

Review article

NAD⁺ metabolism and oxidative stress: the golden nucleotide on a crown of thorns

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In the twentieth century, NAD⁺ research generated multiple discoveries. Identification of the important role of NAD⁺ as a cofactor in cellular respiration and energy production was followed by discoveries of numerous NAD⁺ biosynthesis pathways. In recent years, NAD⁺ has been shown to play a unique role in DNA repair and protein deacetylation. As discussed in this review, there are close interactions between oxidative stress and immune activation, energy metabolism, and cell viability in neurodegenerative disorders and ageing. Profound interactions with regard to oxidative stress and NAD⁺ have been highlighted in the present work. This review emphasizes the pivotal role of NAD⁺ in the regulation of DNA repair, stress resistance, and cell death, suggesting that NAD⁺ synthesis through the kynurenine pathway and/or salvage pathway is an attractive target for therapeutic intervention in age-associated degenerative disorders. NAD⁺ precursors have been shown to slow down ageing and extend lifespan in yeasts, and protect severed axons from degeneration in animal models neurodegenerative diseases.

Keywords: Sirtuins, Oxidative stress, NAD⁺, PARP, Ageing

Introduction

Current thinking regarding the importance of NAD (including NAD⁺ and NADH) metabolism in health and disease stems from the original discovery that niacins were efficacious for the treatment of pellagra.¹ In 1937, Elvehjem² showed that nicotinic acid (NA) and nicotinamide (NM) (the breakdown product of NAD⁺) were effective agents for both the treatment and prevention of black tongue in dogs, and the human equivalent, pellagra. While the disappearance of pellagra in the developed world reduced interest in this life-threatening deficiency disease, a recent observation that several disorders mimic pellagra in its range of symptoms (e.g. dermatitis, diarrhoea, dementia, and death) has re-ignited considerable interest on the mechanism of action of NAD⁺ and its key metabolites.³⁻⁷

Intriguingly, with the understanding of the pharmacological role of niacins was the almost simultaneous discovery of the structure and function of NAD⁺ by four Nobel laureates.⁸ In 1904, Sir Arthur Harden separated Buchner's yeast juice into a high- and low-molecular-weight fraction, neither of which could undergo

fermentation individually. However, recombination of both fractions allowed fermentation to take place. Harden inferred the existence of high-molecular-weight ferment (enzyme) and a low-molecular-weight coferment or 'cozymase'.⁸

The involvement of cozymase in fermentation, respiration, and glycolysis was identified in a variety of organisms in subsequent years. However, due to the low-molecular-weight nature of cozymase, it was difficult to isolate.⁸ It was Hans von Euler-Chelpin⁹ who eventually succeeded in isolating cozymase from yeast extracts in the late 1920s. He determined the dinucleotide structure of NAD⁺ and two other mononucleotides, adenosine monophosphate (AMP) and nicotinamide mononucleotide (NMN).⁹ The central redox function of NAD⁺ was determined by Otto Warburg and Christian in the mid-1930s.¹⁰ He identified the capability of NAD⁺ to transfer hydrogen from one molecule to another.¹⁰ Since then, ongoing investigations have been directed towards the identification of the enzymatic pathways involved in its synthesis and metabolism.¹¹ In 1954, Arthur Kornberg¹² discovered the NAD phosphorylase reaction, the crucial step of NAD⁺ synthesis. He detected the enzymatic activity in yeast extracts which catalysed the

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condensation of ATP with NMN to form NAD.¹² While this reaction is catalysed by nicotinamide mononucleotide adenylyltransferase (NMNAT) [EC 2.7.7.1], it took another 55 years for the primary structure of this enzyme to be discovered.¹³

Another important milestone was the discovery of the NAD⁺-consuming activity associated with the transfer of ADP-ribosyl moieties to protein acceptors which occurred in the 1960s.¹⁴ In the same period, the term 'pyridine nucleotide cycle' was introduced to define several enzymatic reactions involved in the biosynthesis and catabolism of NAD⁺, although the significance of NAD⁺ metabolism remained unclear.¹⁵ Rechsteiner and Catanzarite (1974)¹⁶ showed that NAD⁺ turnover was strongly suppressed in enucleated yeast cells, suggesting that the nucleus is the major compartment responsible for the anabolism and breakdown of NAD⁺. Since then, studies on the enzymology of mammalian NAD⁺ metabolism have increased in line with those investigating the potential involvement of NAD-related metabolites in cellular physiology.^{11,17,18} The involvement of NAD⁺ in several key cellular processes has suggested to some the possibility that NAD⁺ metabolism may be an attractive therapeutic target.^{19,20}

Changes in NAD⁺ metabolism have been associated with several pathologies, including neurodegenerative diseases, cancer, cardiovascular disease, and normal ageing. A number of comprehensive papers have appeared in the literature, which provide a generalized overview of the information in this field.^{11,17,18,21} Beginning with an overview of relevant background research by others, this review provides additional insight into the involvement of NAD⁺ metabolism in neurodegenerative disorders and ageing.

NAD⁺ biosynthesis and salvage pathways

The kynurenine pathway and de novo NAD⁺ synthesis

In mammals, the kynurenine pathway (KP) represents the *de novo* synthesis of NAD⁺ from dietary tryptophan (TRP) (Fig. 1). The KP begins with the oxidative cleavage of TRP to *N*-formylkynurenine by the two enzymes tryptophan 2,3-dioxygenase (TDO) [EC 1.13.1.2] and indoleamine 2,3-dioxygenase (IDO) [EC 1.13.11.17].^{22,23} Both IDO and TDO are haem-requiring enzymes and are considered rate limiting for this pathway. TDO has been localized predominantly in the liver, but has also been found in the brain, and is induced by a number of factors including TRP, glucocorticoids, hydrocortisone, and fasting.^{22,23} IDO, on the other hand, has been identified in several extrahepatic tissues including the brain, and is up-regulated by various cytokines and inflammatory molecules such as lipopolysaccharides,²⁴ amyloid peptides,²⁵ human immunodeficiency virus (HIV)

proteins,²⁶ and tumour cells.²⁷ TDO uses L-TRP exclusively as the substrate, whereas IDO can metabolize L- and D-TRP as well as serotonin and other related indoleamines.

The catabolite of TRP, formylkynurenine is then hydrolysed to form kynurenine (KYN) by the action of kynurenine formamidase (KFase) [EC 3.5.1.9].²⁸ KYN can be transformed to kynurenic acid (KYNA) by the action of kynurenine-oxoglutarate transaminase [EC 2.6.1.7].²⁹ The remaining enzymes of the pathway include kynurenine 3-hydroxylase (K3H) [EC 1.14.13.9];²⁹ and kynureninase [EC 3.7.1.3].³⁰ K3H can convert KYN to 3-hydroxykynurenine (3-HK) by the hydroxylation of the aromatic ring. Kynureninase produces anthranilic acid (AA) by the cleavage of the alanine side chain from KYN. AA can also undergo hydroxylation to 5- and 3-hydroxyanthranilic acid (3-HAA) via non-specific microsomal hydroxylating enzymes. Kynureninase is also involved in the formation of 3-HAA from 3-HK. Furthermore, 3-hydroxyanthranilic acid 3,4-dioxygenase [EC 1.13.11.6] converts 3-HAA to the unstable intermediate, α -amino- β -carboxymuconic- Σ -semialdehyde which cyclizes spontaneously to form quinolinic acid (QUIN). 3-HAA can also be converted to picolinic acid (PIC) by aminocarboxymuconate-semialdehyde decarboxylase [EC 4.1.1.45].^{31,32} Quinolinic acid phosphoribosyltransferase (QPRT) [EC 2.4.2.19] catalyses the formation of nicotinic acid mononucleotide (NAMN) using phosphoribosyl pyrophosphate (PRPP), which is then transformed spontaneously to NAD⁺.³³

In recent years, the KP has generated considerable interest following the observation that some intermediates are endowed with neuroactive properties. Among them is the selective *N*-methyl-D-aspartate (NMDA) receptor agonist and free radical generator, QUIN;³⁴ KYNA, a non-selective antagonist at both the NMDA site and the glycine strychnine-resistant site of the same receptor, which can antagonize the cytotoxic effects of QUIN;^{35,36} PIC, an endogenous metal chelator;³⁷ 3-HK and 3-HAA, which can generate free radicals.^{38,39} Alterations in the endogenous levels of these metabolites has been implicated in the pathogenesis of several inflammatory brain diseases, including AD,⁴⁰⁻⁴² AIDS dementia complex,^{43,44} cerebral malaria,⁴⁵ amyotrophic lateral sclerosis,⁴⁶ MS,⁴⁷ Huntington's disease,^{48,49} schizophrenia,⁵⁰ and Parkinson's disease (PD).⁵¹ The inflammatory state can induce IDO, mainly by interferon gamma (IFN- γ).⁵² IDO induction in immune-activated astrocytes and microglia appears to be a primary site for TRP catabolism via the KP, leading to increased production of the neurotoxic TRP metabolites, whose accumulation in the brain has been implicated in the pathogenesis of the aforementioned disorders.⁵¹

