that leads to spontaneous hepatocellular carcinoma)	liver	~25%	Tummala et al., 2014
Noise injury	cochlea	~40%–50%	Brown et al., 2014
Liver regeneration	liver	~60%–70%	Mukherjee et al., 2017
Atm mutant mice (ataxia telangiectasia model)	cerebellum	~40%	Fang et al., 2016

Values are estimated from graphs when not stated in the cited reference. In most cases, a decrease in NAD⁺ cannot be distinguished from a shift in the NAD⁺/NADH ratio, and in at least some cases, the effect of a redox shift appears to be important; e.g., in the age-related changes reported by Braidy et al. (2011) and in the NAD⁺ decline in human brain (Zhu et al., 2015).

majority of cases in which NMN and NR are effective, it still remains unclear what downstream mechanisms mediate their beneficial effects. NAD⁺ is required as a cosubstrate for PARPs, sirtuins, ADP-ribosyl cyclases, and mono-ADP ribosyltransferases but also serves as a redox cofactor for countless enzymes (Figure 1B). In several cases, deletion of sirtuins has been shown to block key benefits of NAD⁺ supplementation, supporting a role for these enzymes (Brown et al., 2014; Gomes et al., 2013; Guan et al., 2017; Martin et al., 2017). In contrast, pharmacological or genetic inhibition of other key NAD⁺-consuming enzymes, such as PARP1/2 and CD38, is sufficient to confer beneficial effects that have been attributed to increased tissue NAD⁺ availability (Bai et al., 2011a, 2011b; Camacho-Pereira et al., 2016). Given the wide range of benefits that have been reported, and the potentially complex interactions between NAD⁺-dependent processes, a great deal of work will be required to define the precise mechanisms acting downstream of NAD⁺ supplementation to promote health.

It is also critical to carefully assess potentially detrimental side effects for these NAD⁺ intermediates. Although NAD⁺ appears to promote certain pathways related to DNA repair and suppression of inflammation, NAD⁺-depleting drugs are currently under development as cancer chemotherapeutics, and there might be a risk that boosting NAD⁺ could drive tumor growth (Gujar et al., 2016). Similarly, one of the downstream targets that is hypothesized to mediate many of NMN and NR's effects, SIRT1, has been shown to have pro-carcinogenic or anti-carcinogenic effects in different contexts (Chalkiadaki and Guarente, 2015). In addition, we cannot exclude the possibility that increased accumulation of nicotinamide, a potent inhibitor of sirtuins (Avalos et al., 2005; Bitterman et al., 2002), could have detrimental effects in NMN- and NR-treated mice. It is also clear that high-dose supplementation greatly exceeds the body's requirement for niacin equivalents and thus will result in substantial elimination through the urine. One major route to nicotinamide elimination is methylation via nicotinamide N-methyltransferase, and there has been little investigation of whether this can lead to methyl donor depletion over time. Thus, further preclinical and clinical studies are needed to establish the long-term safety of NMN and NR as human therapeutics.

In Vivo Pharmacokinetics of NMN and NR

Despite the growing interest in NMN and NR, their *in vivo* pharmacokinetics remain poorly understood. Only a handful of published studies have carefully investigated the distribution and metabolism of NMN or NR in rodents or humans. It was reported that NMN rapidly appears in plasma, liver, white adipose tissue, and pancreas in wild-type mice within 15 min after one bolus intraperitoneal injection of NMN (500 mg/kg) (Yoshino et al., 2011). Then, NMN is immediately utilized for NAD⁺ biosynthesis, leading to markedly increased NAD⁺ (2- to 3-fold) concentrations in liver over 60 min (Yoshino et al., 2011). Consistently, a recent study using doubly labeled isotopic NMN (C13-D-NMN) shows that orally administered NMN is rapidly absorbed and converted to NAD⁺ in peripheral organs such as liver and skeletal muscle (Mills et al., 2016). In addition, data obtained from blood and urine pharmacokinetics conducted in rats suggest that intraperitoneally administered NMN appears to be retained within the body longer than nicotinamide (Kawamura et al., 2016). Although these findings firmly