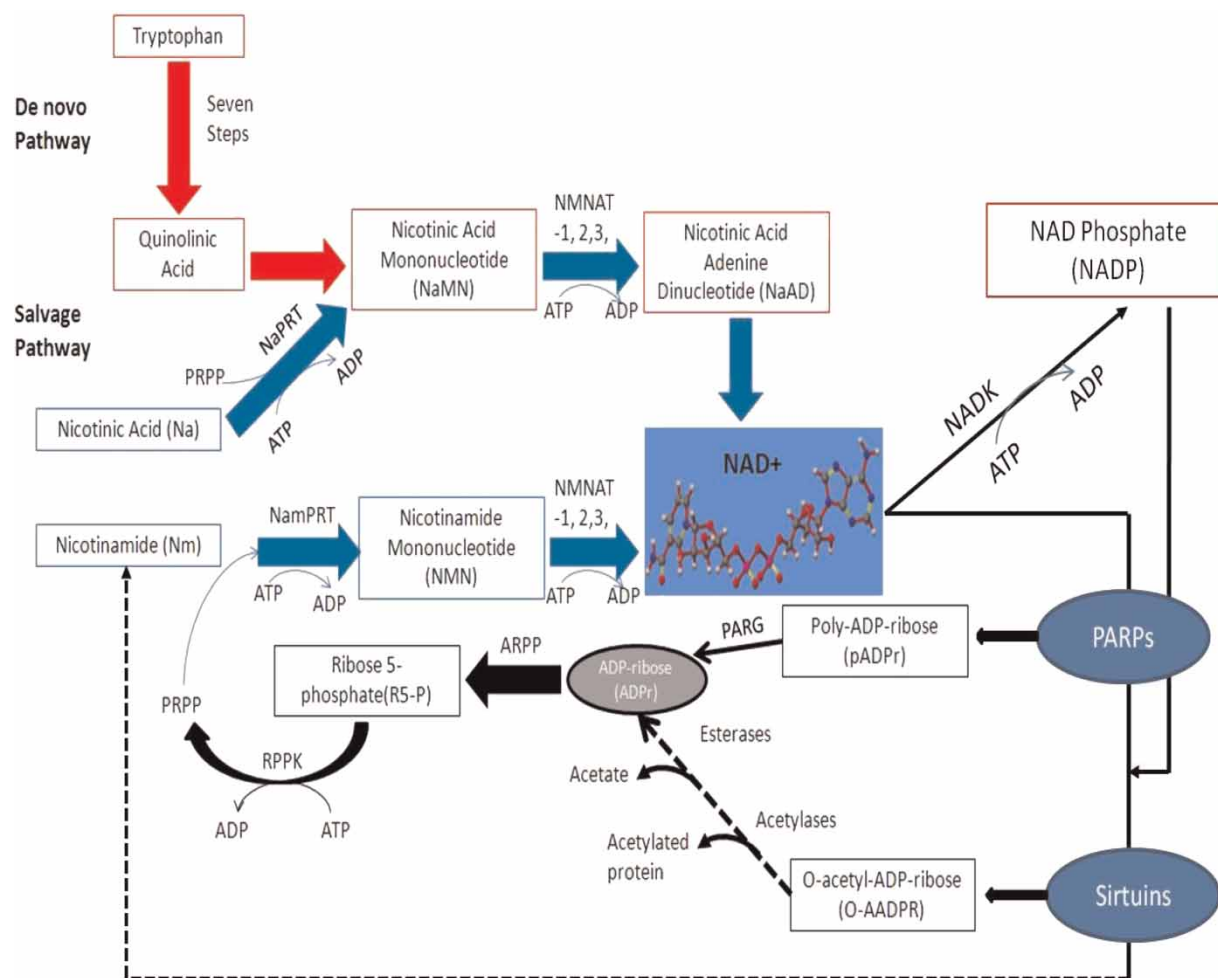


Figure 1 NAD⁺ metabolism in higher eukaryotes. The *de novo* pathway represents the catabolism of the amino acid L-tryptophan to quinolinic acid through the KP. Quinolinic acid is then converted to nicotinic acid mononucleotide (NaMN) which connects to the salvage pathway. The three different salvage pathways start either from nicotinamide (Nm), nicotinic acid (Na), or nicotinamide riboside (NR). Nicotinamide is a by-product of NAD metabolism which gets converted into nicotinamide mononucleotide (NMN) by nicotinamide phosphoribosyl transferase (NamPRT) and then into NAD by the action of nicotinamide mononucleotide adenylyl transferases (Na/NMNAT1, 2, and 3).

Our group has identified a direct involvement of the KP in NAD⁺ synthesis in human brain cells, and that reduced intracellular NAD⁺ synthesis leads to reduced activity of NAD⁺-dependent histone deacetylase function. We have shown that the KP metabolite and NMDA receptor agonist, QUIN is a substrate of NAD⁺ synthesis in nanomolar concentrations in both astrocytes and neurons but is cytotoxic at higher amounts in both cell types. These results also show a clear neuroprotective effect of NMDA receptor antagonism and nitric oxide (NO) synthase inhibition against QUIN-mediated neuronal and glial cytotoxicity, strongly suggesting that QUIN-induced excitotoxicity in astrocytes and neurons is mediated by overactivation of the NMDA receptor. Recently, we showed that natural polyphenolic compounds can attenuate QUIN-mediated neurotoxicity by a number of different mechanisms.

Also, the KP represents a defence mechanism following IDO induction during an immune-mediated response. Up-regulation of IDO expression in infected

tissue can deprive the infected area of the essential amino acid, TRP, and together with the cytotoxic effects of the released KP intermediates, can exert antimicrobial activity.⁵³ Enhanced IDO expression has been reported in rats during endotoxin shock,⁵⁴ or during malarial infection,⁵⁵ suggesting that IDO may be involved in the host response following systemic infections. Moreover, increased IDO expression in dendritic cells and macrophages can deplete TRP levels from the surrounding microenvironment, which can selectively affect surrounding T-cells and inhibit their replication or induce apoptosis. It is well established that IDO induction is associated with the development of T-cell-mediated immune tolerance.⁵³ Indeed, increased IDO expression is involved in the inhibition of T-cell-mediated rejection of allogeneic foetuses,⁵⁶ allografted pancreas islets in mice,⁵⁷ as well as suppression of T-cell-mediated experimental asthma.⁵⁸ IDO induction in tumour cells can also suppress T-cell immunity in the tumour microenvironment leading to tumoural immune resistance.^{59,60} The KP therefore

represents a potential target for the development of novel therapies in several disorders associated with immune function.⁵³ However, the involvement of the KP in maintaining cellular NAD⁺ homeostasis in various tissues, and its potential role in neurodegenerative disease and ageing, remains unclear.

Since 2007, another enzyme with IDO-like activity, IDO-like-protein 1 (INDOL1, IDO2), has been described in both mice and humans. In an exploratory application, Maiwald *et al.* (2011) substantial differences exist between the expression of IDO1 and IDO2 by professional antigen-presenting cells and MSCs (mesenchymal stromal cells) under the influence of IFN- γ and T lymphocyte media (TCM), although the exact mechanisms remain unclear. Further research is needed to delineate the functional differences between IDO1 and IDO2 in degenerative diseases and ageing.

Some studies have observed that IDO activity is directly linked to the maintenance of NAD⁺ levels in diverse cell types. IDO induction due to IFN- γ resulted in a significant increase in *de novo* NAD⁺ synthesis in a mouse macrophage/monocyte cell line RAW 264.7.⁶¹ This suggests that KP activation may play a key role in maintaining NAD⁺ levels during the immune response, mainly due to the activation of poly(ADP-ribose) polymerase (PARP), which consumes a majority of intracellular NAD⁺.⁶² Similarly, IDO induction has been shown to boost NAD⁺ concentrations and reduce cell death following PARP activation in murine astrocytes treated with hydrogen peroxide.⁶² Conversely, inhibition of IDO has been shown to significantly reduce NAD⁺ levels in primary human astrocytes.⁶³ Together, these results suggest that IDO and KP metabolites are an important source of NAD⁺ synthesis particularly under conditions of increased NAD⁺ turnover. Therefore, the use of KP inhibitors as potential therapeutic targets needs to be reconsidered since they can profoundly reduce cellular NAD⁺ levels. Another study reported a decline in intracellular NAD⁺ levels in primary murine macrophages following IDO induction via tumour necrosis factor.⁶⁴ This study shows that activation of primary murine macrophages increases NAD⁺ turnover due to increased oxidative stress.

NAD⁺ salvage pathway

Apart from the *de novo* biosynthesis pathway, NAD⁺ can also be synthesized by the NAD⁺ salvage pathway (Fig. 1). In this pathway, NAD⁺ can be synthesized by one of two routes from NM, the breakdown product of NAD⁺.^{18,65} Firstly, the enzyme nicotinamide phosphoribosyl transferase (NMPRT) [EC 2.4.4.12] converts NM to NMN using PRPP as a cosubstrate, and subsequently to NAD⁺ by the action of NMNAT.⁶⁶ Alternatively, the enzyme

nicotinamide deamidase (NM deamidase) [EC 3.5.1.9] can convert NM to NA, and then to NAMN by the enzyme nicotinic acid phosphoribosyl transferase [EC 2.4.2.11].⁶⁷ NAMN can also be directly converted to nicotinic acid adenine dinucleotide via NMNAT. A further amidation reaction catalysed by NAD synthetase [EC 6.3.5.1] is necessary to achieve the effective NAD⁺ form using glutamine as the nitrogen donor. Genomic analysis suggests that these two pathways are often exclusive: many organisms contain either NMPRT or NM deamidase.⁶⁵

Nicotinamide riboside (NR) represents another precursor for NAD⁺ synthesis. The phosphorylation of NR to NMN is catalysed by nicotinamide riboside kinase (NRK) [EC 2.7.1.22].⁶⁸ In mammalian cells, NR is formed from NMN in a reaction catalysed by NMN 5'-nucleotidase [EC 3.1.3.5].⁶⁸ Apart from its role in NAD⁺ synthesis, NRK is also involved in the phosphorylation of the compounds tiazofurin and, benzamide riboside, which is necessary to enable their further conversion into NAD⁺ analogues (TAD and BAD) by NMNAT.⁶⁸ These analogues have been previously shown to inhibit the action of inosine mononucleotide dehydrogenase (IMPDH), the rate-limiting enzyme involved in guanine nucleotide biosynthesis. Up-regulation of IMPDH activity has been reported in cancer and tiazofurin has been approved as an orphan drug for the treatment of chronic myelogenous leukaemia.⁶⁹ However, since the active forms of these compounds are NAD⁺ analogues, administration of these drugs is associated with some amount of toxicity in the clinic.⁶⁶

It is important to note that both NM and NA appear to be involved in numerous physiological processes. NM can enhance energy metabolism by inhibiting PARP and a new class of NAD⁺-dependent histone deacetylase enzymes known as sirtuins.⁷⁰ Numerous studies have highlighted the potential neuroprotective role of NM in multiple diseases such as cerebral ischaemia.⁷⁰ NA has been shown to significantly affect brain function by inducing glutamate release.⁷¹ Additionally, NR has been shown to extend the replicative lifespan of yeast.⁷²

Oxidative stress in cellular degeneration

Ageing is defined as the time-dependent progressive decline of biochemical and physiological functions, and is associated with increased risk of mortality and morbidity.⁷³ There is a growing awareness that oxidative stress plays a key role not only in the ageing process but also in various other clinical conditions including neurodegenerative diseases such as Alzheimer's disease and PD, cancer, diabetes, chronic inflammation and ischaemia reperfusion injury.⁷⁴ Harman (1956)⁷⁵ was the first to propose the free-radical theory which suggested that

age-related biochemical and physiological decline is associated with an accumulation of reactive oxygen species (ROS) that is generated as a result of normal cellular metabolic processes. These free radicals are thought to have deleterious side effects on the cellular constituents and play an essential role in ageing.⁷⁶ In the last 50 years, Harman's hypothesis has been modified to include other forms of activated oxygen such as aldehydes and peroxides which also contribute to cellular damage. The contemporary version of this tenet is now known as the oxidative stress theory of ageing. While there are several hypotheses that explain how ageing occurs, the oxidative stress theory of ageing is considered a key player during the ageing process and cell senescence.

The oxidative stress theory of ageing suggests that there is an imbalance between the amount of ROS (such as singlet oxygen, superoxide anion, hydrogen peroxide, and NO) that is generated during normal oxidative metabolism and the complex system of endogenous antioxidants (i.e. glutathione, pyridine nucleotides, and retinoic acid), and damage repair mechanisms which counter-act the deleterious effects generated by the antioxidants.⁷⁷ These antioxidants are thus essential to maintain the cells in their balanced state of redox. The gradual shift in the net disparity between the ROS generation and antioxidant system is thought to be an important driving force behind the ageing process.^{77,78}

Although current information from the studies performed on selected *in-vitro* model organisms are in support of the oxidative stress theory of ageing, its exact role in some unicellular organisms is still debatable.⁷⁹ Previous studies in clonal ageing using *Saccharomyces cerevisiae* (a unicellular fungus) found no significant evidence to support the limiting role oxidative stress in reducing lifespan.⁸⁰ However, a unicellular organism would seem a poor choice as a model system for ageing cell division leaves two identical daughter cells, which would result in either population

immortality or extinction. Oxidative stress also appears to be unambiguously limited in hyphae senescence in *P. Anserine*.⁸¹ Considering the strong dose-response between lifespan and oxidative stress in *Drosophilla melanogaster*, a provisional case can be made that oxidative stress appears to play a major role in influencing lifespan, even in wild-type flies under normal physiological conditions. Further studies are necessary to evaluate the role of oxidative stress and ROS production in an ageing human population.

Sources of ROS

Cells are vulnerable to ROS insult from a large variety of both exogenous and endogenous sources.⁸² The central nervous system (CNS) is extremely vulnerable to free radical-mediated destruction due to significant oxidative metabolic activity, lower levels of antioxidants and protective enzymes and an abundance of polyunsaturated fatty membranes.⁸² Since most neurons do not proliferate, the CNS neural network can be readily disrupted.⁸³ Importantly, the aged have an increased susceptibility to oxidative stress due to generally negative changes in dietary patterns, absorption or utilization of nutrients, or underlying infections, which can disrupt the normal antioxidant defence system.⁸³

It is generally accepted that the mitochondrial electron transport chain (ETC) is the major site for the generation of ROS.⁷⁷ The ETC is continuously involved in reducing molecular oxygen to water in a four electron reduction process.⁷⁷ However, only a small percentage of the oxygen consumed escapes the ETC as superoxide anion (O₂⁻), which can generate other endogenous ROS, therefore posing a great threat to aerobic organisms, and their intracellular constituents.⁷⁷ The main forms of ROS are summarized in Table 1.

Superoxide is relatively labile which means it is rapidly dismutated to H₂O₂ in the mitochondrial membrane by superoxide dismutase. H₂O₂ is

Table 1 Main ROS and their effects

Species	Chemical structure	Description	Occurrence	Action
Superoxide radical	O ₂ ⁻	Most potent radical in the induction of cellular damage	Almost all aerobic cells	Majority of reactions as a reducing agent
Hydroxyl radical	OH•	O ₂ ⁻ acid conjugate, highly reactive	Formed through water radiolysis	DNA, proteins, carbohydrates and lipids
Hydrogen peroxide	H ₂ O ₂	It is not a free radical because did not submit electrons paired in the last layer	Reactions for the production of OH•	Proteins and lipids
Singlet oxygen	¹ O ₂	Excited form of molecular oxygen. It is not a free radical, because did not submit electrons paired in the last layer	Generated by phagocytes, luminous induction and catalysed reactions by peroxidases	DNA changes

Adapted from Oliveira MC *et al.* (2010).⁷⁸

detoxified by catalases to H₂O and O₂. Alternatively, transition metals such as ferrous and cuprous ions can reduce H₂O₂ into hydroxyl anion (OH).⁸⁴ O₂⁻ is unable to cross the mitochondrial membrane whereas H₂O₂ can easily diffuse across the membrane, and is thought to have important effects on intracellular signalling pathways in the cytosol, which can affect redox balance, cellular responses, and energy metabolism.⁸⁴

Over the last decade, new insights on the potential role of mitochondrion H₂O₂ have been uncovered. In particular, these studies have shown that H₂O₂ may regulate mitochondrial ROS production through activation of mild mitochondrial uncoupling.⁸⁵ Furthermore, O₂⁻, NO, and other ROS may play a role as important mediators of cell signalling processes at moderate concentrations.⁸⁴ For instance, O₂⁻ can be involved in oxidative stress responses and maintenance of redox homeostasis. These studies support the notion that low levels of ROS are vital to the maintenance of various cell functions. However, detrimental effects on cells are likely if ROS are in excess.⁸⁶

A major exogenous source of ROS in living organisms is exposure to ionizing and non-ionizing irradiation. Exposure of tissue and cells to γ -irradiation results in the formation of several free radical species following the ionization of intracellular water.⁸⁷ ROS such as OH, O₂⁻, and H₂O₂ can also be produced following exposure to non-ionizing irradiation such as ultraviolet B (UV-B).⁸⁸ Haemolytic cleavage of H₂O₂ by UV radiation produces the \cdot OH radical. Air pollutants such as cigarette smoke, car exhaust, and industrial waste containing NO derivatives are another major source of ROS capable of damaging the organism by either inhalation into the lungs or direct interaction with the skin.⁸⁸ Certain drugs may also produce ROS.^{89,90} For instance, the mechanism of action of the antibiotic bleomycin is mediated by ROS production.⁹⁰ Several xenobiotics, such as pesticides and herbicides, may also form ROS as by-products of their metabolism *in vivo*.⁹¹ Additionally, pathogenic organisms may also induce free radical production either by direct release from the pathogen or as an immune response by phagocytes and neutrophils.⁹²

Oxidative damage targets and types

Acute and chronic exposure to ROS from exogenous and endogenous sources can lead to the accumulation of oxidative damage to cellular components, and a significant impairment in normal cellular function. Among the biological targets vulnerable to oxidative damage are proteins, lipid membranes, and DNA.⁸⁶

The phospholipid bilayer of all cellular membranes is extremely vulnerable to oxidation due to their higher

concentrations of polyunsaturated fatty acids.⁹³ The general process of oxidative damage to lipids consists of three stages: initiation, propagation, and termination. The first phase involves hydrogen ion abstraction. Several ROS species are capable of abstracting a hydrogen atom from the methylene group in the lipid. Following hydrogen abstraction, the remaining fatty acid radical retains a single electron and is stabilized following the rearrangement of the molecular structure of the newly formed conjugate diene. The fatty acid can react to form the peroxy radical (ROO⁻) in the presence of oxygen in the surrounding microenvironment during the propagation phase. The ROO⁻ can become a lipid hydroperoxide that can further breakdown to form reactive aldehyde products, including malondialdehyde, 4-hydroxy-2-nonenal, 4-hydroxy-2-hexenal, and acrolein in the presence of reduced metals or ascorbate (Fig. 2).⁹² Lipid peroxidation can disrupt the normal assembly of the cellular membrane leading to alterations in fluidity and permeability, dysregulation of ion transport, and inhibition of normal metabolic processes. Lipid peroxidation is one of the major outcomes of ROS-mediated damage to cells and tissues.^{86,92,93}

Proteins, which also represent major components of the cellular membrane, are also potential targets for ROS-mediated damage. H₂O₂ appears to have a weak effect on proteins.^{86,94,95} However, proteins containing the sulfhydryl (-SH) group can undergo oxidation following interaction with H₂O₂. Protein oxidation products can include aldehydes, ketone compounds, and carbonyls.^{86,94,95} A major adduct that can be easily detected as a marker for protein oxidation is 3-nitrotyrosine (3-NT). 3-NT is formed following the interaction of the peroxynitrite (ONOO⁻) and other reactive nitrogen species with the amino