demonstrate the bioavailability of NMN *in vivo*, the mechanism of NMN uptake into cells or tissues is currently unclear and under debate. Two mechanisms have been proposed and discussed to account for NMN uptake (Figure 2): One is the direct uptake of NMN presumably through specific transporter(s). The other is extracellular dephosphorylation of NMN into NR by ectonucleotidases (e.g., CD73) before uptake. The first possibility is supported by the fact that intraperitoneal or oral administration of NMN (300–500 mg/kg) immediately (within 5 min) increases NMN in plasma and then NAD⁺ content in peripheral organs (Mills et al., 2016; Yoshino et al., 2011), suggesting the presence of an active NMN uptake system in the gut and other organs. This hypothesis was challenged by a recent report using *Nrk1* knockout mice. These knockout mice cannot phosphorylate NR to NMN, thus showing a lack of effective increases in NAD⁺ biosynthesis in kidney and brown adipose tissue after one bolus intraperitoneal injection of NMN (500 mg/kg), compared with wild-type mice (Ratajczak et al., 2016). However, in these *Nrk1* knockout mice, NMN still results in a robust increase in NAD⁺ content in the liver (~130% increase relative to basal values), suggesting that NMN is utilized for hepatic NAD⁺ biosynthesis in an NRK1-independent manner. Several *in vitro* studies show that chemical or genetic inhibition of 5'-nucleotidase or *Nrk1/2* abolishes the utilization of NMN in cultured cells (Fletcher et al., 2017; Grozio et al., 2013; Nikiforov et al., 2011; Ratajczak et al., 2016; Sociali et al., 2016). It is possible that extracellular NMN is transported into cells or tissues and utilized for NAD⁺ biosynthesis in a tissue- and cell-type-specific manner. For example, NRK1 is barely detectable in heart and white adipose tissue (Ratajczak et al., 2016). Nonetheless, systemic NMN administration has been shown to increase NAD⁺ biosynthesis in both heart and white adipose tissue (Karamanlidis et al., 2013; Stromsdorfer et al., 2016; Yamamoto et al., 2014; Yoshino et al., 2011), implying a significant contribution of direct NMN uptake or substantial breakdown to nicotinamide and resynthesis after uptake into these organs. Further studies using tissue-specific *Nrk1/2* knockout mice in conjunction with isotopically labeled NMN should clarify these models.

Information regarding *in vivo* NR pharmacokinetics is also limited. A recent study by Trammell et al. (2016a) used targeted NAD⁺ metabolomics approaches and provided a comprehensive analysis of the effects of oral NR administration on peripheral blood mononuclear cells (PBMCs) in humans and on hepatic NAD⁺ metabolism in mice. In this study, NR increased the concentrations of all detected NAD⁺ metabolites in PBMCs except for nicotinamide. Interestingly, nicotinic acid adenine dinucleotide (NaAD⁺), which is not expected to be produced from NR, appeared with a slight delay relative to other metabolites but with a very large fold change owing to a low background level, making it potentially an attractive biomarker for NAD⁺ biosynthesis. In mice, hepatic levels of nicotinamide, NMN, and NAD⁺ were increased approximately 4-fold 6 hr after oral administration of NR (185 mg/kg). NR was not detected in human PBMCs or mouse liver, presumably due to rapid conversion of NR to NMN by NRKs. To fully understand the pharmacokinetics and uptake mechanisms of NR and NMN, it will be of great importance to evaluate their stability in the blood circulation. Indeed, data obtained from a recent *in vivo* study using doubly

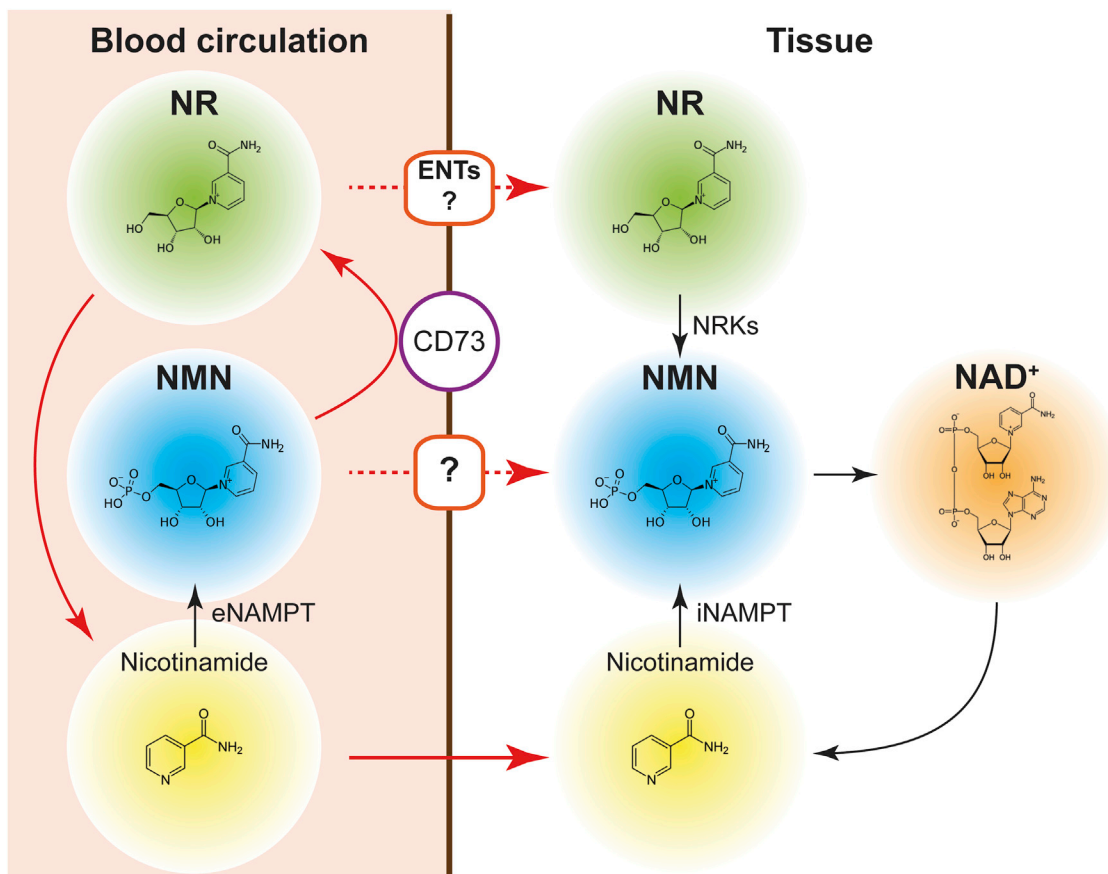


Figure 2. Uptake of NMN and NR *In Vivo*

Extracellular conversion and degradation of NMN and NR and their possible uptake mechanisms are indicated. See details in the text. eNAMPT, extracellular nicotinamide phosphoribosyltransferase; iNAMPT, intracellular NAMPT; ENTs, equilibrative nucleoside transporters.

labeled isotopic NR (C13-D-NR) demonstrate that oral NR administration (200 mg/kg) markedly increases circulating nicotinamide concentrations without a major effect on whole-blood NR concentrations (Frederick et al., 2016). Doubly labeled NR and NAD⁺ were detected in the liver, but not in skeletal muscle, suggesting a significant effect of first-pass metabolism and/or limited half-life in the blood. Furthermore, it has recently been reported that NR, but not NMN, is unstable and quickly degrades into nicotinamide in murine plasma or fetal-bovine-serum-containing culture medium (Ratajczak et al., 2016). Although *in vitro* studies demonstrate that NR can be directly transported through equilibrative nucleoside transporters (ENTs) and utilized for intracellular NAD⁺ biosynthesis (Nikiforov et al., 2011; Ratajczak et al., 2016), a substantial fraction of orally administered NR is likely converted into nicotinamide by first-pass metabolism in the liver or by hydrolysis in the blood circulation before its uptake into other tissues *in vivo* (Figure 2). Indeed, Rowen and Kornberg (1951) reported the efficient degradation (the “phosphorolysis”) of NR into nicotinamide and ribose-1-phosphate by liver extracts in 1951. Similarly, NMN may be subjected to first-pass metabolism or converted into NR by ectonucleotidases and then degrades into nicotinamide before uptake (Figure 2). Importantly, NMN and NR fail to increase NAD⁺ but nicotinamide still can in some tissues of *Nrk1* knockout mice, strongly supporting the

conclusion that at least part of the compounds administered reaches the target tissues before being degraded to nicotinamide.