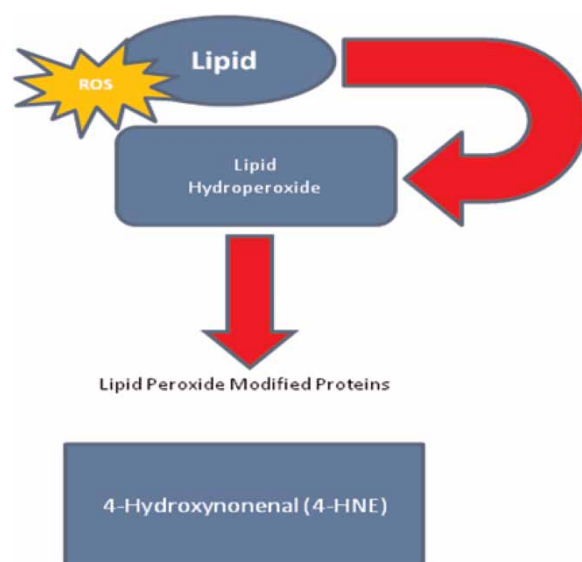


Figure 2 Formation of lipid hydroperoxides. A great variety of compounds are formed during lipid peroxidation of membrane phospholipids.

acid tyrosine.⁹⁶ Proteins can undergo direct and indirect damage following exposure to ROS, including peroxidation, damage to amino-acid residues, denaturation of the tertiary protein structure, and fragmentation, leading to loss of function.^{86,94,95}

A major factor associated with age-related diseases is the increase in oxidative DNA damage. ROS can interact with DNA leading to the modification of DNA bases, loss of purines, and single- and double-strand DNA breaks. The main ROS capable of causing damage to DNA is the $\cdot\text{OH}$ radical.^{86,97,98} Following exposure of DNA to OH, several adducts, such as the oxidation product, 8-hydroxydeoxyguanosine are formed at the C-8 position.⁹⁹ $\cdot\text{NO}$ and O_2^- can also lead to the formation of ONOO^- , which can also cause DNA damage similar to the $\cdot\text{OH}$ radical. More recently, ROS induced DNA double-stranded breaks have been shown to induce histone H2AX phosphorylation on serine 139 (Fig. 3).^{100,101} DNA damage may affect the expression of a variety of genes involved in the regulation of cell proliferation or inhibit expression of other genes associated with DNA repair. Many cell types do not show time-related degeneration due to rapid turnover. However, some mammalian cells, particularly neurons change considerably with age.¹⁰² Elevated levels of $\cdot\text{OH}$ mediated DNA and RNA damage are consistently observed in mutations, carcinogenesis, degenerative and other diseases, inflammation, ageing and during development.^{86,97,98}

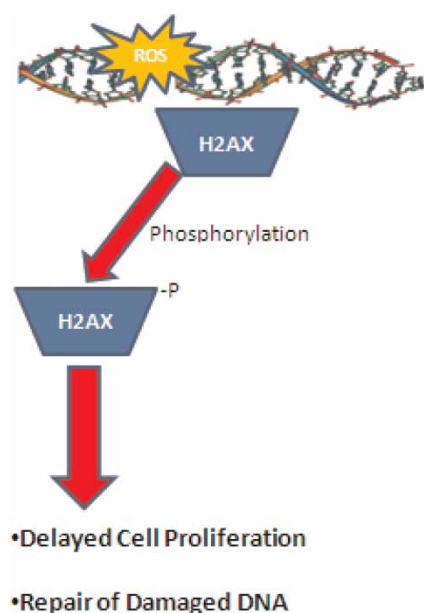


Figure 3 Phosphorylation of H2AX at Serine 139 following ROS damage to DNA. Under mild-to-moderate oxidative damage, phosphorylation of H2AX can lead to temporary cessation of cellular function and DNA repair. However, under extreme conditions, hyperphosphorylation of H2AX can lead to cell death via an apoptotic mechanism.

Importance of maintaining intracellular NAD⁺ levels

NAD⁺ has now been identified as a ubiquitous molecule which plays a critical role in several biological processes, including cellular respiration, DNA repair, and transcriptional regulation.¹⁰³ NAD⁺ and its reduced form NADH have enormous importance in cell biology, and are generally considered core components in redox reactions.¹⁰⁴ NAD⁺ plays a critical role in an increasingly diverse range of cellular processes, including signal transduction, DNA repair, and post-translational protein modifications and apoptosis.¹⁰⁴ NAD⁺ and NADH are used during cellular respiration during the process of oxidative phosphorylation and ATP production. Therefore, ATP synthesis and redox potential is directly proportional to intracellular NAD⁺ concentrations.¹⁰⁴ In addition to its role in cellular metabolism, NAD⁺ is also important for a number of ADP-ribosylation reactions associated with cell regulation and repair mechanisms which are discussed later in this paper.

NAD⁺ in energy metabolism

As mentioned previously, NAD⁺ and NADH serve as key regulators of glycolysis by acting as important cofactors for GAPDH in the cytosol.¹⁰⁵ Cytosolic NAD⁺ and NADH also mediate other energy metabolism-related reactions in the cytosol, including the lactate dehydrogenase-catalysed lactate–pyruvate conversions and the PDHC, which converts pyruvate to acetyl-CoenzymeA, a substrate for the tricarboxylic cycle (TCA).¹⁰⁶ Moreover, cytosolic NADH may also affect oxidative phosphorylation, since the reducing equivalents of NADH can enter the mitochondria through NADH shuttles.¹⁰⁷

NAD⁺ and NADH also play pivotal roles in the TCA and mitochondrial electron transport chain (NAD⁺ and NADH serve as cofactors for at least three rate-limiting enzymes in the TCA).¹⁰⁸ NADH is also one of the major electron donors in the electron transport chain, mediated by cytochrome *c* and mitochondrial cytochrome oxidase.¹⁰⁹ Cytosolic NADH is transferred to cytochrome *c* by the NADH-cytochrome *b*₅ oxido-reductase complex on the external mitochondrial membrane, and the cytochrome *c* transfers the electron to mitochondrial complex IV (cytochrome oxidase) at the respiratory site. Subsequently, molecular oxygen is reduced with generation of the electrochemical membrane potential. This process has been shown to occur in the early stage of apoptosis, and may also occur under physiological conditions, as constitutive release of mitochondrial cytochrome *c* to the cytosol does occur.^{109,110} This biological process may act not only to enhance removal of excessive NADH but also to promote cell survival when the first three respiratory complexes are impaired.

Poly(ADP-ribose) polymerase and NAD⁺ depletion

In genomic DNA repair, NAD⁺ is the sole substrate for the DNA nick sensor, PARP. The PARP family of enzymes is DNA-binding enzymes activated by ROS-initiated breaks to the DNA and is critical to the base excision repair (BER) process.¹¹¹ While many proteins are involved in repairing DNA damage, the majority of lesions are repaired by BER.¹¹¹ For many years, PARP1 was thought to be the only enzyme catalysing poly-ADP-ribosylation. PARPs now constitute a new class of protein, consisting of six members in humans.¹¹²

Several reports indicate that PARP activity represents the main NAD⁺ catabolic process in the cell, thereby forcing the cell to continuously synthesize NAD⁺ from the *de novo* or salvage pathway to maintain cellular viability following oxidative damage.^{113–115} The fast recycling is consistent with the short half-life of NAD⁺, which is estimated to be around 1–2 hours.¹¹⁶ Activation of PARP catalyses the cleavage of NAD⁺ into adenosine 5'-diphosphoribose (ADPR) and nicotinamide, and the covalent attachment of polymers of ADP-ribose to histones and other nuclear proteins, including PARP itself (Fig. 4).¹¹⁷ PARP1, which accounts for the majority of the ADPR synthesized, is found at the highest concentrations in the nucleus.

PARP1 activation leads to DNA repair and recovery of normal cellular function. Experimental studies have shown that PARP1 is activated in response to

free radical-mediated injury to DNA after brain ischaemia and reperfusion.¹¹⁸ Hyperactivation of PARP1 following DNA strand breaks can rapidly consume intracellular NAD⁺ pools, resulting in a loss of ability to synthesize ATP, and the cessation of all energy-dependent functions and consequent cell death (Fig. 5).^{112,119,120}

PARP-mediated NAD⁺ depletion has been implicated in the pathogenesis of AD, with one study showing that poly(ADP-ribose) (PAR) polymers accumulate at higher concentrations in the temporal and frontal cortex in the brains of AD patients compared with control brains.¹²¹ This indicates that PARP1 is over expressed in the AD brain and implies an excessive NAD⁺ turnover in susceptible neuronal cells.¹²¹ Over-activation of PARP1 has also been reported in diabetes, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced parkinsonism, traumatic brain injury, hypoglycaemic brain disease, and shock.^{73,112,122–124}

Sirtuin activity

In addition to its role in PARP activity, another essential factor that is greatly altered by changes in intracellular NAD⁺ levels are the sirtuins, or the silent information regulators of gene transcription.¹²⁵ Sirtuins are a highly conserved family of class III deacetylase proteins which catalyse a unique reaction in which NAD⁺ is used to remove an acetyl group from the lysine residue, releasing nicotinamide and acetyl-ribose as end products (Fig. 6).¹²⁶ At least