These findings indicate that NMN and NR have distinct *in vivo* behavior and kinetics, leading us to the following three outstanding questions: First, how are NMN and NR metabolized in the liver and blood circulation after systemic administration? Second, are there any tissue- or cell-specific mechanisms responsible for uptake of NMN and NR? Lastly, are there any particular conditions in which one works more efficiently than another? Additional studies are needed to compare NMN and NR side by side by using isotopically labeled tracers to determine the spatiotemporal pharmacokinetics of NAD⁺ intermediates and metabolites in blood and tissues. Elucidating *in vivo* pharmacokinetics of NMN and NR is critical to better understand the mechanisms of their pharmacological actions. However, what is their pathophysiological relevance in mammalian systems? In the next section, we discuss this important issue, focusing on the function of extracellular NAMPT (eNAMPT), a key regulator of systemic NAD⁺ biosynthesis.

Systemic NAD⁺ Biosynthesis

One peculiar but interesting feature related to NMN is that NAMPT, the enzyme responsible for the production of NMN, has two different forms in mammals—namely, intracellular

and extracellular NAMPT (iNAMPT and eNAMPT, respectively) (Figure 1B). Considerable amounts of eNAMPT exist in blood circulation in mice (Revollo et al., 2007; Yoon et al., 2015) and humans (Friebe et al., 2011), as well as in human cerebrospinal fluid (Hallschmid et al., 2009) and seminal plasma (Riammer et al., 2016). It has also been reported that many different cell types, including adipocytes (Revollo et al., 2007; Yoon et al., 2015), hepatocytes (Schuster et al., 2014), leukocytes (Friebe et al., 2011), cardiomyocytes (Pillai et al., 2013), glia cells (Zhao et al., 2014), epithelial cells (Zhao et al., 2014), and monocytes (Yano et al., 2015), produce eNAMPT. Although it is unclear whether all these cell types have actively regulated mechanisms for eNAMPT production, both fully differentiated white and brown adipocytes actively secrete eNAMPT, which possesses a significantly higher enzymatic activity compared with iNAMPT (Revollo et al., 2007). Interestingly, iNAMPT is acetylated, and SIRT1-dependent deacetylation of iNAMPT at lysine 53 (K53) predisposes the protein to secretion in adipocytes and enhances its enzymatic activity (Yoon et al., 2015). Fasting-induced enhancement of eNAMPT secretion from adipose tissue is completely abrogated in whole-body and adipose-tissue-specific *Sirt1* knockout mice, demonstrating the physiological importance of this SIRT1-mediated eNAMPT secretion from adipose tissue. A surprise came when adipose-tissue-specific *Nampt* knockout and knockin (ANKO and ANKI) mice were found to show reciprocal changes in hypothalamic NAD⁺, SIRT1 activity, and physical activity, as well as changes in circulating eNAMPT levels (Yoon et al., 2015). Consistently, systemic injection of an NAMPT-neutralizing antibody significantly reduces hypothalamic NAD⁺ levels *in vivo*, whereas purified eNAMPT can enhance NAD⁺, SIRT1 activity, and neuronal activity in *ex vivo* hypothalamic explants (Yoon et al., 2015). Collectively, these findings provide compelling evidence that eNAMPT plays a crucial, systemic role in remotely regulating hypothalamic NAD⁺ biosynthesis and function. In addition, it has recently been shown that monocyte-derived eNAMPT plays a critical role in preserving myocardial NAD⁺ levels and SIRT1 activity in pressure-overloaded mouse hearts (Yano et al., 2015), providing another line of evidence for the pathophysiological importance of eNAMPT.

For NAMPT to function as an NAD⁺ biosynthetic enzyme, it forms a dimer. Indeed, the crystal structure of NAMPT clearly demonstrates that this protein belongs to the dimeric class of type II phosphoribosyltransferases (Kim et al., 2006; Wang et al., 2006). Based on its size, circulating eNAMPT appears to be a dimer under normal conditions (Körner et al., 2007). However, it has recently been reported that serum levels of monomeric eNAMPT are selectively elevated in high-fat-diet-fed diabetic mice (Kieswich et al., 2016). It seems that monomeric eNAMPT contributes to metabolic impairment in a diabetic condition via NAD⁺-independent proinflammatory effects, whereas dimeric eNAMPT or its product NMN mediates protective effects against metabolic dysfunction. These findings likely provide an important clue to resolve a current controversy around the pathophysiological importance of eNAMPT as a systemic NAD⁺ biosynthetic enzyme versus a proinflammatory cytokine (Garten et al., 2015; Hara et al., 2011) and also suggest that circulating dimeric eNAMPT contributes to NMN synthesis to maintain normal NAD⁺ biosynthesis at a systemic level. Indeed, NMN has been detected in mouse and human plasma (Mills et al.,