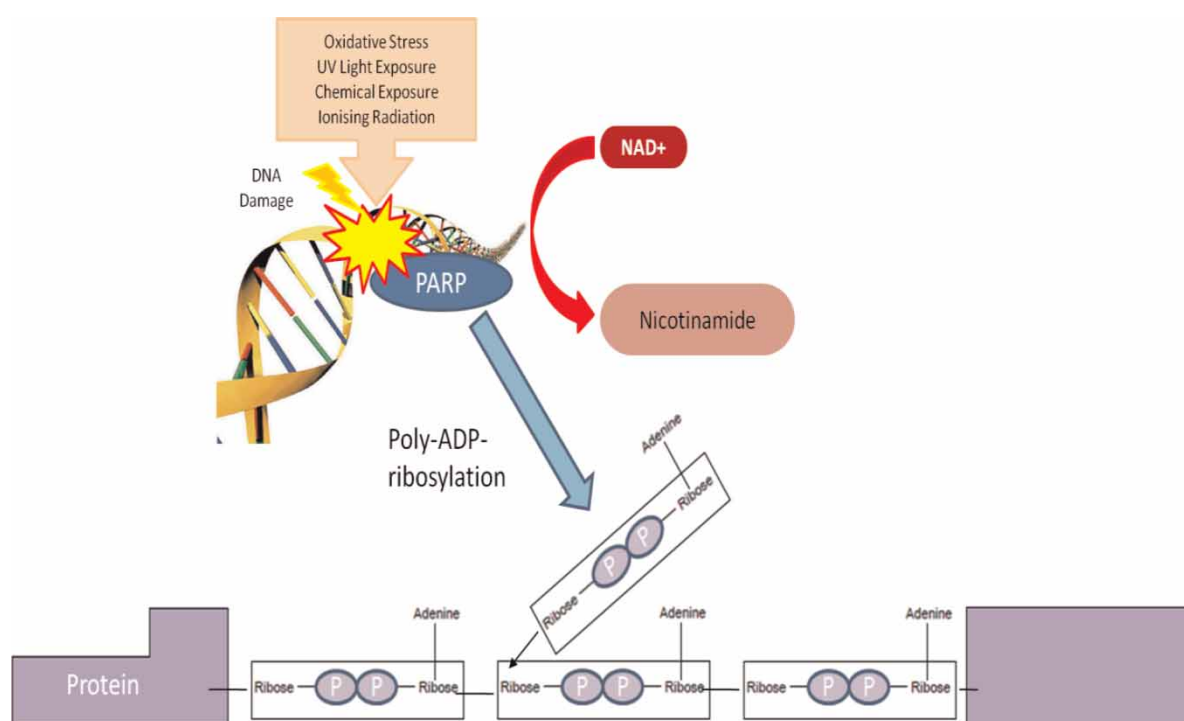


Figure 4 Schematic representation of poly(ADP-ribose) synthesis. Poly(ADP-ribose) polymerases breaks the bond between nicotinamide and ribose in NAD⁺ leading to the formation of ADP-ribosyl moiety. Repeated reaction triggers the formation of PAR chains.

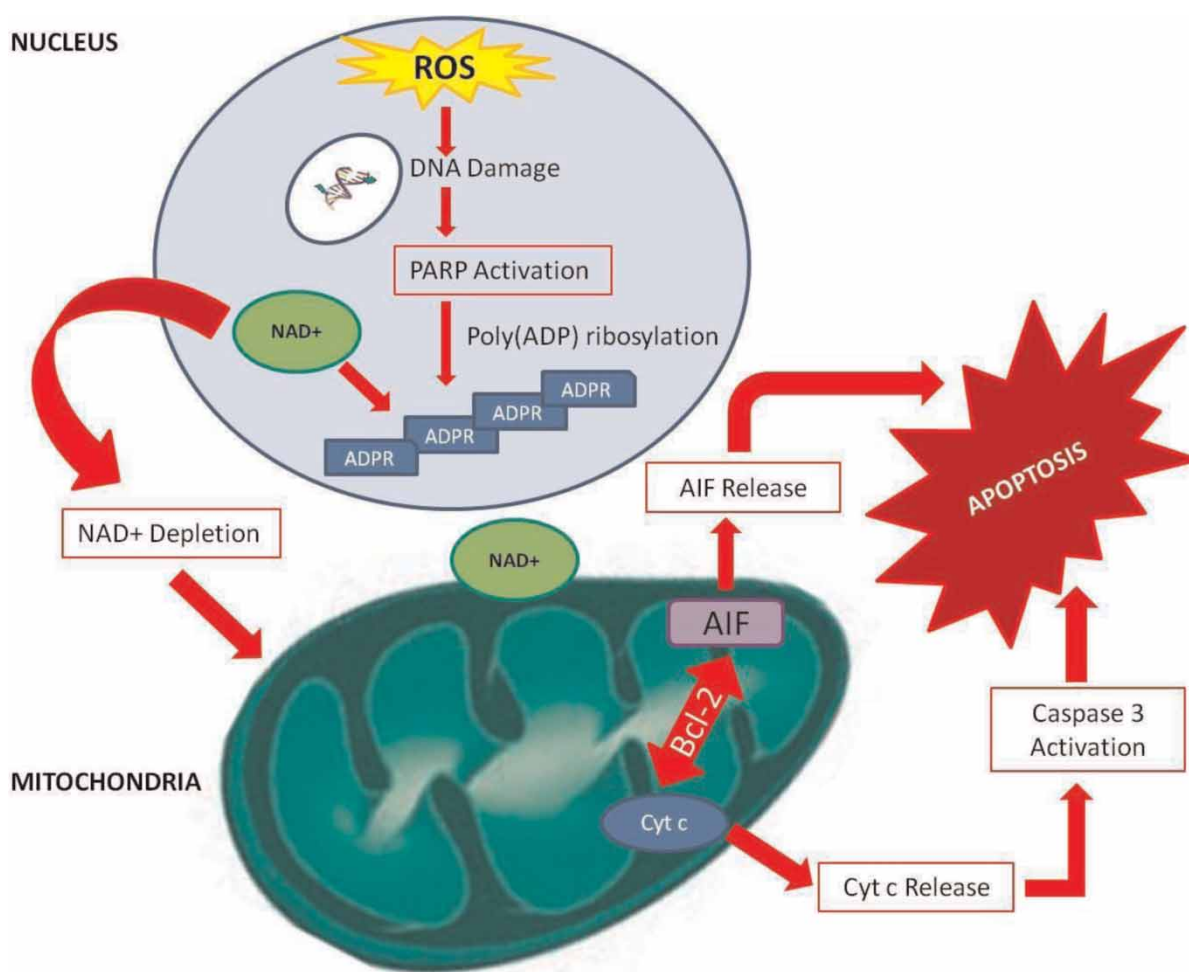


Figure 5 PARP-1-dependent signalling in apoptosis. Excessive oxidative stress leads to hyperactivation of PARP-1 in the nucleus. PAR formation and NAD⁺ depletion can trigger a cascade of events which can lead to the release of apoptotic inducing factor (AIF). AIF can migrate to the nucleus where it can trigger cell death. AIF can also promote release of cytochrome c (cyt c) through upregulation of Beclin-2 protein (Bcl-2) which can cause apoptosis via a downstream signalling through caspase activation.

seven classes of sirtuins (SIRT1–7) have been identified, each of which exhibit a wide range of fascinating biological functions, including the control of gene expression, cell cycle regulation, apoptosis, DNA repair, metabolism, and ageing.^{127,128} SIRT1 is located in the nucleus and is thought to play a vital role in ageing and cell longevity.

In mammals, seven homologues of Sir2 have been described, namely SIRT1–7, which are ubiquitously expressed. SIRT1, SIRT6, and SIRT7 are nuclear proteins involved in the regulation of chromatin structure and gene expression.¹²⁹ In contrast, SIRT2 is localized in the cytoplasm where it mediates gene expression by deacetylating transcription factors which shuttle from the cytoplasm to the nucleus.¹³⁰ The remaining members of the sirtuin family (SIRT3, SIRT4, and SIRT5) are mitochondrial protein.¹²⁹

SIRT1 is the most characterized mammalian sirtuin,²⁰ and is activated in response to energy stress, such as fasting,¹³¹ exercise,¹³² or low glucose availability which also serves to increase intracellular NAD⁺

levels.¹³³ As well, SIRT1 modulates the acetylation status of a number of important transcription factors, including the peroxisome proliferator-activated receptor- γ (PPAR γ), tumour suppressor protein (p53), and the FOXO forkhead family of transcription factors, all of which are key metabolic regulators.¹³⁴ Consistent with observations in lower-order organisms, a number of beneficial effects have been observed in mice that over-express SIRT1.^{127,128} Bordone and colleagues (2007) found that these transgenic mice displayed phenotypes similar to mice on a calorie-restricted diet, including reduced body weight, greater metabolic activity, reduced blood cholesterol, adipokines, insulin and fasted glucose, and were more glucose tolerant.^{127,128} Not surprisingly, the life-enhancing properties of sirtuins go hand in hand with those of NAD⁺ metabolism, suggesting a causal relationship where SIRT1 translates alterations of NAD⁺ levels into transcriptional events.^{103,135}

Although less is known about the cellular roles of other sirtuins on NAD⁺ levels, some interesting

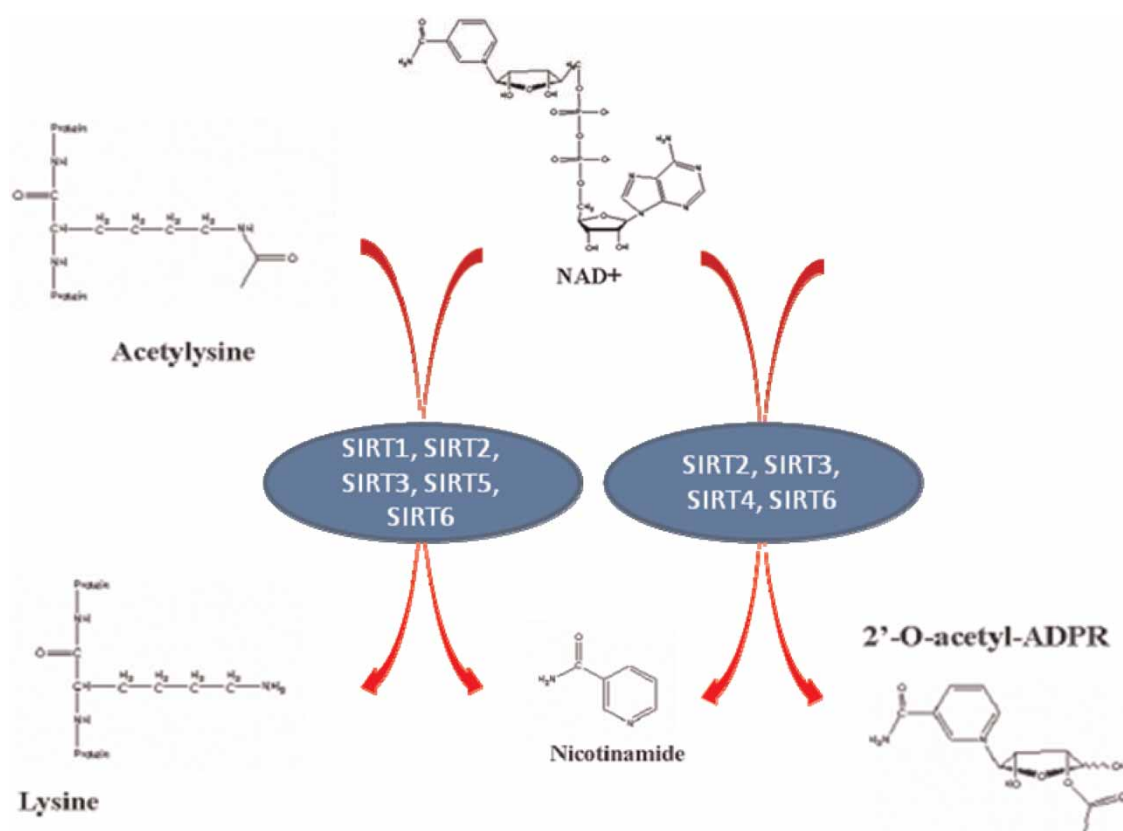


Figure 6 Sirtuin enzymatic activities. Sirtuins display two different NAD⁺ consuming activities, both of which render NM as a product.

functions have been associated with these sirtuins which are worth mentioning. SIRT2 is a tubulin deacetylase which is down-regulated in gliomas.¹³⁶ Ectopic expression of SIRT2 in glioma cell lines has been shown to decrease colony formation, suggesting that SIRT2 may have a tumour suppressor role.¹³⁶ Furthermore, SIRT2 also acts as a mitotic checkpoint which maintains chromosomal stability in the early metaphase.¹³⁷ Recently, SIRT2 has been shown to promote myelination in oligodendrocytes.¹³⁸

SIRT2 and SIRT1 have been thoroughly investigated in regard to ageing. Activation can mimic the effect of calorie restriction (CR) and extend the lifespan of yeast, worms, and flies. Moreover, overexpression of SIRT2 has been shown to extend lifespan in *Caenorhabditis elegans*.¹³⁹ Recently, Pearson *et al.* (2008)¹⁴⁰ showed that resveratrol (a putative SIRT1 activator) improved metabolism and significantly enhanced the health and survival of mice on high-calorie diet. In addition, both overexpression of SIRT1 and administration resveratrol have been shown to have neuroprotective effects.^{140–142} Collectively, these studies raise the possibility that activation of human sirtuins may slow down the ageing process. However, additional studies in the human population are required in order to elucidate the involvement of sirtuins in human population health.

Impaired SIRT1 activity due to PARP-1-mediated NAD⁺ depletion stimulates the activity of several

apoptotic effectors such as p53,¹⁴³ therefore, sensitizing cells to apoptosis. Both human and mouse SIRT1 are thought to promote cell survival by deacetylating and thus deactivating p53 tumour suppressor gene hence enhancing p53 degradation.^{20,144} Adequate NAD⁺ levels are therefore critical to maintaining SIRT1 activity which can delay apoptosis and provide vulnerable cells with additional time to repair even after the repeated oxidative stress insult.

SIRT3 has been linked to adaptive thermogenesis,¹⁴⁵ mitochondrial function,¹⁴⁵ energy homeostasis,¹⁴⁶ and cellular viability following genotoxic insult.¹⁴⁷ On the other hand, despite the presence of a conserved sirtuin domain, SIRT4 does not appear to exhibit deacetylase activity *in vitro*. Instead, SIRT4 appears to target protein activities through ADP-ribosylation.¹⁴⁸ Like SIRT3 and SIRT4, SIRT5 is a mitochondrial sirtuin with deacetylase activity, although the exact role in maintaining cellular homeostasis remains unknown.¹⁴⁹ Upcoming research will shed some light in our understanding of SIRT5 biology.

While SIRT6 was initially suggested to possess ADP-ribosylation activity only,¹⁵⁰ it recently has been shown to deacetylate histones and DNA polymerase β , a DNA repair enzyme.¹⁵¹ Several lines of evidence suggest that SIRT6 regulates genomic stability and DNA repair. SIRT6-deficient mice die prematurely, and exhibit severe defects, such as

lymphopenia, decreased bone mineral density, and impaired glucose homeostasis. This phenotype mimics multiple pathologies observed in elderly humans, suggesting that SIRT6 could play an essential role in maintaining organ integrity during ageing and development.^{150,151}

Finally, SIRT7 is localized in the nucleolus where it positively regulates transcription of ribosomal DNA during elongation, which can account for up to 60% of total transcription in metabolically active states.^{152,153} Over-expression of SIRT7 increases RNA polymerase transcription in an NAD-dependent manner, whereas down-regulation of SIRT7 can reduce cell proliferation and trigger apoptosis.^{152,153} In addition, the tumorigenic potential of several cell lines inversely correlates with SIRT7 expression.¹⁵⁴

Interactions between PARPs and sirtuins

There is now emerging evidence indicating an association between the PARP-1 and SIRT1 pathways, which have been previously independently studied. As previously described, overactivation of PARP-1

following extensive DNA damage can lead to cell death, since prolonged PARP-1 activation can deplete the essential substrate NAD⁺, leading to a subsequent increase in the byproduct, nicotinamide. The decline in NAD⁺ and the rise in nicotinamide may downregulate the SIRT1 deacetylase activity.¹⁵⁵ It is conceivable, therefore, that poly(ADP)-ribosylation by PARP-1, as induced by DNA damage, can modulate SIRT1 protein deacetylation via the NAD⁺/nicotinamide connection (Fig. 7).

Subcellular distribution of NAD⁺ and its metabolism

Compartmentation of NAD⁺

A major complication associated with the assessment of intracellular NAD⁺ levels is the subcellular compartmentation of NAD⁺ in mammalian cells. There are two major independent NAD⁺ pools: cytosolic and mitochondrial.¹¹⁹ It is estimated that the relative number of mitochondria within a tissue corresponds to the share of mitochondrial NAD⁺. The mitochondrial fraction of NAD⁺ is therefore significantly

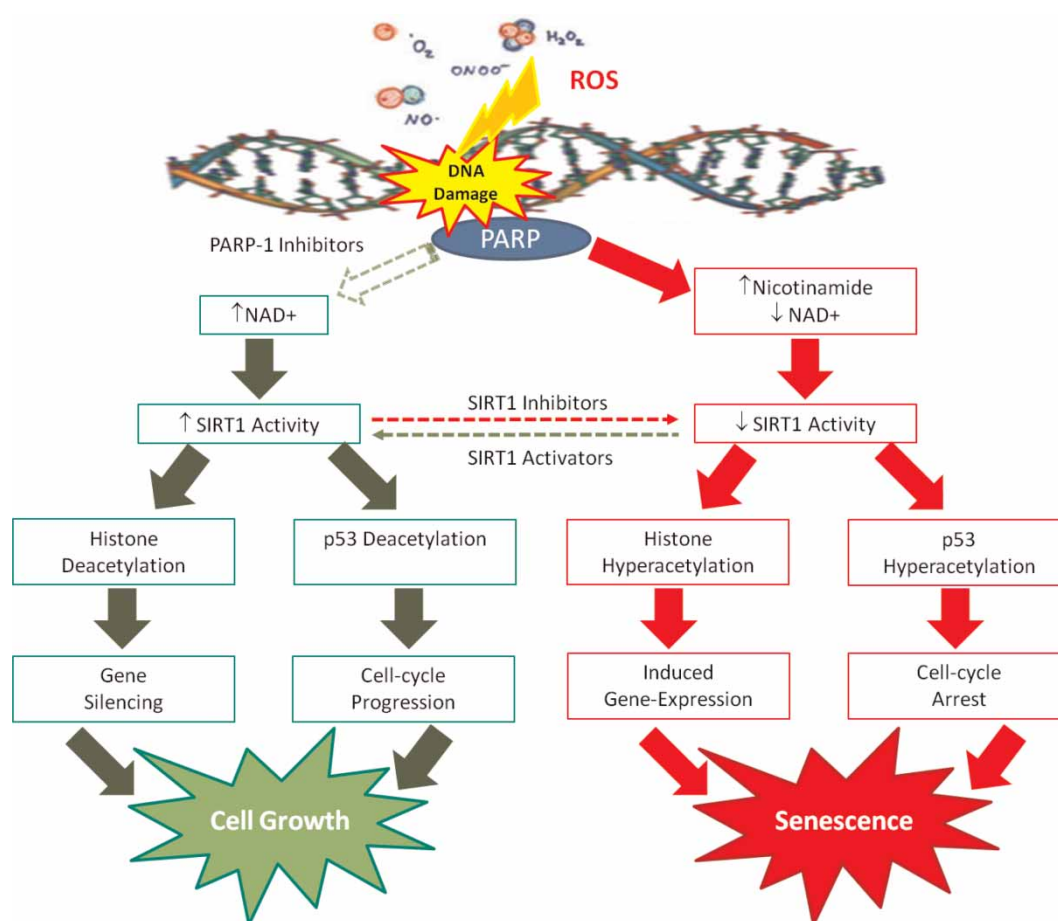


Figure 7 NAD⁺/nicotinamide levels may serve as converging points for interaction of PARP-1 and SIRT1 pathways. It is conceivable that poly(ADP-ribose) metabolism can downregulate SIRT1 through NAD⁺ depletion and nicotinamide production during oxidative stress. Reciprocally, regulation by SIRT1 deacetylation for expressing genes related to apoptosis or longevity may depend on PARP-1 activity. PARP-1 inhibitors maintain NAD⁺ levels and suppress the nicotinamide surge, and therefore may indirectly serve as SIRT1 enhancers. The interactions of PARP-1/SIRT1 pathways provide a network for multicellular eukaryotes to effectively deal with nutritional supply and oxidative stress.