2016; Ramsey et al., 2008; Revollo et al., 2007). However, additional studies are required to carefully evaluate where and how exactly circulating eNAMPT synthesizes NMN. It is not known whether NR and NRKs exist in blood circulation, although some cultured human cells might be capable of secreting NR (Kulikova et al., 2015). It will certainly be of great interest to examine the dynamics of blood and extracellular NMN and NR metabolism under various pathophysiological conditions.

Given the pathophysiological importance of eNAMPT and the pharmacokinetic features of NMN, an interesting concept, named the “NAD World,” has been proposed for the systemic regulatory mechanism of mammalian aging and longevity. The original concept of the NAD World was proposed in 2009 as a systemic regulatory network that connects NAD⁺ metabolism, biological rhythm, and aging and longevity control in mammals (Imai, 2009a, 2009b, 2010). SIRT1 and NAMPT are two key components that comprise the NAD World: SIRT1 functions as a critical metabolic regulator in many different organs and tissues in response to changes in NAD⁺ availability, whereas NAMPT functions as a pacemaker that produces circadian oscillatory NAD⁺ production (Nakahata et al., 2009; Ramsey et al., 2009) and fine-tunes SIRT1 activity at a systemic level. In this concept, both eNAMPT and NMN play an important role in regulating NAD⁺ biosynthesis systemically. The availability of NMN is particularly important for tissues and organs that possess very low levels of iNAMPT, such as pancreatic β cells and central neurons. Recently, this concept has been reformulated with new findings over the past 8 years, now called NAD World 2.0 (Imai, 2016). NAD World 2.0 proposes the importance of the inter-tissue communications among three key tissues, namely, the hypothalamus, adipose tissue, and skeletal muscle, for mammalian aging and longevity control. In this concept, NMN is hypothesized to function as a systemic signaling molecule that maintains biological robustness of the NAD World.

It will be of great interest to examine whether NR and NRKs play a critical role in regulating systemic NAD⁺ biosynthesis. Given that NAD⁺ levels decline with age at a systemic level (Braidly et al., 2011; Camacho-Pereira et al., 2016; Frederick et al., 2016; Gomes et al., 2013; Massudi et al., 2012; Stein and Imai, 2014; Yoshino et al., 2011) (Table 3), supplementing NMN or NR to enhance systemic NAD⁺ biosynthesis could be an effective anti-aging intervention to maintain physiological functions and delay the aging process in mammals. Several clinical trials are currently being conducted to evaluate the effects of NMN and NR on a wide variety of physiological outcomes, including glucose metabolism, insulin sensitivity, cardiovascular function, immune function, and cognitive function (<https://clinicaltrials.gov>). Therefore, it may not be so long until we know whether NMN and NR can effectively mitigate physiological decline or have therapeutic effects in humans.

Concluding Remarks

In this new era of NAD⁺ biology, we are now revisiting many old questions and, at the same time, producing many new questions as well. One such question in the former category is about the compartmentalization of NAD⁺ biosynthesis. For example, subcellular compartments, such as the nucleus and mitochondria, appear to possess distinct machinery to synthesize NAD⁺. But, are NAD⁺ intermediates shared among different subcellular

compartments? Does NMN or NR actually go into mitochondria? If so, how? Another interesting question in the latter category is whether specific transporters exist for NMN and NR. If so, where are they expressed? And what is their physiological and pathophysiological importance in aging? Are there any tissue specificities for NMN and NR transport? Intensive investigations are currently under way to address both old and new questions. As Gholson (1966) predicted more than 50 years ago, “an important, but as yet unknown, function” for NAD⁺ and even for NAD⁺ intermediates could be discovered. With all these exciting developments in the field of NAD⁺ biology, the door is fully open for new breakthroughs and translation of these remarkable preclinical results to effective interventions in humans within our life time.

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