higher in the liver and other mitochondria-rich tissue such as the brain and heart. The cytosolic pool of NAD⁺ appears to be freely exchangeable with the nucleus. As no physiological means of exchange have been identified between the mitochondria and cytosolic NAD⁺ compartments, these two pools are considered independent of each other.^{119,156,157} di Lisa and Ziegler (2001),¹¹⁹ however, suggested that mitochondrial permeability transition (MPT) pore opening can lead to mitochondrial NAD⁺ release that is metabolized by NAD-dependent dehydrogenases in the heart. However, it is unclear as to whether MPT-dependent NAD⁺ release also takes place in neurons and glial cells.

The NAD⁺:NADH ratio

The NAD⁺:NADH ratio plays a pivotal role in regulating the intracellular redox state, and is hence considered to be a measure of the metabolic state.¹⁴⁴ As previously mentioned, several enzymes appear to be regulated by the NAD⁺:NADH ratio, such as GAPDH and dehydrogenase reactions.¹⁵⁸ Several studies have shown that the NAD⁺:NADH ratio fluctuates in response to a change in metabolism.^{159–163}

If NAD⁺ is a metabolic regulator, then the ratio of the intracellular NAD⁺ to NADH is close to 1. Therefore, the NAD⁺:NADH is regulated by small changes in the NAD⁺ concentration.¹⁶¹ For instance, if the ratio is very high (e.g. 600), then one would assume that the NAD⁺:NADH ratio will be more sensitive to a change in the NADH concentration, and not NAD⁺.¹⁴⁴ Table 2 shows the reported ratios of total intracellular NAD⁺:NADH. These values suggest that NAD⁺ is a key metabolic regulator of the NAD⁺:NADH ratio in a variety of tissues.

Compartmentation of NAD⁺ metabolism in mammalian cells

Interestingly, the key enzymes involved in the biosynthesis of NAD⁺ are localized within the nucleus.¹⁶⁴ In humans, the predominant isoform of NMNAT (NMNAT1) is located within the nucleus (Fig. 1.17). The recent discovery of nuclear NAD⁺-dependent signalling pathways such as poly-ADP-ribosylation and sirtuin-mediated histone deacetylation suggests that

nuclear NAD⁺ production is necessary to compensate for the high rate of NAD⁺ turnover. Indeed, the inhibitory effect of NMNAT1 on PARP activity has been previously described to counteract NAD⁺ and ATP depletion following hyperactivation of PARP in response to oxidative stress.¹⁶⁵

Recent studies have indicated the presence of two additional isoforms of human NMNATs – NMNAT2 and NMNAT3, which are located in the Golgi complex and mitochondria, respectively.^{166,167} These findings, together, with discovery of NAD-dependent tankyrases in Golgi complex and NAD⁺-consuming enzymes in the mitochondria, provide further evidence to support the existence of independent NAD⁺ synthesizing machinery in the nucleus, Golgi complex, and mitochondria.¹⁶⁸

Therapeutic potential of NAD⁺ metabolism NAD⁺ in neurodegeneration

Considering the importance of NAD⁺ in energy metabolism, DNA repair and transcriptional regulation, maintaining intracellular NAD⁺ reserves emerges as a major therapeutic target for the treatment of several age-related degenerative diseases, including AD.¹⁶⁹ In particular, increased nuclear NAD⁺ biosynthesis and consequent activation of SIRT1 has been shown to protect mouse neurons from mechanical and chemical injury.¹⁷⁰ Restoring cellular NAD⁺ levels have been shown to protect against axonal degeneration in the experimental autoimmune encephalomyelitis animal model for MS in humans.¹⁹

Axonopathy is a critical feature of several neurodegenerative diseases and often precedes the death of neuronal bodies in AD, PD, and MS.¹⁷¹ As axonal deficits are central to the patient's neurological disability, therapies that prevent axonal degradation are of great therapeutic importance.¹⁷² Increased NMNAT1 activity has also been shown to protect against axonal degeneration in Wallerian degeneration slow (Wld^s) mice. Exogenous administration of NAD⁺ prior to axotomy also delayed axonal degeneration, but to a lesser extent in NMNAT1 expressed mice, further indicating the importance of maintaining intracellular NAD⁺ pools as a preventive measure against axonal degradation. In the absence of exogenous NAD⁺, PARP inhibition increased the survival of dorsal root ganglion cultures following mechanical injury. No protective effect on Wld^s mice was observed following PARP inhibition in the presence of exogenous NAD⁺.^{170,172} This suggests that maintaining adequate intracellular NAD⁺ levels can promote neuronal survival.

Another link between neurodegeneration and NAD⁺ metabolism lies in the fact that many neurodegenerative disorders, including AD, PD, and MS, are

Table 2 Baseline ratio of cytosolic NAD⁺:NADH ratio in various species

Species	Tissue type	NAD ⁺ :NADH ratio	References
Mice	Liver	4	160
Mice	Kidney	2.6	160
Mice	Brain	0.26	159
Mice	Blood	0.03	159
Mice	Pancreas	0.1	163
Swine	Liver	0.07	162

associated with mitochondrial dysfunction due to extensive oxidative damage.¹⁷³ Therefore, it is possible that activation of PARP1 can mediate the neuronal cell death observed in these pathological states.⁷³ Indeed, increased PARP expression has been reported in the brains and peripheral cells in post-mortem patients with AD, PD, and MS.^{121,174,175} Further work is necessary to identify the mechanism(s) underlying the major differences in PARP expression in these neurodegenerative diseases.

As previously mentioned, the KP represents the major route of TRP catabolism leading to the synthesis of a number of neuroreactive compounds.¹⁷⁶ Among those, QUIN appears to be involved in the pathogenesis of several neuroinflammatory disorders.¹⁷⁷ Therefore, KP inhibitors have been developed to reduce QUIN levels in the CNS.¹⁷⁸ It remains unresolved whether KP inhibition will also result in decreased NAD⁺ biosynthesis in the brain.

NAD⁺ in ageing

As chronic oxidative stress and associated nuclear damage can promote NAD⁺ catabolism, the NAD⁺ metabolic pathway has been implicated as a potential therapeutic target to promote longevity.^{113,114,179} Owing to the importance of oxidative stress in ageing, it is highly likely that PARPs may play major roles in ageing by promoting NAD⁺ depletion.^{73,123} Grube and Burkle (1992)¹²⁴ showed that PARP-1 activity in mononuclear blood cells increases with ageing in at least thirteen mammalian species, and this is likely to be associated with its important DNA repair function. Consistent with its role as the major player in the OS theory of ageing, the mitochondrial dysfunction which can occur in the ageing process is associated with changes in NAD⁺ levels.¹⁸⁰ Although a number of studies have demonstrated elevated levels of oxidative stress-mediated damage in aged tissue, to our knowledge, no study has yet reported on changes in NAD⁺ levels during the human ageing process. Braidy *et al.* (2011)¹⁸¹ recently reported a parallel increase in p53 acetylation with age, closely correlating with increased levels of lipid peroxidation, protein cross-linking, and DNA damage in the heart, lung, liver, and kidney, or aged female wistar rats. However, the potential for increased NAD⁺ catabolism appears to be involved in ageing, the impact of this process and its contribution on NAD⁺ in extending lifespan needs to be clarified.

It has been well established that CR is one of the best strategies to improve lifespan in several species. The hypothesis that increased mitochondrial function upon CR is associated with the beneficial effects on lifespan has been recently extended to humans, in which a general increase in metabolism occurred

during CR.²⁰ The beneficial effects of CR appear to be NAD⁺ dependent and partly mediated by SIRT1/Sir2 activity.¹⁸² In *C. elegans*, extra copies of the SIRT2 orthologue *sir-2.1* extended lifespan.¹³⁹ Both human and mouse SIRT2 have been shown to function as NAD-dependent p53 deacetylases, and deacetylation of p53 by SIRT2 can promote cell survival under stress.^{20,144} Recently, Pearson *et al.* (2008)¹⁴⁰ showed that activation of SIRT1 using resveratrol in mice fed a high-calorie diet improved metabolism and significantly enhanced the lifespan of these animals. Previous work from our laboratory showed for the first time that resveratrol does have a novel function in specifically upregulating NAD⁺ synthesis. Additionally, SIRT3, SIRT6, and SIRT7 have been linked with a longer lifespan in mice,^{151,154,183} while SIRT1 gene polymorphisms can affect lifespan by stimulating energy release.¹⁸⁴ Additional human studies are required to clarify the involvement of sirtuins in human population health.

However, Anderson *et al.* (2003)¹⁴⁷ suggested an alternative mechanism by which CR and Sir2 may mediate longevity in yeast. Pyrazinamide/nicotinamide (PNC1) encodes an enzyme which converts NM to NA, thereby promoting Sir2 activity and depleting the endogenous Sir2 inhibitor, NM.¹⁴⁷ PNC1 appears necessary for the lifespan extension mediated by CR. Within the NAD⁺ biosynthesis pathway NMPRT has been shown to regulate the ageing process.¹⁸⁵ Reduced NMPRT expression resulted in premature senescence in ageing human smooth muscle cells, whereas over-expression of NMPRT delayed senescence while promoting enhanced antioxidant defence.¹⁸⁵

Due to the important role of oxidative stress in ageing, it is conceivable that PARPs may play significant roles in ageing by increasing demand for its substrate, NAD⁺.^{73,123} PARP1 activities in mononuclear blood cells strongly correlate with longevity in at least 13 mammalian species, which may be associated with its important DNA repair function.¹²⁴ Moreover, PARP1 has been shown to inhibit the catalytic activities of the protein of Werner syndrome, a human disease of premature ageing.¹⁸⁶ While the NAD⁺ metabolic pathway appears to be involved in ageing, the exact role of NAD⁺ in extending lifespan is not so clear-cut. Further research in this field is necessary to determine whether NAD⁺ is an effective target to increase lifespan.

Conclusion

Pellegra, a syndrome caused by a diet deficient in either NA or TRP can lead to psychotic symptoms leading to presenile dementia likely due to upregulation of IDO, which can deplete neurons of the essential amino acid, TRP causing neurodegeneration.⁶

Administration of the NAD⁺ precursors, NA or NM previously improved the neurological state of dementia patients in the 1930s.⁶ Pharmacological doses of either NA and NM have also provided dramatic therapeutic benefits for other diseases, including rheumatoid arthritis, type I diabetes, colitis, MS, and schizophrenia in animal models and in the clinical setting.⁴ Among these precursors, NA appears to specifically activate the G-protein coupled receptor, GPR109, leading to the release of the prostaglandins, PGE₂ and PGD₂.¹⁸⁷ These prostaglandins exert potent anti-inflammatory effects through endogenous signaling mechanisms involving PPAR γ .¹⁸⁷ While NM can prevent MS in animal models, it is also an inhibitor of sirtuins, and may therefore prove detrimental on long-term cell survival and longevity.^{147,188}

There is growing evidence suggesting that NAD⁺ administration may also reduce cellular injury in multiple diseases.¹¹⁵ NAD⁺ treatment has been shown to reduce PARP1-induced astrocyte death.¹⁸⁹ It has been also shown to prevent PARP1-mediated NAD⁺ depletion in cardiac myocytes in the presence of H₂O₂.¹⁹⁰ PARP1 has been implicated in the pathogenesis of several diseases including diabetes, AD and PD (Ying, 2006, 2008).¹¹⁵ Since supplementation with NAD⁺ can protect against PARP1 mediated cell death, NAD⁺ administration may improve cell viability in these diseases by at least partially ameliorating PARP1 toxicity. *In vitro* studies have shown that NAD⁺ remains protective even when administered at 3–4 hours following PARP1 activation, suggesting that NAD⁺ administration has a long window period for reducing cellular injury.¹⁸⁹ In addition, NAD⁺ may also improve cell viability by enhancing sirtuin activities and/or improving energy metabolism (Ying, 2006, 2008).¹¹⁵

Resveratrol is a polyphenol with major health benefits that is thought to operate through direct activation of the ‘antiageing’ enzyme SIRT1.¹⁹¹ However, recent reports have challenged this ‘direct-activation’ hypothesis,¹⁹² suggesting that the mechanism by which resveratrol increases SIRT1 function is still unknown. Previous work from our group has shown for the first time that resveratrol induces a dose-dependent increase in activity of the NAD⁺ synthetic enzyme nicotinamide mononucleotide adenylyl transferase (NMNAT1) (unpublished data). As SIRT1 requires NAD⁺ as a substrate to perform its gene-silencing function, higher NAD⁺ levels will enhance SIRT1 activity.¹⁷² This finding suggests that resveratrol may promote SIRT1 function by enhancing NAD⁺ synthesis in whole cell systems without requiring direct activation.

Our observation that resveratrol increases NAD⁺ levels in primary human brain cells by acting on NMNAT, together with the neuroprotective effects

of green tea polyphenols against QUIN-mediated excitotoxicity, supports the view that polyphenols have considerable therapeutic potential, particularly for the treatment of neurodegenerative diseases.¹⁹³ As NMNAT can accelerate NAD⁺ synthesis from all three substrates, QUIN, NA, and NM (Berger *et al.*, 2005),^{17,167} NMNAT activation by resveratrol represents an ideal natural therapeutic to replenish NAD⁺ levels. Maintenance of higher cellular NAD⁺ will enhance SIRT1 activity and other NAD⁺-dependent pathways, impacting positively on cell viability and longevity. This work has therefore formed the basis of relevant patent applications.

While the potential involvement of NAD⁺ metabolic pathways in energy metabolism and mitochondrial function have been known for quite some time, suggestions of the involvement of NAD⁺ in DNA repair and longevity have grown at a rapid rate in the last decade.⁸ Characterization of the NAD⁺ synthetic pathways has not only made these advancements possible, but also contributed extensively to the understanding of the diverse roles of pyridine nucleotides in cellular biology.⁸ Despite this, information regarding the fundamental roles of NAD⁺ in neurodegeneration and ageing remains limited. Further investigations are necessary in this increasingly interesting field. Research in the following areas may be of particular interest.

Firstly, maintaining intracellular NAD⁺ levels in human brain cells such as astrocytes and neurons is crucial for the retention of cellular viability during conditions of chronic oxidative stress and immune activity through the promotion of oxidative phosphorylation (ATP production), DNA repair (PARP activity), and gene expression (sirtuin activity). Therefore, characterization of the effect of varying degrees of immune activation on *de novo* NAD⁺ synthesis in selected brain cells is necessary to validate the role of NAD⁺ metabolism as a primary contributor to neuronal dysfunction and cell death during neuroinflammatory disorders.

Secondly, as NAD⁺ is an essential molecule for all living organisms, it is not surprising that numerous cell types may possess a number of different strategies to generate NAD⁺, particularly under conditions of acute and chronic oxidative stress and NAD⁺ depletion.¹⁹⁴ For instance, post-mitotic cells such as neurons may rely on different pathways than those actively in dividing cells such as astrocytes.¹⁹⁴ Further studies may therefore need to be aimed at identifying the preferred substrate for NAD⁺ synthesis in neuronal cells under various conditions.

Thirdly, additional NAD⁺ regulates diverse pathways which may control lifespan. The importance of NAD⁺ is further underscored by recent work providing genetic evidence for the existence of several

pathways necessary for NAD⁺ synthesis. For example, Belenky *et al.* (2007)⁷² recently demonstrated that a newly identified NAD⁺ precursor, NR, can contribute to NAD⁺ synthesis by at least two unique pathways in the yeast *S. cerevisiae*. Both pathways require the nicotinamide ring for entry into the previously established pathways for NAD⁺ synthesis.⁷² Future studies are required to address the importance of NR in human health and disease, and whether it can be effectively used to replenish lowered NAD⁺ levels in age-related diseases, such as AD.

Fourthly, the essential cofactor PRPP is an important regulator for the *de novo* NAD⁺ synthetic enzyme, QPRT, which catalyses the conversion of the excitotoxin QUIN to NAMN^{11,18,166}. PRPP concentration has been positively correlated with cytosolic NAD⁺ and ATP levels in whole animals.¹⁹⁵ Therefore, the availability of PRPP for QPRT activity may be compromised during increased NAD⁺ turnover. This may occur in neurodegenerative disorders and ageing due to ROS-mediated DNA damage.¹⁹⁶ Increased QUIN secretion into the CSF may be due to increased flux through the KP parallel to reduced QPRT activity associated with an increased demand for PRPP for NAD⁺ synthesis in damaged cells. Additional studies are required to investigate the effect of PRPP on *de novo* NAD⁺ synthesis during neuroinflammation and ageing. Fifthly, the roles of different sirtuins in brain function need to be further investigated under different physiological and pathological conditions. Numerous studies have highlighted the importance of sirtuins as key regulators in ageing.¹⁹⁷ However, the precise roles of different sirtuin isoforms (SIRT1-7) and their response to varying NAD⁺ concentrations in brain function remain unclear. Future work may be aimed at establishing the roles of sirtuins in brain function as a therapeutic target for the treatment of a variety of brain disorders and increasing lifespan.

Sixthly, NAD⁺ plays a key role in regulating intracellular calcium homeostasis by acting as the substrate for NAD-dependent glycohydrolase (NADase) (see Section 1.4.5). While PARP1 is a major NAD-consuming enzyme in the cell, recent studies have raised the possibility that NADase may play a key role in NAD⁺ metabolism under physiological conditions.⁸ For instance, increased NAD⁺ levels have been reported in the brain, lung, and kidney in NADase-deficient mice.¹⁹⁸ Moreover, NADase activity was absent in the plasma membranes, mitochondria, sarcoplasmic reticulum, and nuclei in NADase-deficient mice.¹⁹⁹ These studies suggest that NADase is a key regulator of cellular NAD⁺ levels under physiological conditions, while PARP1 is a key factor determining intracellular NAD⁺ levels when significant oxidative stress and DNA damage occurs.¹¹⁵ Owing to the

critical roles of NADase and calcium in cellular function, it is warranted to further examine the roles of NAD⁺-dependent changes in calcium homeostasis not only in normal brain function but also in brain ageing and neurological disorders, in general.

Seventhly, while the current investigations reported herein focussed on PARP1 in cellular degeneration, the role of other PARPs such as tankyrases in cellular function remains largely unknown. Since NAD-dependent tankyrases are main mediators of telomerase activity, it is highly likely that NAD⁺ may also affect the ageing process through regulation of tankyrase activity.²⁰⁰ It would therefore be intriguing to study the effects of tankyrases and telomerases on certain biological functions, including neurogenesis, which might affect the ageing brain.

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